Running head: SUBIA postpones dark-induced senescence in rice

Corresponding author: Julia Bailey-Serres
Address: Department of Botany and Plant Sciences, University of California, Riverside, CA 92521
Telephone: 951-827-3738
Email: serres@ucr.edu

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The submergence tolerance gene \textit{SUB1A} delays leaf senescence under prolonged darkness through hormonal regulation in rice

Takeshi Fukao\textsuperscript{1}, Elaine Yeung, and Julia Bailey-Serres* 
Department of Botany and Plant Sciences, Center for Plant Cell Biology, University of California, Riverside, California, 92521
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1. Present address: Department of Crop and Soil Environmental Sciences, Virginia Tech, Blacksburg, Virginia, 24061.

*Corresponding author; email serres@ucr.edu.
SUMMARY
Leaf senescence is a natural age-dependent process that is induced prematurely by various environmental stresses. Typical alterations during leaf senescence include breakdown of chlorophylls, a shift to catabolism of energy reserves, and induction of senescence-associated genes, all of which can occur during submergence, drought, and constant darkness. Here, we evaluated the influence of the submergence tolerance regulator, SUB1A, in the acclimation responses during leaf senescence caused by prolonged darkness in rice (Oryza sativa L.). SUB1A mRNA was highly induced by prolonged darkness in a near-isogenic line containing SUB1A. Genotypes with conditional and ectopic overexpression of SUB1A significantly delayed loss of leaf color and enhanced recovery from dark stress. Physiological analysis revealed that SUB1A postpones dark-induced senescence through maintenance of chlorophyll and carbohydrate reserves in photosynthetic tissue. This delay allowed leaves of SUB1A genotypes to recover photosynthetic activity more quickly upon re-exposure to light. SUB1A also restricted transcript accumulation of representative senescence-associated genes. Jasmonate and salicylic acid are positive regulators of leaf senescence, but ectopic overexpression of SUB1A dampened responsiveness to both hormones in the context of senescence. We found that ethylene accelerated senescence stimulated by darkness and jasmonate, however SUB1A significantly restrained dark-induced ethylene accumulation. Overall, SUB1A genotypes displayed altered responses to prolonged darkness by limiting ethylene production, and responsiveness to jasmonate and salicylic acid, thereby dampening breakdown of chlorophyll, carbohydrates, and accumulation of senescence-associated mRNAs. A delay of leaf senescence conferred by SUB1A can contribute to enhancement of tolerance to submergence, drought, and oxidative stress.
INTRODUCTION
Leaf senescence is a natural developmental process which occurs near the end of leaf development. Senescence of leaves is also triggered prematurely by various environmental stresses such as constant darkness, submergence, drought, salinity, and extreme temperature. Leaf senescence is a genetically programmed process which is actively regulated at various levels. Major physiological alterations include an increase in the breakdown of chloroplasts and a switch of carbon assimilation to catabolism of energy resources such as proteins, lipids, and nucleic acids. Recent molecular studies identified hundreds of senescence-associated gene (SAG)-encoded mRNAs, which are differentially expressed during leaf senescence in *Arabidopsis thaliana* and rice (Lee et al., 2001; Gepstein et al., 2003; Lin and Wu, 2004; Liu et al., 2008). Consistent with physiological changes, genes involved in photosynthesis and other anabolic processes are down-regulated in senescing leaves, whereas genes responsible for macromolecule catabolism and transport are up-regulated. Genome-wide comparison of gene transcript data revealed that natural and induced senescence regulates overlapping but different sets of genes in Arabidopsis rosette leaves (Buchanan-Wollaston et al., 2005; van der Graaff et al., 2006). Interestingly, the transcript profile of suspension culture cells exposed to sucrose deficiency is more similar to that of senescing leaves during constant darkness than natural age-dependent senescence, suggesting that carbohydrate starvation can trigger alterations in transcript accumulation under the two conditions.

A number of genetic and biochemical studies revealed that various phytohormones are involved in the onset and progression of leaf senescence. Ethylene is a positive regulator of leaf senescence as well as flower senescence and fruit ripening. Transcriptome analysis revealed that genes associated with ethylene biosynthesis and signaling including *ACC SYNTHASE (ACS)*, *ACC OXIDASE*, *ETHYLENE INSENSITIVE 3 (EIN3)*, and *ETHYLENE RESPONSIVE FACTOR (ERF)1a* are up-regulated in Arabidopsis leaves during age-dependent and dark-induced senescence (van der Graaff et al., 2006). Application of ethylene decreases chlorophyll content and photochemical efficiency in Arabidopsis leaves (Woo et al., 2001; Jing et al., 2002). In addition, leaf senescence was delayed in ethylene insensitive mutants, *erecta, etr1*, and *ein3*, whereas ethylene hypersensitive mutants, *hpr1* and *edr1* exhibited an early senescence phenotype (Zacarias and Reid, 1990; Grbić and Bleecker, 1995; Wawrzynska et al., 2008; Pan et al., 2012).
Methyl jasmonate (MeJA) and its precursor jasmonate (JA) also play an important role in the regulation of natural and stress-induced leaf senescence. For example, the level of endogenous JA significantly increases during natural and dark-induced senescence in Arabidopsis rosette leaves (He et al., 2002; Seltmann et al., 2010). Exogenous application of MeJA leads to a rapid reduction in chlorophyll content and induction of several SAGs in Arabidopsis and rice (Schenk et al., 2000; He et al., 2001; Woo et al., 2001; Kong et al., 2006). Moreover, leaf senescence is significantly delayed in JA-defective and insensitive Arabidopsis mutants such as coi1, drr1, jaw-D, mkk9, and mpk6, emphasizing the significance of the JA pathway in the regulation of leaf senescence (He et al., 2002; Schommer et al., 2008; Zhou et al., 2009; Morquecho-Contreras et al., 2010). However, age-dependent and dark-induced senescence was not influenced by reduced accumulation of JA in RNAi-knockdown lines of lipoxygenase-2 (Settmann et al., 2010), suggesting that leaf senescence is also modulated through a JA-independent pathway.

Salicylic acid (SA) is a positive regulator of leaf senescence. The concentration of endogenous SA is significantly greater in senescing leaves than mature green leaves in Arabidopsis and the level of several SAG transcripts are reduced in SA defective and insensitive mutants during leaf senescence (Morris et al., 2000). A global-scale gene expression analysis confirmed down-regulation of a subset of SAGs in a SA defective transgenic NahG (Buchanan-Wollaston et al., 2005). Low light- and salinity-induced senescence is accelerated in the Arabidopsis saull1 mutant that over-accumulates SA and SAG transcripts under the stress (Vogelmann et al., 2012). An early senescence phenotype in the mutant was rescued in the SA deficient pad4 background, indicating that SA is necessary for saull1-dependent premature senescence. Overall, these data indicate that multiple hormones are involved in the regulation of leaf senescence at molecular and physiological levels. However, the interplay of these hormones in the onset and progression of leaf senescence remains unknown.

Leaf senescence is a major visible symptom in plants exposed to prolonged submergence. For example, the loss of leaf color is clearly observed in Arabidopsis and rice plants after 5-7 days of complete submergence (Fukao et al., 2006; Lee et al., 2011, Vashisht et al., 2011). A rapid reduction in carbohydrate reserves also occurs in leaves of rice, *Rumex palustris*, and *Rorippa* species (Fukao et al., 2006; Chen et al., 2010; Akman et al., 2012; Barding et al., 2012). In rice, these physiological alterations are restrained by the submergence tolerance regulator,
SUBMERGENCE 1A (SUB1A), an ERF domain-containing transcription factor, in rice. Only limited rice cultivars possess the SUB1A gene, and more specifically the SUB1A-1 allele, which is highly induced during submergence (Xu et al., 2006; Fukao et al., 2006). Genotypes containing SUB1A-1 (henceforth referred to as SUB1A) can endure complete submergence for prolonged periods with considerably less severe leaf senescence and recommence the development of new leaves and tillers after de-submergence. Functional characterization of SUB1A revealed that its submergence-induced expression restricts further ethylene production and dampens gibberellic acid responsiveness, causing shoot tissue to dampen carbohydrate consumption, chlorophyll breakdown, amino acid accumulation, and elongation growth (Fukao et al., 2006; Fukao and Bailey-Serres, 2008; Barding et al., 2012). This quiescence response to submergence aids the maintenance of carbohydrate reserves and capability for photosynthesis. We hypothesized that avoidance of carbohydrate starvation may be involved in the less severe leaf senescence manifested during submergence in varieties bred to have SUB1A. In addition to submergence tolerance, SUB1A also enhances recovery from dehydration stress through activation of reactive oxygen species detoxification and induction of stress-specific transcription factors and downstream genes (Fukao et al., 2011).

Prolonged darkness has been used as a procedure to initiate synchronous senescence in leaves because it effectively induces expression of SAGs, breakdown of chlorophyll, and catabolism of energy reserves (Lee et al., 2001; Buchanan-Wollaston et al., 2005; van der Graaff et al., 2006; Seltmann et al., 2010). To dissect SUB1A-dependent regulatory mechanisms underlying stress-induced senescence in leaves, we evaluated the contribution of SUB1A to physiological and molecular adaptations to prolonged darkness. The role of SUB1A in the responsiveness and interaction of senescence-regulating hormones, ethylene, JA, and SA was also analyzed. The results demonstrate that SUB1A coordinates chlorophyll degradation, photosynthetic activity, and carbohydrate consumption via hormonal regulation, resulting in a significant postponement of leaf senescence during prolonged darkness.

RESULTS
Prolonged darkness increases the transcript levels of the three SUB1 locus genes
The multigenic locus SUB1 encodes three ERF domain-containing transcription factors, SUB1A, SUB1B, and SUB1C, all of which are submergence-inducible (Fukao et al., 2006; Xu et al, 2006).
To discern whether prolonged darkness affects the levels of *SUB1* gene transcripts, relative levels of the three mRNAs were monitored in aerial tissue of plants exposed to complete darkness for up to 12 d in the near-isogenic M202 and M202(*Sub1*) lines, non-transgenic Liaogeng (LG), and the *SUB1A* overexpression line *Ubi:*SUB1A (Fig. 1). Our quantitative RT-PCR (qRT-PCR) analysis revealed that the abundance of *SUB1A* transcript was considerably elevated by 1 d of darkness and the transcript level gradually decreased in a time dependent manner in M202(*Sub1*). *SUB1B* and *SUB1C* mRNA levels increased in response to the stress in both M202 and M202(*Sub1*). The levels of these transcripts were elevated or maintained during the stress period, but the degree of the induction was significantly lower for these genes than *SUB1A*. In the *SUB1A* overexpression line, *SUB1A* mRNA constitutively accumulated under non-stressed conditions, but exposure to prolonged darkness decreased the level of the transcript. Consistent with the observation in M202 and M202(*Sub1*), the abundance of *SUB1B* and *SUB1C* transcripts increased in response to prolonged darkness with similar trends in LG and *Ubi:*SUB1A. The level of *SUB1C* mRNA was significantly lower in *Ubi:*SUB1A at multiple time points during dark treatment, confirming previously reported *SUB1A*-dependent negative regulation of *SUB1C* mRNA accumulation (Xu et al., 2006; Fukao and Bailey-Serres, 2008; Fukao et al., 2011).

**SUB1A enhances survival of prolonged darkness through maintenance of chlorophyll and carbohydrate reserves in aerial tissue**

Prolonged darkness discontinues energy conversion by photosynthesis, resulting in acceleration of carbohydrate catabolism and chlorophyll degradation. To evaluate the contribution of *SUB1A* to survival of dark stress, rice plants were transferred to complete darkness for 18 d and recovered under regular growth conditions for 14 d (Fig. 2A). The green leaf color of both M202 and LG plants turned to pale green or yellow after 14 d of dark treatment, whereas the color of M202(*Sub1*) and *Ubi:*SUB1A leaves was maintained. By day 18, most leaves of M202 and LG were yellowish and wilted, but the two *SUB1A*-containing genotypes sustained green leaves. During the recovery period, significantly more plants established new leaves from apical meristems in M202(*Sub1*) and *Ubi:*SUB1A as compared to M202 and LG (Fig. 2B). Notably, almost all *Ubi:*SUB1A plants (93.3%) recovered from 18 d of complete darkness. These data
indicate that SUB1A delays leaf senescence promoted during prolonged darkness and significantly enhances survival of dark stress.

To quantify the alteration in chlorophyll content during dark treatment, the abundance of chlorophyll a and b was monitored in aerial tissue of plants exposed to complete darkness (Fig. 3A). Both were sustained for 3 d of darkness and then gradually decreased until day 12 in all four genotypes tested. However, more chlorophyll a and b were maintained in the genotypes with SUB1A during dark treatment, consistent with the visual inspection. Better maintenance of chlorophylls was also observed in SUB1A genotypes treated with prolonged submergence and oxidative stress (Fukao et al., 2006; Fukao et al., 2011). Many environmental stresses trigger an increase in the level of anthocyanins in vegetative tissue, which is an indicator of cellular damage. We found that the abundance of anthocyanins increased 6 d after complete darkness in aerial tissue of M202 and M202(Sub1), with greater accumulation in the genotypes lacking SUB1A (Fig. 3B).

To determine whether SUB1A affects photosynthetic activity after prolonged darkness, CO2 assimilation by photosynthesis was monitored in dark-treated plants (Fig. 3C). There was no significant difference in photosynthetic activity between M202 vs. M202(Sub1) and LG vs. Ubi:SUB1A plants under regular growth conditions. After more than 3 d of complete darkness, the rate of photosynthesis was below the detection limit in the four genotypes and over 2 h of recovery in light was required to quantify CO2 assimilation. M202 plants could not recover after 9 d of darkness, whereas photosynthesis recommenced in M202(Sub1) after 24 h of recovery. A similar trend was observed in LG and Ubi:SUB1A, with quicker recovery after 9 d of darkness for the SUB1A overexpression line. These results indicate that SUB1A slows chlorophyll breakdown thereby enabling resumption of photosynthesis following a sub-lethal period of prolonged darkness.

Under the conditions that carbohydrate production through photosynthesis is unavailable, proper management of carbohydrate reserves is a key for survival. To evaluate the influence of SUB1A in carbohydrate consumption during prolonged darkness, the amount of starch, glucose, fructose, and sucrose was monitored in aerial tissue (Fig. 4). Before stress treatment, the levels of starch, glucose, fructose, and glucose were similar in aerial tissue of M202 and M202(Sub1). The same trend was also observed in the starch content of LG and Ubi:SUB1A. By contrast, overexpression of SUB1A significantly increased the accumulation of glucose, fructose, and
sucrose under non-stress conditions. Complete darkness rapidly decreased the abundance of carbohydrates in all genotypes, but M202(Sub1) and Ubi:SUB1A plants maintained more starch, glucose, fructose, and sucrose during the stress. This SUB1A-dependent maintenance of carbohydrate reserves was also observed in aerial tissue of plants exposed to prolonged submergence (Fukao et al., 2006; Barding et al., 2012).

Transcript accumulation of senescence-associated genes are dampened by SUB1A

Leaf senescence occurs when plants are exposed to stressful conditions for prolonged periods. A subset of the genes involved in age-dependent senescence are induced by a variety of stresses such as prolonged darkness, carbohydrate deficiency, and submergence in rice and Arabidopsis (Lee et al., 2001; Buchanan-Wollaston et al., 2005; Rolland et al., 2006; Lim et al., 2007; Wang et al., 2007; Jung et al., 2010; Mustroph et al., 2010). To discern whether SUB1A affects the accumulation of genes associated with senescence, mRNA levels of representative SAGs were monitored in aerial tissue of plants exposed to complete darkness by qRT-PCR (Fig. 5). STAY-GREEN (SGR) encodes a novel chloroplast-located protein which is necessary for chlorophyll degradation in light-harvesting complex II (LHCII) in Arabidopsis and rice (Jiang et al., 2007; Park et al., 2007; Hötensteiner, 2009). Red chlorophyll catabolite reductase (RCCR) functions at the last step of chlorophyll degradation in chloroplasts (Pružinská et al., 2007). Of the three RCCR genes in rice, RCCR1 is most highly induced by dark-induced and age-dependent senescence in leaves (Tang et al., 2011). NON-YELLOW COLORING 1 and 3 (NYC1 and NYC3) were isolated through mutant screening of a stay green phenotype in rice (Kusaba et al., 2007; Morita et al., 2009). NYC1 encodes chlorophyll b reductase, which catalyzes the conversion of chlorophyll b to chlorophyll a, whereas NYC3 is a chloroplast-located α/β hydrolase family protein involved in chlorophyll degradation. OsI85 encodes isocitrate lyase which is highly induced by prolonged darkness, natural senescence, sugar starvation, and submergence in leaves and suspension cultured cells of rice (Lee et al., 2001; Wang et al., 2007, Mustroph et al., 2010). Our qRT-PCR studies revealed that transcript abundance of these senescence-regulating genes was elevated in response to prolonged darkness in all four genotypes (Fig. 5). However, accumulation of these transcripts was significantly repressed in those encoding SUB1A. Notably, the overexpression of SUB1A constitutively restricted accumulation of SAG mRNAs.
DELAY OF THE ONSET OF SENESCENCE (DOS) has been identified as a negative regulator of leaf senescence in rice (Kong et al., 2006). DOS encodes a nuclear-localized CCCH-type zinc finger protein and its mRNA level declines during age-dependent leaf senescence. In contrast with SAGs, overexpression of DOS delays chlorophyll degradation during prolonged darkness and age-dependent senescence. We found that the abundance of DOS mRNA was elevated in response to complete darkness, with higher accumulation in genotypes containing SUB1A. The data demonstrate SUB1A enhances up-regulation of the key senescence-regulating gene, concomitant with delaying senescence during prolonged darkness.

SUB1A enhances responsiveness to the senescence regulatory hormones JA and SA

JA and SA are key phytoregulators of molecular and biochemical processes of leaf senescence (Lim et al., 2007; Balbi and Devoto, 2008; Pauwels et al., 2009). Application of a derivative of JA, methyl jasmonate (MeJA), significantly decreased the basal level of SUB1A mRNA in aerial tissue of M202(Sub1) plants grown under control conditions (Fig. 6A). By contrast, the level of ectopically expressed SUB1A mRNA was minimally affected by MeJA in Ubi:SUB1A. MeJA treatment slightly repressed the basal level of SUB1B transcript, whereas there was little effect of the hormone on SUB1C mRNA in all genotypes. Similar trends in SUB1 gene accumulation were observed in SA treated plants (Fig. 6B).

Since MeJA and SA reduce basal levels of SUB1A transcript in M202(Sub1), we focused our attention on the influence of SUB1A on mRNA accumulation of representative senescence-regulating genes in MeJA- or SA-treated Ubi:SUB1A and LG plants. SGR, NYC3, and Osl85 were induced by application of MeJA (Fig. 7A). However, overexpression of SUB1A significantly restricted the accumulation of these transcripts regardless of the hormone treatment. The abundance of NYC1 and RCCR1 and was constitutively repressed in Ubi:SUB1A although the two genes were not influenced by MeJA. DOS mRNA was highly accumulated in mock- and MeJA-treated Ubi:SUB1A. To discern whether SUB1A modulates responsiveness to MeJA, inhibition of shoot elongation by the hormone was assayed at the seedling stage (Fig. 7B). Application of MeJA repressed elongation growth of seedling shoots in LG and Ubi:SUB1A, but overexpression of SUB1A significantly dampened the inhibition mediated by MeJA. The effect of SUB1A on transcript accumulation of senescence-regulating genes was also evaluated in plants treated with SA (Fig. 7C). SGR, RCCR1, NYC1, NYC3, and Osl85 were responsive to SA
in LG and *Ubi:SUB1A*. However, overexpression of *SUB1A* significantly restrained mRNA abundance of these genes in mock and SA-treated plants. Conversely, *DOS* mRNA constitutively accumulated in *Ubi:SUB1A*. As seen with MeJA, shoot elongation was repressed by SA treatment in LG and *Ubi:SUB1A* (Fig. 7D), but the growth inhibition was significantly lower in the *SUB1A* overexpression line. Together, these data indicate that constitutive overexpression of *SUB1A* diminishes responsiveness to MeJA and SA.

**Ethylene promotes dark- and JA-induced senescence, but *SUB1A* restricts ethylene accumulation during prolonged darkness**

Ethylene is an endogenous regulator of leaf senescence (Lim et al., 2007; Bleecker and Kende, 2000). Previously, we reported that *SUB1A* mRNA accumulation was significantly up-regulated by ethylene, although *SUB1A* repressed ethylene production under submergence (Fukao et al., 2006). To investigate whether this *SUB1A*-mediated restriction occurs during prolonged darkness, the rate of ethylene evolution was quantified in plants exposed to the stress (Fig. 8A). No difference in ethylene production was evident in M202 or M202(*Sub1*) at the 0 and 3 d time points. However, 6 d of darkness clearly increased the level of ethylene evolved by M202 but not M202(*Sub1*). LG also significantly increased ethylene production within 3 d of darkness (*P* < 0.001). However, the overexpression of *SUB1A* restricted ethylene evolution under dark conditions and also during standard growth conditions. These data indicate that *SUB1A* down-regulates ethylene production during constant darkness, as documented for submergence (Fukao et al., 2006).

To evaluate the effect of ethylene on dark-induced senescence, leaf segments were incubated on a half-strength MS medium containing the immediate precursor of ethylene, 1-aminocyclopropane-1-carboxylic-acid (ACC) in the dark (Fig. 8B). In the absence of ACC, the greenness of leaves was gradually lost over the 9 d of dark treatment in M202 and LG. In the genotypes carrying *SUB1A*, leaf color was unchanged after 9 d, consistent with the observation at the whole plant level (Fig. 2A). Addition of ACC accelerated dark-induced senescence in M202 and LG, but had no visible effect on M202(*Sub1*) or *Ubi:SUB1A*. Interaction of ethylene with MeJA and SA in leaf senescence was also evaluated in the same experimental system using LG and *Ubi:SUB1A*. M202 and M202(*Sub1*) were not tested because of the two hormones reduced rather than increased basal *SUB1A* mRNA levels (Fig. 6A, B). MeJA induced a leaf color
change in both genotypes, but the loss of greenness was slower in the SUB1A genotype (Fig. 8C). In LG, MeJA-mediated senescence was further promoted by addition of ACC. In both genotypes, SA promoted cell death along the cut edge of the leaf segments, consistent with a functional hypersensitive response. SA stimulated leaf senescence more severely in control genotypes than SUB1A-containing genotypes (Fig. 8D). However, ACC did not influence SA-mediated senescence. Overall, it appears that SUB1A delays dark and JA-induced senescence through restriction of an ethylene response pathway.

**DISCUSSION**

Complete submergence imposes multiple environmental stimuli due to a 10,000-fold reduction in the diffusion rate of O₂, CO₂, and ethylene as well as a restriction of light and nutrient availability. A combination of these environmental factors induces a reduction in photosynthesis and aerobic respiration and increase in catabolism of energy reserves including carbohydrates, proteins, and lipids, resulting in carbohydrate starvation, chlorophyll degradation, and leaf senescence. Previously, our studies demonstrated that SUB1A restricts carbohydrate consumption and chlorophyll breakdown through regulation of ethylene production and gibberellin responsiveness during submergence (Fukao et al., 2006; Fukao and Bailey-Serres, 2008). To further dissect the role of SUB1A in adaptation to the complex stress, we investigated physiological and molecular responses of vegetative-stage plants to prolonged darkness, which also provokes common environmental alterations to plants.

An early physiological response to constant darkness is a reduction in carbohydrate reserves of aerial tissue. We observed the abundance of starch, glucose, fructose, and sucrose quickly decreased after 1 d of dark treatment (Fig. 4), followed by chlorophyll degradation and anthocyanin accumulation after 6 d of stress (Fig. 3) in all four genotypes. However, the decline in carbohydrate reserves and chlorophylls was significantly dampened in genotypes carrying SUB1A as compared to non-SUB1A lines (Fig. 3A and 4), consistent with the observations during submergence (Fukao et al., 2006, Barding et al., 2012). Notably, the major soluble carbohydrates were constitutively higher in aerial tissue of Ubi:SUB1A plants even under normal growth conditions, presumably due to SUB1A-mediated restriction of carbohydrate catabolism, since CO₂ assimilation by photosynthesis was not altered by overexpression of SUB1A (Fig. 3C and 4). Photosynthesis and carbohydrate catabolism are regulated by light/dark transitions and the
circadian clock (Lu et al., 2005; Farré et al., 2012). In Arabidopsis, levels of starch are most abundant at the end of the light cycle, and decline significantly over the course of each night to fuel rapid growth (Graf et al., 2010). If the night is prolonged or the central oscillator of the circadian clock (CCA1/LHY4) is disrupted, starch is consumed more rapidly during the night to the detriment of overall biomass of the plant. Previously, we monitored the level of SUB1A mRNA in aerial tissue of M202(Sub1) every 3 h for 24 h, but its abundance was unaltered by the light/dark transition (Peña-Castro et al., 2011), indicating that SUB1A may not be regulated by the depletion of carbohydrate reserves during the anticipated nighttime. However, SUB1A mRNA levels rose dramatically (>90-fold) by 24 h of darkness initiated at mid-day, correlating with a rapid decline in carbohydrate reserves (Figure 1 and 4). This pronounced but transient accumulation of SUB1A mRNA could be due to the decline of carbohydrate reserves beyond that which occurs overnight. Overall, our results indicate that dark-induced SUB1A enables more conservative carbon use to prolong maintenance of cellular homeostasis under conditions of prolonged darkness or submergence.

The catabolism of chlorophyll and chloroplast proteins is actively regulated during natural leaf senescence, which remobilizes nutrients or energy resources to storage organs or seeds (Lim et al., 2007). It is feasible that this genetically-coordinated process is also a part of acclimation response to prolonged darkness and submergence. However, excessive breakdown of chlorophyll and chloroplast proteins prevents the recommencement of photosynthesis during recovery. Here, we show that the induction of two chlorophyll catabolic enzyme genes, NYC1 and RCCR1, is significantly reduced by SUB1A during constant darkness, especially at the latter time points (Fig. 5). Recently, it was shown that direct or indirect interaction of SGR with NYC1 and RCCR is necessary for recruitment of these enzymes into senescing chloroplasts (Sakuraba, 2012). It seems that SUB1A-dependent regulation of the key regulators for chlorophyll degradation limits catabolism of chlorophyll and chloroplast proteins under stress conditions. Consistent with this, our previous 1H-NMR spectrometry study of metabolites demonstrated that SUB1A represses accumulation of nine amino acids, which were elevated during submergence (Barding et al., 2012). Here we found that genotypes carrying SUB1A better retained chlorophyll content during constant darkness and recovered photosynthetic activity more quickly after re-illumination (Fig. 3C). Despite the impediment of gas exchange and light availability, the degree of underwater photosynthesis influences survival of terrestrial wetland plants including rice
(Colmer et al., 2011). It follows that maintenance of chlorophylls in SUB1A genotypes may benefit photosynthetic energy production during submergence stress and upon recovery.

We demonstrated that transcript accumulation of representative SAGs was significantly restricted during constant darkness in genotypes carrying SUB1A, indicating that SUB1A functions as a repressor of these senescence-inducible genes (Fig. 5). By contrast, a negative regulator of leaf senescence, DOS, was further induced in the presence of SUB1A during prolonged darkness. This gene was also induced by submergence, with higher level in M202(Sub1) than M202 (Jung et al., 2010; Mustroph et al., 2010). DOS encodes a nuclear-localized CCCH-type zinc finger protein which regulates expression of a subset of genes associated with JA biosynthesis and signaling in rice (Kong et al., 2006). Genetic analysis revealed that responsiveness to JA is elevated in DOS knockdown lines, but repressed in DOS overexpression lines. Thus, DOS acts upstream of the JA pathway to restrain JA-dependent leaf senescence. In accordance with the observations in DOS overexpression lines, SUB1A-mediated constitutive accumulation of DOS mRNA displayed diminished responsiveness to JA as well as postponed dark and JA-induced leaf senescence (Fig. 7B and 8). It seems that SUB1A potentiates DOS up-regulation to dampen responsiveness to JA, thereby limiting JA-promoted senescence. Further investigation will be required to elucidate the regulatory mechanism of DOS mRNA accumulation governed by SUB1A.

In Arabidopsis, SA is specifically involved in age-induced but not dark- or starvation-induced senescence. Microarray analysis revealed that a subset of genes down-regulated in a SA defective transgenic line are induced in age-dependent senescence, but not in dark- and starvation-induced senescence in Arabidopsis (Buchanan-Wollaston et al., 2005). Additionally, age-dependent senescence was significantly delayed in the SA defective line, but dark-induced senescence occurred similarly in wild type and the transgenic line. Based on our results, SA may be important in dark-induced senescence in rice. Here, we show that constitutive expression of SUB1A restricts accumulation of mRNAs induced by SA and represses SA-mediated inhibition of shoot elongation (Fig. 7C), indicating that responsiveness to SA is down-regulated by constitutive expression of SUB1A. Previously, we reported a marked delay of seed maturation as well as vegetative growth and flowering in the two independent SUB1A overexpression lines (Fukao and Bailey-Serres, 2008). Thus, SUB1A-dependent reduction of SA responsiveness may also delay of seed maturation, a developmental senescence process.
Previously, we reported that submergence-induced \textit{SUB1A} down-regulates underwater ethylene production and accumulation of ethylene-inducible genes which are associated with elongation and anaerobic metabolism (Fukao et al., 2006). We found that \textit{SUB1A} also diminishes the induction of ethylene production during constant darkness in both M202(\textit{Sub1}) and \textit{Ubi:SUB1A} (Fig. 8A). Overexpression of \textit{SUB1A} also repressed dark- and JA-induced senescence promoted by application of ACC (Fig. 8B and 8C). Thus, ethylene modulates GA-mediated processes during submergence (Fukao and Bailey-Serres, 2008), and ethylene stimulates JA-mediated process which promote stress-induced senescence. Both of these ethylene-induced responses promote consumption of energy reserves and are inhibited by \textit{SUB1A}. Identification of direct targets of \textit{SUB1A} and functional characterization of the downstream ramifications will aid elucidation of the integrated molecular mechanisms underlying \textit{SUB1A}-mediated hormonal regulation conferring multiple stress tolerance to rice.

\textbf{CONCLUSION}

Plants encounter similar physiological alterations under submergence and constant darkness, such as extensive catabolism of carbohydrate reserves, breakdown of chlorophyll, and recycling of chloroplast proteins. These acclimation responses provide nutrient resources under the conditions where energy production through photosynthesis is limited or not available. However, prolonged exposure to the stress eventually exhausts energy resources and causes death. The present study demonstrated that the key regulator of submergence tolerance, \textit{SUB1A}, coordinates physiological and molecular responses to prolonged darkness, resulting in enhanced survival of the stress. Prolonged darkness places constraints on carbohydrate availability for growth and stimulates accumulation of ethylene and JA (Lim et al., 2007; Seltmann et al., 2010). Ethylene accelerates leaf senescence mediated by JA. In the genotype with an endogenous \textit{SUB1A}, ethylene induces \textit{SUB1A} mRNA accumulation, however, which restricts ethylene production. As a result, JA-mediated senescence responses are restrained, enhancing survival of prolonged darkness. Stress-induced senescence occurs as a consequence of prolonged exposure to a variety of biotic and abiotic stresses including submergence and drought. Further investigation of regulatory mechanism underlying stress-induced senescence may provide additional strategies to improve the resilience of crops to the extremes in weather associated with climate change. A question raised by these studies is whether or not the influence of \textit{SUB1A} on JA and SA
responsiveness associated with senescence may affect innate immunity to pathogens. To date, there is no evidence that Sub1 rice varieties grown in farmers’ fields are altered in pathogen resistance relative to non-Sub1 control lines (D. Mackill and Abdelbagi Ismail, personal communication).

MATERIALS AND METHODS

Plant materials
Rice (Oryza sativa L.) cv. M202 (SUB1B-2, SUB1C-2), cv. Liaogeng (LG) (SUB1B-2, SUB1C-2), the SUB1 introgression line M202(Sub1) (SUB1A-1, SUB1B-1, SUB1C-1), and the SUB1A overexpression line Ubi:SUB1A-3 (SUB1A-1, SUB1B-2, SUB1C-2) were used in this study (Xu et al., 2006). Ubi:SUB1A-3 is one of two well-characterized representative transgenic lines with constitutively expressed SUB1A-1 in LG background, referred as to Ubi:SUB1A (Fukao and Bailey-Serres, 2008; Fukao et al., 2011). Sterilized seeds were placed on wet filter paper for 3 d at 25°C in the light (50 µmol m⁻² s⁻¹) and germinated seeds were transplanted into soil-containing plastic pots (W:L:H, 10 x 10 x 10 cm). Plants were grown in a greenhouse (30°C day, 20°C night) for 14 d (M202, M202(Sub1), and LG) or 21 d (Ubi:SUB1A) under natural light conditions. Ubi:SUB1A exhibits a semi-dwarf phenotype, but all four genotypes are developmentally matched at the ages used in these analyses (Fukao and Bailey-Serres, 2008). The plants have three fully opened leaves under our growth conditions. All experiments were performed at this developmental age unless otherwise indicated.

Dark and hormone treatments
All dark and hormone treatments were replicated in at least three independent experiments. For dark treatment, 14- or 21-d-old plants were transferred to a growth chamber at midday and subjected to complete darkness at 25 °C for up to 18 d. Aerial tissue was harvested at midday on the day specified under green light in the darkroom to avoid a light response. For MeJA treatment, the entire aerial tissue was excised at the base of the stem and immediately placed into 20 ml of mock (0.1% (v/v) DMSO) or MeJA solution (5 or 50 µM in 1% (v/v) DMSO) in a 250 ml glass beaker for 24 h in the light (50 µmol m⁻² s⁻¹). For salicylic acid treatment, deionized water and salicylic acid (1 or 20 mM) were used as mock and hormone solutions, respectively. After each treatment, collected tissue was immediately frozen in liquid nitrogen and stored in -
80°C until use. To observe the effect of darkness and hormones on leaf senescence, the fully expanded uppermost leaves were cut into pieces (8 mm length) and the leaf segments were floated on a half-strength MS medium in the dark or containing hormone solution in the light (50 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)) at 25 °C for up to 9 d. To monitor hormonal effect on seedling growth, sterilized seeds are incubated on wet filter paper containing MeJA (1, 5, 25 \( \mu \text{M} \)) or Salicylic acid (5, 10, 25 mM) at 25 °C in the light (50 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)) and the length of each shoot was recorded after 6 d.

**Chlorophyll and anthocyanin assays**

Chlorophyll \( a \) and \( b \) contents were quantified from 50 mg of tissue in 5 mL of 100% methanol as described in Porra (2002). After centrifugation at 4 °C for 20 min at 16,800 g, the absorbance of the supernatant was measured at 652.0 and 665.2 nm with a spectrophotometer (DU800, Beckman). Anthocyanin content was assayed following the method of Jeong et al. (2010). Aerial tissue (50 mg) was homogenized in 600 µL of 1% (v/v) HCl in methanol on ice and then incubated for 16 h at 4 °C in the dark with gentle shaking. After incubation, the extract was mixed with 400 µL water and 400 µL chloroform. Following centrifugation at 4 °C for 2 min at 16,800g, \( A_{530} \) and \( A_{657} \) was measured.

**Carbohydrate assays**

Glucose, fructose, and sucrose contents were measured by the method of Guglielminetti et al. (1995). Aerial tissue (50 mg) was homogenized in 1 ml of 80% (v/v) ethanol and incubated at 80 °C for 20 min. Following centrifugation for 10 min at 16,800g, the supernatant was collected and the extraction process repeated twice, with the three tissue extracts combined and dried under a vacuum. After rehydration in 0.5 mL water, the samples were subjected to coupled enzymatic methods with a spectrophotometer. Glucose was assayed in a reaction mixture (1 mL) containing 50 \( \mu \text{L} \) extract, 100 mM Tris-HCl (pH 7.6), 8 mM MgCl\(_2\), 1 mM ATP, 1.5 mM NADP, 1 unit hexokinase (Sigma-Aldrich), and 1 unit glucose-6-phosphate dehydrogenase (Sigma-Aldrich). The mixture was incubated at 37 °C for 30 min and the increase in \( A_{340} \) was measured. For fructose, 6 units of phosphoglucone isomerase (Sigma-Aldrich) were added to the glucose assay mixture. For sucrose, a reaction mixture (100 \( \mu \text{L} \)) containing the extract (50 \( \mu \text{L} \)), 15 mM sodium acetate (pH 4.6), and 40 units of invertase (Sigma-Aldrich) was incubated at 37 °C for 15 min.
and the resulting glucose was quantified as described above. Starch content was measured following the method of Fukao et al. (2006). The pellet obtained after ethanol extraction was washed with water, re-suspended in 1 mL water containing 10 units of heat-resistant α-amylase (Sigma-Aldrich), and incubated at 95 °C for 15 min. After cooling, the suspension was adjusted to 25 mM sodium citrate (pH 4.8) and 5 units of amyloglucosidase (Sigma-Aldrich) were added. Following incubation at 55 °C for 30 min, the reaction mixture was centrifuged at 16,800 g for 30 min and the supernatant was subjected to glucose assay as described above. The reaction efficiency of each method was validated by analyzing known amount of each carbohydrate.

**Photosynthetic activity measurement**

To analyze photosynthetic light response, maximum rates of net CO₂ assimilation was quantified with a portable photosynthesis analysis system (Model 6400; Li-Cor) equipped with a red-blue light source (Model 6400-02B, no. SI-710; Li-Cor) as described in Santiago (2007). Uppermost expanded leaves of eight plants were measured at 370 µmol mol⁻¹ CO₂ and 1200 µmol m⁻² s⁻¹ irradiance at noon. Since plants treated with prolonged darkness required recovery in the light to produce a detectable amount of CO₂, plants were placed in the light (50 µmol m⁻² s⁻¹) at 25 °C for 2-24 h. Following photosynthetic measurements, leaf area was recorded and used for standardization.

**Quantitative RT-PCR**

Total RNA was extracted from frozen aerial tissue using the RNeasy Plant mini kit (Qiagen). Genomic DNA was removed by the on-column digestion method described in the manufacture protocol. cDNA was synthesized from 2 µg total RNA following the method of Fukao et al. (2006). Real-time PCR was performed in a 20 µL reaction using iQ SYBR Green Supermix (Bio-Rad) in CFX96 real-time PCR detection system (Bio-Rad). PCR efficiency (95-105%) was verified as described in Schmittgen and Livak (2008). Amplification specificity was validated by melt-curve analysis at the end of each PCR reaction. Relative transcript abundance was calculated using the comparative CT method (Livak and Schmittgen, 2001). ACTIN1 or α-TUBULIN was used as a normalization control. Sequences and annealing temperatures of primer pairs are listed in Supplemental Table 1 online.
Ethylene measurement

The rate of ethylene production was quantified as described in Larsen and Cancel (2004). Dehulled seeds were sterilized in 70% (v/v) ethanol for 10 min and in 2.5% (v/v) sodium hypochlorite solution for 20 min. After rinsing with sterilized water thoroughly, each seed was cultured on a half-strength of MS medium in a test tube for 9 d (M202, M202(Sub1), and LG) or 12 d (Ubi:SUB1A) (16 h light / 8 h dark; light level, 100 µmol m⁻² s⁻¹). For dark treatment, the test tubes were placed in the dark at 25 °C for up to 6 d. Following stress treatment, each tube was tightly closed with a rubber serum stopper and incubated in the dark for 2 h. The accumulated gas sample (0.9 mL) was withdrawn from each tube with a 1 mL syringe and assayed by a gas chromatograph (6850 Series; Hewlett Packard) equipped with an alumina-based capillary column (Agilent Technologies).

Accession Numbers

Sequence data from this article can be found in the Michigan State University Rice Genome Annotation Project database (http://rice.plantbiology.msu.edu) under the following accession numbers: Actin1 (LOC_Os03g50890), SUB1A (DQ011598b*), α-tubulin (LOC_Os07g38730) SUB1B (LOC_Os09g11480), SUB1C (LOC_Os09g11460), SGR (LOC_Os09g36200), RCCR1 (LOC_Os10g25030), NYC1 (LOC_Os01g12710), NYC3 (LOC_Os06g24730), Osll85 (LOC_Os07g34520), DOS (LOC_Os01g09620). To obtain GenBank sequences, which are linked to RAP identification numbers, please see http://rapdb.dna.affrc.go.jp/tools/converter to convert from MSU identification numbers (LOC_Os00g00000) to Rice Annotation Project identification numbers (Os00g00000). * GenBank/EMBL accession number; this gene is absent from Nipponbare and therefore has no LOC number.

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FIGURE LEGENDS

Figure 1. Relative levels of SUB1 gene transcripts in aerial tissue under prolonged darkness. Developmentally matched 14-d-old (M202, M202(Sub1), and LG) and 21-d-old (Ubi:SUB1A) plants were exposed to complete darkness for up to 12 d and aerial tissue was analyzed by quantitative RT-PCR using gene specific primers. Relative level of each mRNA was calculated by comparison to the non-stressed control (M202(Sub1) or Ubi:SUB1A at day 0 for SUB1A; M202 or LG at day 0 for SUB1B and SUB1C). The data represent means ± SE from three biological replicates. Asterisks indicate significant difference between the two genotypes (*P < 0.05; **P < 0.01).

Figure 2. SUB1A enhances tolerance to prolonged darkness. A, Photos of M202, M202(Sub1), LG, and Ubi:SUB1A plants exposed to complete darkness for up to 18 d and recovered under regular growth conditions for 14 d. B, Viability of plants treated with 18 d of complete darkness. The survival of each genotype was evaluated 14 d after recovery. Plants were counted as viable if new leaves appeared during recovery. The data represent means ± SD from three biological replicates (n = 75). Asterisks indicate significant difference between the two genotypes (P < 0.01).

Figure 3. SUB1A restricts leaf senescence and maintains photosynthesis capability after prolonged darkness. A, Chlorophyll contents in aerial tissue during prolonged darkness. The levels of chlorophyll a and b were analyzed in aerial tissue of plants exposed to complete darkness. The data represent means ± SD (n = 3). B, Anthocyanin contents in aerial tissue during...
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Figure 4. Carbohydrate contents in aerial tissue during prolonged darkness. The levels of starch (A), glucose (B), fructose (C), and sucrose (D) were monitored in aerial tissue of plants treated with complete darkness for up to 12 d. The content of starch was quantified after digestion into glucose. The data represent means ± SD (n=3) and asterisks indicate significant difference between the two genotypes (*P < 0.05; **P < 0.01). FW; fresh weight.

Figure 5. Relative mRNA levels of genes associated with leaf senescence. Transcripts of representative genes were quantified in aerial tissue of plants exposed to complete darkness by quantitative RT-PCR. Relative level of each mRNA was calculated by comparison to non-stressed M202 or LG. The data represent means ± SE from three biological replicates. Asterisks indicate significant difference between the two genotypes (*P < 0.05; **P < 0.01).

Figure 6. Relative transcript levels of SUB1 genes following MeJA or SA treatment. The levels of SUB1A, SUB1B, and SUB1C mRNAs were compared between (A) M202 vs. M202(Sub1) and (B) LG vs. Ubi:SUB1A plants treated with MeJA or SA by quantitative RT-PCR. Relative level of each mRNA in aerial tissue was calculated by comparison to the non-stressed control (M202(Sub1) or Ubi:SUB1A at day 0 for SUB1A; M202 or LG at day 0 for SUB1B and SUB1C). The data represent means ± SE from three biological replicates. Asterisks indicate significant difference between the two genotypes (*P < 0.05; **P < 0.01).

Figure 7. Constitutive expression of SUB1A diminishes responsiveness to MeJA and SA. A, C, Relative mRNA levels of senescence-regulated genes following MeJA (A) or SA (C) treatment. The transcript levels of representative genes were monitored in aerial tissue of plants treated with
MeJA or SA by quantitative RT-PCR. Relative level of each mRNA was calculated by comparison to non-stressed LG. The data represent means ± SE from three biological replicates. Asterisks indicate significant difference between the two genotypes (*P < 0.05; **P < 0.01). B, D, MeJA (B) or SA (D)-inhibited shoot elongation in germinating seeds. Seeds were incubated on wet filter paper containing a series of MeJA or SA solutions for 6 d. Relative shoot elongation was calculated by comparison to the non-treated seeds of individual genotypes. The data represent means ± SE (n = 25) and asterisks indicate significant difference between the two genotypes (*P < 0.05; **P < 0.01).

Figure 8. SUB1A limits dark- and MeJA-induced senescence promoted by ethylene. A, Ethylene evolution during dark treatment. Developmentally matched nine-day-old (M202, M202(Sub1) and LG) or 12-d-old (Ubi:SUB1A) plants grown in test tubes were exposed to complete darkness for up to 6 d. Following treatment, ethylene accumulated in the test tube was quantified by gas chromatography. The data represent means ± SD (n=5) and asterisks indicate significant difference between the two genotypes **P < 0.01). B, C, D, Photos of leaf segments treated with prolonged darkness (B), MeJA (C), or SA (D) with and without 1-aminocyclopropane-1-carboxylic-acid (ACC). Leaf segments of uppermost leaves in 14-d-old (M202, M202(Sub1), and LG) and 21-d-old (Ubi:SUB1A) plants were incubated on half-strength MS medium containing hormone solution with and without ACC (100 µM) for up to 9 d (n = 10).
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Figure 8

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