Salt Stress and Ethylene Antagonistically Regulate Nucleocytoplasmic Partitioning of COP1 to Control Seed Germination

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Seeds germination, a critical stage initiating the life cycle of a plant, is severely affected by salt stress. However, the underlying mechanism of salt inhibition of seed germination (SSG) is unclear. Here, we report that the Arabidopsis (Arabidopsis thaliana) CONSTITUTIVE PHOTOMORPHOGENESIS1 (COP1) counteracts SSG. Genetic assays provide evidence that SSG in loss of function of the COP1 mutant was stronger than this in the wild type. A GUS-COP1 fusion was constitutively localized to the nucleus in radicle cells. Salt treatment caused COP1 to be retained in the cytosol, but the addition of ethylene precursor 1-aminocyclopropane-1-carboxylate had the reverse effect on the translocation of COP1 to the nucleus, revealing that ethylene and salt exert opposite regulatory effects on the localization of COP1 in germinating seeds. However, loss of function of the ETHYLENE INSENSITIVE3 (EIN3) mutant impaired the ethylene-mediated rescue of the salt restriction of COP1 to the nucleus. Further research showed that the interaction between COP1 and LONG HYPOCOTYL5 (HY5) had a role in SSG. Correspondingly, SSG in loss of function of HY5 was suppressed. Biochemical detection showed that salt promoted the stabilization of HY5, whereas ethylene restricted its accumulation. Furthermore, salt treatment stimulated and ethylene suppressed transcription of ABA INSENSITIVE5 (ABI5), which was directly transcriptionally regulated by HY5. Together, our results reveal that salt stress and ethylene antagonistically regulate nucleocytoplasmic partitioning of COP1, thereby controlling Arabidopsis seed germination via the COP1-mediated down-regulation of HY5 and ABI5. These findings enhance our understanding of the stress response and have great potential for application in agricultural production.

Seed germination is a critical stage in the initiation of plant life cycles, and it has received much attention due to its vital importance in sustainable agriculture (Weitbrecht et al., 2011). Under favorable conditions, seeds initiate germination with water uptake for imbibition, completing the process with radicle protrusion and elongation through the covering layers (Finch-Savage and Leubner-Metzger, 2006; Holdsworth et al., 2008). Increasing evidence suggests that seed germination is regulated by a lot of factors, including light, salt, and phytohormones (Finch-Savage and Leubner-Metzger, 2006; Holdsworth et al., 2008; Weitbrecht et al., 2011). For instance, light is a major environmental factor that affects seed germination (Borthwick et al., 1952; Seo et al., 2009). DE-ETIOLATED1 regulates the stabilization of LONG HYPOCOTYL IN FAR-RED1 (HFR1) and PHYTOCHROME INTERACTING FACTOR1 (PIF1), and the PIF1-PIF complex inversely modulates the entire genomic transcriptional network in phytochrome B-dependent seed germination (Shi et al., 2013, 2015). CONSTITUTIVE PHOTOMORPHOGENESIS1 (COP1), a central repressor of light signaling, is a ring finger E3 ligase (Ang et al., 1998; Torii et al., 1998; Jang et al., 2010; Osterlund et al., 2000; Yi and Deng, 2005), which itself is regulated by multiple factors (Zhong et al., 2009; Yu et al., 2013). Nuclear COP1 levels are attributed to dark-light transitions and are correlated with the repression of photomorphogenesis (von Arnim and Deng, 1994). In the dark, COP1 translocates to the nucleus and is responsible for the proteasome-mediated degradation of photomorphogenesis-promoting factors, such as ELONGATED HYPOCOTYL5 (HY5). In contrast, in the presence of light, COP1 is removed from the nucleus, and its activity is repressed (Torii et al., 1998). HY5 has been reported to mediate the abscisic acid (ABA)
RESULTS

COP1 Negatively Regulates SSG

Because ethylene improves seed germination under salt stress (Negm and Smith, 1978; Chang et al., 2010; Lin et al., 2012; Wilson et al., 2014), we applied the phytohormone ethylene or its precursor 1-aminoacyclopropane-1-carboxylate (ACC) to suppress SSG. Our results showed that the addition of ACC did not obviously increase seed germination under control conditions but significantly promoted NaCl inhibition of seed germination compared to the corresponding controls (Supplemental Fig. S1). Ethylene overproduction mutants (eto1/2/3) and an ethylene constitutive response mutant (ctr1) promoted seed germination under NaCl stress, but ethylene signaling mutants etr1-1 (a gain-of-function mutation) and ein2, ein3-1, and ein3 eil1 (loss-of-function mutations) showed a decrease in seed germination under NaCl stress (Supplemental Fig. S2). In addition, after treatment with aminooxyxyl-Gly or silver nitrate (AgNO3), inhibitors of ethylene biosynthesis and signaling pathway, respectively, eto2 displayed lower tolerance to NaCl than the controls (Supplemental Fig. S3). These results confirmed previous findings that ethylene suppresses SSG (Cao et al., 2008; Lin et al., 2012; Wang et al., 2007, 2008).

Because light is a major factor controlling seed germination (Borthwick et al., 1952; Seo et al., 2009) and COP1 is the master regulator of light-associated development (Osterlund et al., 2000), we questioned whether COP1 affects SSG. To test this hypothesis, we first determined that germination in Col-0 and cop1-4, which lacks the WD-40 repeat domain with a hyperthermostable domain, is lower than Col-0 (Fig. 1C). Additionally, ein3-1 (EIN3ox in Col-0 background; Zhong et al., 2009) and cop1-4, which lacks COP1, exhibited lower germination, as did the corresponding controls (Fig. 1, C and D). However, cop1-4 was more sensitive to NaCl than Col-0, and ACC did not improve the germination of cop1-4 under salt stress (Fig. 1A), indicating that COP1 is a negative regulator of SSG and is required for the suppression of SSG by ethylene, distinct from the regulation in photomorphogenic development.

To investigate the relationship between COP1 and ethylene signaling in SSG, our data showed that eto2× cop1-4, similar to cop1-4, had lower seed germination under salt stress than eto2 and Col-0 (Fig. 1B), further demonstrating that COP1 is downstream of ethylene in the regulation of SSG. We next evaluated the germination of ein3-1×COP1ox and cop1-4×EIN3ox under salt stress. Transgenic lines overexpressing COP1 (COP1ox in Nos [No] background; von Arnim and Deng, 1994) and EIN3 (EIN3ox in Col-0 background; Zhong et al., 2009) were more tolerant to salt than their corresponding controls (Fig. 1, C and D). However, cop1-4×EIN3ox, which lacks COP1, exhibited lower germination, as did cop1-4, than Col-0 (Fig. 1C). Additionally, ein3-1×COP1ox, which lacks EIN3, exhibited higher germination, as did COP1ox, than No×Col-0 (Fig. 1D), indicating that COP1 functions genetically downstream of EIN3 in ethylene-promoted seed germination under salt stress.

Salt Promotes Cytosolic Partitioning of COP1

Some of the seeds completed germination within 2 d after imbibition, but this process was delayed approximately 1 to 2 d by the addition of 100 mEq NaCl (Supplemental Fig. S4). We then used 1- to 1.5-d

response through ABA INSENSITIVE5 (ABI5) during seed germination and early seedling growth (Chen et al., 2008), revealing the integration of light signaling with seed germination.

It is estimated that more than 6% of the world’s total land and approximately 20% of irrigated land are greatly affected by salt stress, which restrains plant growth and severely reduces crop yield (Munns and Tester, 2008). Salt has long been considered a key factor that affects seed germination (Darwin, 1856). In the field, seed germination occurs in soil. In a soil environment, salinity has a strong impact on seed germination and impairs the establishment of seedlings in many species (Almansouri et al., 2001; Debez et al., 2004; Zapata et al., 2004). However, the mechanism of salt inhibition of seed germination (SSG) remains largely unknown. Specifically, it is unclear whether photomorphogenic factors, such as COP1, are involved in SSG. In addition, if COP1 does regulate SSG, its direct targets and its behavior in response to salt in germinating seed tissue remain unclear.

Extensive studies have reported that ethylene signaling components participate in salinity responses during seed germination and seedling growth (Cao et al., 2008). For example, Arabidopsis (Arabidopsis thaliana) ETHYLENE RECEPTOR1 (ETR1) and ETHYLENE INSENSITIVE4 (EIN4) inhibit germination during salt stress, whereas ETR2 stimulates it (Wilson et al., 2014). The CONSTITUTIVE RESPONSE1 (CTR1) mutant ctr1-1 exhibits a high survival rate under salt and osmotic stress treatments, whereas loss-of-function mutants ein2 and ein3-1 eil1-1 (double mutations of EIN3 and EIN3-LIKE1 [EIL1]) exhibit remarkably reduced tolerance to salt (Achard et al., 2006; Cao et al., 2007; Lei et al., 2011). In addition, salinity-induced ethylene promotes the tolerance of Arabidopsis to soil salinity by enhancing sodium/potassium homeostasis (Jiang et al., 2013). Ethylene activates the expression of salt-induced and EIN3/EIL1-dependent peroxidase genes by increasing EIN3/EIL1 abundance to prevent the accumulation of reactive oxygen species and enhance tolerance to salt stress (Peng et al., 2014), which demonstrates the essential role of ethylene in regulating the salt response.

In this study, through the use of ethylene to improve seed germination under salt stress, we discovered that COP1 counteracts SSG, distinctive from the regulation in photomorphogenic development. Ethylene antagonistically modulates this process through affecting nucleocytoplasmic partitioning of COP1, thereby controlling Arabidopsis seed germination via the COP1-mediated down-regulation of HY5 and ABI5 in the nucleus. This finding is a novel and important contribution to our understanding of the stress response, with great potential for application in agricultural production.

Salt Promotes Cytosolic Partitioning of COP1

Some of the seeds completed germination within 2 d after imbibition, but this process was delayed approximately 1 to 2 d by the addition of 100 mEq NaCl (Supplemental Fig. S4). We then used 1- to 1.5-d
germinating seeds with emerging radicles for gene expression and protein detection in the following assays. In photomorphogenesis, COP1 activity is correlated with the nucleocytoplasmic partitioning of COP1. In the dark, COP1 is mainly located in the nucleus, mediating the degradation of its target proteins, such as HY5. However, light can inhibit this activity by reducing the nuclear abundance of COP1 and allowing the accumulation of HY5 to promote photomorphogenesis (von Arnim and Deng, 1994; Osterlund et al., 2000; Yu et al., 2013). To determine whether COP1 movement plays a role in SSG, we quantified the nuclear and cytoplasmic localization of COP1 in a transgenic line that constitutively expressed a GUS-COP1 fusion protein. In the protruding radicles, GUS-COP1 was localized mainly to the nucleus in both light and darkness (Fig. 2A), which was consistent with a previous report in Arabidopsis roots (von Arnim and Deng, 1994). However, NaCl treatment maintained the localization of GUS-COP1 to the cytosol in both light and darkness (Fig. 2A), revealing that NaCl prevented the translocation of GUS-COP1 from the cytosol to the nucleus.

Additionally, ACC treatment did not obviously increase the amount of nucleus-enriched GUS-COP1 (Fig. 2A), which was similar to the effect of ACC on seed germination under control conditions. Interestingly, this retentive effect of NaCl on the cytosolic localization of GUS-COP1 was impaired by the addition of ACC in the presence of NaCl, and the amount of nucleus-enriched GUS-COP1 recovered to a level that was similar to that in the samples without NaCl treatment (Fig. 2A). These data indicate that ACC and NaCl might have opposite effects on the movement of COP1 between nucleus and cytosol.

In addition, we performed cell fractionation experiments to directly detect the levels of COP1 in the nucleus and cytosol using COP1 antibodies. To exclude the influences of de novo protein production and degradation during the process, we supplemented the samples with cycloheximide (CHX) and MG132, with or without ACC and NaCl, in light or darkness for 16 h. Consistent with the observations shown in Figure 2A, COP1 was mainly localized to the nucleus in both light and darkness. Additionally, the repression of...
translocation to the nucleus by NaCl was reversed by the addition of ACC (Fig. 2B). Thus, our results demonstrate that ethylene and salt inversely modulate the localization of COP1 during seed germination, independent of the transition between light and darkness.

We then examined the localization of GUS-COP1 in the ein3-1 background. With loss of function of EIN3 in the ein3-1 x GUS-COP1 hybrid, although NaCl treatment still attenuated the translocation of GUS-COP1 to the nucleus, ACC treatment did not reverse this process (Fig. 2C). Cell fractionation experiments using ein3-1 eil1 further confirmed the above observation, i.e. the amount of COP1 protein localized in the nucleus was decreased by NaCl treatment, and the addition of ACC did not recover salt-restrained COP1 localization in cytosol (Fig. 2D), strongly suggesting that the reversal of ethylene on salt-induced COP1 movement is inactivated by the removal of EIN3 and EIL1.

Interaction between COP1 and HY5 Plays a Role in the Regulation of SSG

COP1 is an E3 ligase that targets multiple proteins, including HY5, for degradation via protein-protein interactions. COP1 contains three structural modules: an N-terminal RING-finger, a predicted coiled-coil domain, and C-terminal WD-40 repeats, which are all important for interaction (Ang et al., 1998; Torii et al., 1998; Osterlund et al., 2000). In the above research, we revealed that the transgenic line overexpressing full length of COP1 displayed inhibition of SSG (Fig. 1D), but the overexpression of defective COP1 lacking the coiled-coil domain (ΔCoil) or lacking both the coiled-coil and RING-finger domains (ΔRΔC) in Col-0 background did not improve SSG, showing a similar seed germination to Col-0 under salt stress (Fig. 3, A and B). Combined with the promotion of SSG in the loss-of-function cop1-4
mutant, which lacks the WD-40 repeat domain (Fig. 1, A–C), our analyses reveal that the three HY5-interacting domains in COP1 play important roles in the regulation of SSG. The results are consistent with their photomorphogenic phenotypes, i.e. COP1ox shows suppression of photomorphogenic development, whereas the photomorphogenic changes are very weak or absent in ΔRAC (Torii et al., 1998), implying that the HY5-interacting domains of COP1 that function in light signaling are also functional in SSG.

It has been reported that the N-terminal 77 amino acids of HY5 are essential for the interaction of HY5 with COP1 (Ang et al., 1998; Torii et al., 1998). The seed germination of transgenic lines containing a deletion of the N-terminal 77 amino acids of HY5 (HY5-AN77) in a Col-0 or loss-of function mutant hy5 background showed similar trends to Col-0 under control conditions but were more sensitive to salt stress than Col-0 (Fig. 3, A and B), which was consistent with their phenotype in photomorphogenic development (Ang et al., 1998; Yu et al., 2013). These results demonstrate that HY5 takes part in the COP1-regulated SSG.

To further investigate how HY5 participates in SSG, we then showed that hy5 and eto2 mutants were more tolerant, whereas an overexpression line of HY5 (3SS::HY5) was more sensitive to salt stress than Col-0 during seed germination. However, the hybrid eto2×3SS::HY5 displayed lower seed germination than eto2 and hy5, similarly to the transgenic line 3SS::HY5 (Fig. 4A). Thus, HY5 is a positive regulator of SSG and participates in ethylene-suppressed SSG.

To determine whether HY5 functions downstream of EIN3 in ethylene-suppressed SSG, similarly to COP1, we next observed the seed germination of the double mutant ein3-1 hy5 under salt stress. Our results showed that ein3-1 displayed lower germination than Col-0, whereas the double mutant ein3-1 hy5 had a similar germination to hy5, showing higher salt tolerance than Col-0 (Fig. 4B). These results demonstrate that both COP1 and HY5 function genetically downstream of EIN3 to orchestrate ethylene-suppressed SSG.

**Figure 3. Interaction between COP1 and HY5 plays a role in SSG.** A and B, Time course of seed germination without (Control) or with 100 mM NaCl treatment (Salt treatment). The data show the rate of germinated seeds compared to the corresponding controls on the same day of germination. Each value shown represents the mean ± SD of three independent biological experiments.

**Salt Improves the Stabilization of HY5 Protein**

To determine how HY5 is involved in SSG, we first determined HY5 expression in ethylene mutants with or without NaCl treatment. Although NaCl greatly induced HY5 expression, the transcript levels did not show obvious differences between the ethylene mutants and Col-0, suggesting that ethylene does not transcriptionally modulate HY5 expression in the salt response (Supplemental Fig. S5). Interestingly, we found that HY5 protein was greatly decreased in the endogenous ethylene overproduction mutant eto2 and the ethylene constitutive response mutant ctr1 but was not changed in ethylene signaling component mutants (Fig. 5A), providing biochemical evidence that endogenous ethylene restrains HY5 protein accumulation. We then examined the protein stability of HY5 with or without NaCl or ACC treatment in samples pretreated with the protein synthesis inhibitor CHX to exclude de novo protein synthesis. We found that HY5 protein gradually decreased with time under control conditions, but NaCl treatment greatly preserved HY5 accumulation (Fig. 5B), revealing that salt stress stabilizes HY5 protein. The addition of ACC promoted the degradation of HY5 (Fig. 5B), further demonstrating the negative regulatory function of ethylene on HY5 accumulation.

To further examine the regulation of HY5 by ethylene and salt, we next examined the accumulation of HY5 protein by treating Col-0 germinating seeds with 10 μM ACC plus 100 mM NaCl after pretreatment with 50 μM CHX. Our data showed that ACC greatly restrained the accumulation of HY5 induced by NaCl (Fig. 5B). Those data indicate that ethylene and salt oppositely regulate HY5 protein levels posttranscriptionally, supporting the antagonistic regulation by ethylene and salt of COP1 translocation in the germinating radicle. This conclusion was further supported by the detection of HY5 protein in the ethylene-insensitive double mutant ein3 eil1. NaCl treatment still increased HY5 protein accumulation, whereas the inhibitory effect of ACC was
disabled in ein3 eil1 (Fig. 5C). Taken together, these results indicate that ethylene and salt antagonistically regulate HY5 protein levels, thus affecting the salt response during seed germination.

The Expression of ABI5, Transcriptionally Activated by HY5, Participates in SSG

ABI5, a well-known transcription factor that affects seed germination, is transcriptionally activated by the photomorphogenic factor HY5 (Finkelstein and Lynch, 2000; Chen et al., 2008). To address whether the expression of ABI5 was associated with SSG, we first demonstrated that seed germination in different genotypes of ABI5 exhibited no obvious differences under control conditions (Fig. 6A). However, loss of function of ABI5 (abi5-8) displayed better germination, identical to the function of hy5 in SSG, than Col-0 under salt stress. Furthermore, the overexpression lines of ABI5 in Col-0 or hy5 background showed worse germination than Col-0 under salt stress (Fig. 6B), demonstrating that ABI5 acts as a positive regulator for SSG.

We then investigated whether ABI5 expression was controlled by ethylene. Our analyses showed that ABI5 expression was inhibited in ethylene overproduction mutants (eto1/2/3) and was upregulated in loss-of-function mutants (ein2/3) under control conditions. Although salt greatly increased ABI5 expression in all of the tested genotypes, ABI5 transcripts were significantly reduced in eto mutants compared with Col-0 but were enhanced in ein mutants (Supplemental Fig. S6), revealing the transcriptional regulation of ABI5 transcripts by endogenous ethylene. In addition, the expression of ABI5 induced by NaCl was repressed by the treatment of ACC in Col-0 (Fig. 7A), consistent with the effects of ethylene and salt on HY5 protein levels.

Figure 5. Salt and ethylene oppositely modulate the stabilization of HY5 protein. A, HY5 accumulation in different phenotypes. B, HY5 stability in response to NaCl and/or ACC treatment in Col-0. C, HY5 protein levels in response to NaCl and/or ACC treatment in ein3 eil1. Germinating seeds (1 d) were pretreated with 100 nM CHX for 12 h and then treated with or without 100 mM NaCl and/or 10 μM ACC for the indicated time. Protein extracts were prepared from germinated seeds. Immunoblotting was performed with anti-HY5 and antiaitin antibodies. Numbers indicate the relative levels of HY5 protein.
Furthermore, the inhibitory effect of ACC on ABI5 expression was disabled in the ein3 eil1 mutant, indicating that ABI5 participates in ethylene-suppressed SSG.

We next conducted the investigation to detect whether ABI5 expression was modulated by COP1 and HY5 in ethylene-promoted seed germination under salt stress. Our results showed that the expression of ABI5 in cop1-4 was higher than in Col-0, and ACC treatment failed to repress the ABI5 transcripts induced by salt treatment, similarly to these in ein3 eil1 (Fig. 7A). Correspondingly, the expression of ABI5 in the hy5 mutant was maintained at a lower level than in Col-0 no matter if it was with or without the treatment of ACC and/or NaCl (Fig. 7A), providing evidence that the expression of ABI5 might be controlled by HY5, consistent with the transcriptional activation of HY5 on ABI5 in seed germination and early seedling growth (Chen et al., 2008). Thus, our analyses demonstrate that COP1 and HY5 oppositely regulate ABI5 transcription in ethylene-suppressed SSG.

It has been reported that HY5 binds to the G-box CACGTG in the ABI5 and RbcSA promoters (Chattopadhyay et al., 1998; Chen et al., 2008). We next conducted chromatin immunoprecipitation (ChIP) assays using 35S:HY5-HA plants to verify the binding of HY5 to the ABI5 promoter in germinating seeds. Possible binding regions in the promoters of ABI5 and RbcSA (as a positive control) were selected for PCR amplification (Fig. 7B). HY5 was directly associated with the ABI5 promoter (Fig. 7C), indicating that HY5 directly activates ABI5 expression during seed germination.

**DISCUSSION**

In agricultural production, the rate of seed germination determines the planting density per unit area and subsequently affects crop yield. In the soil environment, salinity exhibits a strong impact on seed germination, impairing the establishment of seedlings. It is important to improve seed germination to maintain the sustainable development of agriculture (Bewley, 1997; Finch-Savage and Leubner-Metzger, 2006; Holdsworth et al., 2008; Weitbrecht et al., 2011). Moreover, photomorphogenic regulators are involved in light-initiated seed germination in Arabidopsis (Shi et al., 2013, 2015) and participate in phytohormone-controlled seedling growth (Chen et al., 2008; Seo et al., 2009; Yu et al., 2013). In addition, increasing numbers of studies have revealed that ethylene suppresses SSG (Negm and Smith, 1978; Chang et al., 2010; Lin et al., 2012); however, it remains unclear whether the light signaling factor COP1 participates in SSG. In this study, we found that the COP1 negatively regulates SSG. Moreover, salt stress and ethylene antagonistically regulate the nucleocytoplasmic partitioning of COP1, which suppresses the accumulation of HY5 protein, subsequently resulting in the transcriptional repression of ABI5 and the increase of seed germination. Thus, our data reveal the molecular mechanism by which photomorphogenic factors are involved in SSG. This finding is novel and important for our understanding of the stress response, and it has high potential for application in agricultural production.

Light mediates plant development, in part, by orchestrating multiple signaling cascades. In Arabidopsis hypocotyl cells, nuclear GUS-COP1 levels change in response to dark-light transitions and are quantitatively correlated with the extent of the repression of photomorphogenic development (von Arnim and Deng, 1994). Ethylene suppresses hypocotyl growth by improving GUS-COP1 movement into the nucleus, even under light conditions (Yu et al., 2013). Increasing evidence has revealed that light is a major environmental factor affecting seed germination (Borthwick et al., 1952; Seo et al., 2009; Shi et al., 2013, 2015). In this study, we discovered that COP1 counteracts SSG. Importantly, we reveal that the retention of COP1 in the cytosol following NaCl treatment is fine-tuned by the addition of ACC, which enhances the movement of

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**Figure 6.** ABI5 mutation enhances seed germination under salt stress. A and B, Time course of seed germination without (Control) and with 100 mM NaCl treatment (Salt treatment). The data show the rates of germinated seeds compared to the corresponding controls on the same day of germination. Each value shown represents the mean ± so of three independent biological experiments.

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COP1 into the nucleus. Correspondingly, HY5, a downstream regulator of COP1, participates in SSG; salt maintains the stability of HY5, whereas ethylene reduces it. Thus, our results demonstrate that the antagonistic regulation of ethylene and salt in seed germination is mediated by the control of COP1 translocation, which subsequently results in dynamic changes in HY5 protein and ABI5 transcripts, providing novel evidence that improves our understanding of SSG.

Soil cover is associated with the increased evolution of ethylene, which improves seed germination and seedling establishment (Gomez-Jimenez et al., 2001; Achard et al., 2006; Zhong et al., 2014). Components of the ethylene signaling pathway, including the receptors CTR1, EIN2, and EIN3, play critical roles in early plant development under salt stress (Achard et al., 2006; Cao et al., 2007, 2008; Lei et al., 2011; Wilson et al., 2014). In this study, we found a connection between the photomorphogenic factor HY5 and SSG. Our research showed that ethylene determines whether seeds will initiate germination under salt stress. This fine regulation occurred in ethylene-insensitive mutants (etr1-1, ein2, and ein3-1), in which HY5 protein accumulated and germination was restrained; however, in ethylene overproduction and constitutive expression mutants...
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(ctrl1-1 and eto2), HY5 was reduced, and seed germination was promoted. Moreover, the opposite regulation of seed germination by ethylene and salt was attenuated in the ein3 eil1 mutant. HY5 has also been reported to regulate ABA responses by directly activating the expression of transcription factor ABI5 (Chen et al., 2008). Antagonistic interactions between ABA and ethylene during germination have been demonstrated in numerous species (Beaudoin et al., 2000; Ghassemian et al., 2000). Therefore, HY5 might integrate the ethylene and ABA signaling pathways during seed germination under salt stress. As stress hormones, ABA and ethylene increase under saline conditions and regulate seed germination in distinct ways. ABA specifically promotes the binding of HY5 to the ABI5 promoter, whereas ethylene enhances the degradation of HY5 protein by promoting the COP1 enriched in nuclei. Both the level and the activity of HY5 protein determine the expression of ABI5, whose accumulation inhibits seed germination. Thus, our research demonstrates that HY5, as an integrator, participates in the interaction between ethylene and ABA in seed germination under salt stress.

Previous study has showed that loss-of-function of COP1 enhances photomorphogenic development (Deng et al., 1991). In this research, we found that COP1 counteracts SSG, indicating that COP1 might have different mechanisms responsible for seed germination and photomorphogenesis. Moreover, the functional activity of COP1 is attributed to dark-light transitions in photomorphogenesis (von Arnim and Deng, 1994; Torii et al., 1998). In germinating seeds, COP1 is constitutively localized to the nucleus, which is not affected by light-dark transitions, but affected by salt stress, demonstrating a novel function of COP1 in SSG. In addition, the E3 ligase COP1 directly combines with HY5 to cause the degradation of HY5, and the translocation of COP1 from the nucleus to the cytoplasm has been shown to be sufficiently rapid to participate in light-induced responses (Osterlund et al., 2000; Pacín et al., 2013, 2014). In this study, we found that COP1 also functions through its interaction with HY5, revealing that the COP1-HY5 complex is a key regulator of SSG. Moreover, we found that salt reduced the translocation of COP1 into the nucleus. ACC addition reversed the localization of COP1 to the nucleus, and this regulation was dependent on EIN3, showing that ethylene and salt inversely regulate the localization of COP1. Thus, the evidence presented here demonstrates that COP1 localization directly results in fluctuations in the HY5 protein and ABI5 expression levels in SSG. Although it has been shown that CSN1 contributes to the shuttling of COP1 between the nucleus and cytoplasm (Wang et al., 2009), the mechanism by which the localization of COP1 is regulated by ethylene and salt requires further investigation.

CONCLUSIONS

Based on previous reports (Chen et al., 2008; Zhong et al., 2014) and the results of this study, we propose that the cascade of COP1-HY5-ABI5 might play a central role in SSG (Fig. 8). Once seeds have imbibed, salt stress prevents the entry of COP1 into the nucleus, subsequently causing HY5 protein accumulation, promoting ABI5 expression, and restraining seed germination. In response to the mechanical pressure of water uptake, radicle protrusion, and/or soil cover, imbibed seeds are stimulated to increase ethylene production, which promotes the localization of COP1 to the nucleus to destroy HY5. Finally, the expression of ABI5 is reduced to facilitate seed germination.

MATERIALS AND METHODS

Plant Materials and Chemicals

All Arabidopsis (Arabidopsis thaliana) transgenic lines and mutants were generated in Col-0 background, with the exception of the line overexpressing GUS-COP1 in Nossen (No) background. Homozygous lines of abi5-8 (SALK_013163), ctrl1-1 (CS8057), ctrl1-1 (CS237), ein3 (CS8844), ein3-1 (CS8052), eto1 (CS8072), eto2 (CS8059), eto3 (CS8060), and hy5 (SALK_096651) were obtained from the Arabidopsis Biological Resource Center.

The following transgenic plants and mutants were generated previously: cop1-4 (McNeill et al., 1994); ein3-1 eil1, EIN3ox, and EIN3ox×cop1-4 (Zhong et al., 2009); SALK_013163; ctrl1-1 (CS8057), ctrl1-1 (CS237), ein3 (CS8844), ein3-1 (CS8052), eto1 (CS8072), eto2 (CS8059), eto3 (CS8060), and hy5 (SALK_096651) were obtained from the Arabidopsis Biological Resource Center. The following transgenic plants and mutants were generated previously: cop1-4 (McNeill et al., 1994); ein3-1 eil1, EIN3ox, and EIN3ox×cop1-4 (Zhong et al., 2009); SALK_013163; ctrl1-1 (CS8057), ctrl1-1 (CS237), ein3 (CS8844), ein3-1 (CS8052), eto1 (CS8072), eto2 (CS8059), eto3 (CS8060), and hy5 (SALK_096651) were obtained from the Arabidopsis Biological Resource Center.

Seed Germination

All seeds were used for the assays were planted and harvested at the same time and dried at room temperature for 3 months to break dormancy. Seeds were surface-sterilized and sown on Murashige and Skoog (MS) medium containing 3% Suc and 0.5% Phytagel with the indicated supplementation (10 μM ACC, 100 mM NaCl, 5 μM aminooxyethylyl-Gly, and 5 μM AgNO3). The plates were then chilled at 4°C for 3 d to stratify and improve the uniformity of germination before being moved to 22°C under a 16-h-white-light (50 μmol/m2/s)-h dark cycle. The percentage of seed germination was scored every day in three replicates, and more than 50 seeds each were grown in each plate. In our assays, the tested genotypes did not display obvious differences in seed germination under control conditions. Thus, previously reported data on ethylene-promoted seed germination are not shown in the text, whereas seed germination data on genotypes that had not been previously investigated are presented as control.

RNA Extraction and Quantitative Real-Time PCR

Total RNA was extracted from germinating seeds using the Plant Total RNA Purification Kit (GeneMark), and 2 μg of total RNA was used for the reverse transcription of cDNA with the FastQuant RT kit (Tiengan) according to the manufacturer’s instructions. Gene expression was measured by qPCR analysis using SYBR Premix (Takara) in the IQ-5 real-time system (Bio-Rad). The expression levels were normalized to TUB4. The primers used for qPCR are listed in Supplemental Table S1. Each qPCR was repeated independently three times and the average was calculated.

Western-Blot Analysis

Protein was extracted from germinating seeds with 100 μl of extraction buffer (150 μM NaCl, 1 mM EDTA, 50 μM Tris, pH 7.5, 2 mM sodium orthovanadate, 10 mM NaF, 25 mM β-glycerophosphate, 10% glycerol, 1 mM phenyl methyl sulfonyl fluoride, and 0.1% Tween 20). Ten micrograms of total protein was separated on 12% PAGE gels containing SDS, and western blotting was performed as previously described (Osterlund et al., 2000) using anti-H5x or anti-CP1 antibodies (1:1,000). Actin (1:5,000) was used as a loading control. The relative HY5 protein levels were evaluated by Image Gauge V3.12 (Fujifilm). The values of Actin signal in Col-0 without treatment were normalized to 1.0.
Subcellular Localization of GUS-COP1

Transgenic GUS-COP1 seeds were germinated on MS medium for 1 d. After treatment with or without 10 μM ACC and 100 mM NaCl for 16 h in darkness or in light, the germinating seeds were fixed using cold 90% acetone followed by the GUS staining assay as previously described (Yu et al., 2013). To quantify the nuclear-cytoplasmic localization of GUS-COP1, the percentages of cells in which GUS-COP1 staining was stronger in the nucleus than in the cytoplasm were calculated. At least 30 germinating seeds with 20 cells per seed were analyzed for each GUS fusion.

Microscopy

For GUS activity assays, images were acquired using a Zeiss Axios Imager M1 microscope and a Zeiss AxioCam HRc camera.

Nuclear Protein Extraction

Nuclear proteins were extracted at 4°C using a CellLyte PN extraction kit (Sigma-Aldrich) as previously described (Jang et al., 2010; Yu et al., 2013). The samples grown on MS medium for 1 d were pretreated with 100 μM CHX and 5 μM MG132 (to exclude de novo protein production and degradation during the process) for 12 h and then treated with or without supplementation with 10 μM ACC and 100 mM NaCl in the light for 16 h. The nuclear and cytosol protein fraction were obtained according to the manufacturer’s instructions and analyzed by western blot.

ChIP-qPCR Analysis

The EpisQuik Plant ChIP kit (Epigentek) was used to perform the ChIP assays. IgG was used as a negative control, and the interaction between the RbcSA promoter and HY5 was used as a positive control. The primers used for qPCR are listed in Supplemental Table S1.

Accession Numbers

The sequence data from this study can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: COPI (At2g32950), HY5 (At5g03730), ETR1 (At1g67090), EIL1 (At5g02890), EIN3 (At3g20770), EIL1 (At2g27050), ABI5 (At2g36270), and RbcSA (At1g67090).

Supplemental Data

The following supplemental materials are available.

Supplemental Table S1. The primers used in this study.

Supplemental Figure S1. Application of ACC improves seed germination under salt stress.

Supplemental Figure S2. Endogenous ethylene enhances seed germination under salt stress.

Supplemental Figure S3. Blockage of ethylene biosynthesis or signaling reduced ethylene-promoted seed germination under salt stress.

Supplemental Figure S4. Salt stress inhibits seed germination.

Supplemental Figure S5. Ethylene does not transcriptionally activate the expression of HY5.

Supplemental Figure S6. Ethylene transcriptionally activates the expression of ABI5.

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