The Submergence Tolerance Gene *SUB1A* Delays Leaf Senescence under Prolonged Darkness through Hormonal Regulation in Rice[1][W][OA]

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Leaf senescence is a natural age-dependent process that is induced prematurely by various environmental stresses. Typical alterations during leaf senescence include breakdown of chlorophyll, 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, *SUBMERGENCE1A* (*SUB1A*), in the acclimation responses during leaf senescence caused by prolonged darkness in rice (*Oryza sativa*). *SUB1A* messenger RNA 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 the maintenance of chlorophyll and carbohydrate reserves in photosynthetic tissue. This delay allowed leaves of *SUB1A* genotypes to recover photosynthetic activity more quickly upon reexposure to light. *SUB1A* also restricted the 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, although *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 the breakdown of chlorophyll, carbohydrates, and the accumulation of senescence-associated messenger RNAs. A delay of leaf senescence conferred by *SUB1A* can contribute to the enhancement of tolerance to submergence, drought, and oxidative stress.

Leaf senescence is a natural developmental process that 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 that 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 (*Arabidopsis thaliana*) and rice (*Oryza sativa*; 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 downregulated in senescing leaves, whereas genes responsible for macromolecule catabolism and transport are upregulated. 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 Suc 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*, *ACC OXIDASE*, *ETHYLENE INSENSITIVE3* (*EIN3*), and *ETHYLENE RESPONSIVE FACTOR1a* (*ERF1a*), are

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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 the ethylene-insensitive mutants *erecta*, *ethylene receptor1*, and *ein3*, whereas the ethylene-hypersensitive mutants *hyperrecombination protein1* and *enhanced disease resistance1* 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 important roles 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 the 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 *coronatine insensitive1, decanamide resistant root1, jagged and wavy-D, mitogen-activated protein kinase6*, and *mapk kinase9*, 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 RNA interference-knockdown lines of *lipoxygenase2* (Seltmann 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 in mature green leaves in Arabidopsis, and the levels 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 the down-regulation of a subset of *SAGs* in SA-defective transgenic *Naphthalene hydroxylase G* (Buchanan-Wollaston et al., 2005). Low-light- and salinity-induced senescence is accelerated in the Arabidopsis *senescence-associated ubiquitin ligase1* (*saul1*) mutant that overaccumulates SA and *SAG* transcripts under the stress (Vogelmann et al., 2012). An early-senescence phenotype in the mutant was rescued in the SA-deficient *phytoalexin deficient4* background, indicating that SA is necessary for *saul1*-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 to 7 d 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* spp. (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 *SUBMERGENCE1A* (*SUB1A*), an ERF domain-containing transcription factor. Only limited rice cultivars possess the *SUB1A* gene, and more specifically the *SUB1A-1* allele, which is highly induced during submergence (Fukao et al., 2006; Xu 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 desubmergence. 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 the 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 the activation of reactive oxygen species detoxification and the 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 the expression of SAGs, the breakdown of chlorophyll, and the 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 the 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, nontransgenic Liaogeng (LG), and the *SUB1A* overexpression line *Ubi*:*SUB1A* (Fig. 1). Our quantitative reverse transcription (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 induction was significantly lower for these genes than *SUB1A*. In the *SUB1A* overexpression line, *SUB1A* mRNA constitutively accumulated under nonstressed conditions, but exposure to prolonged darkness decreased the level of the transcript. Consistent with the observations 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).

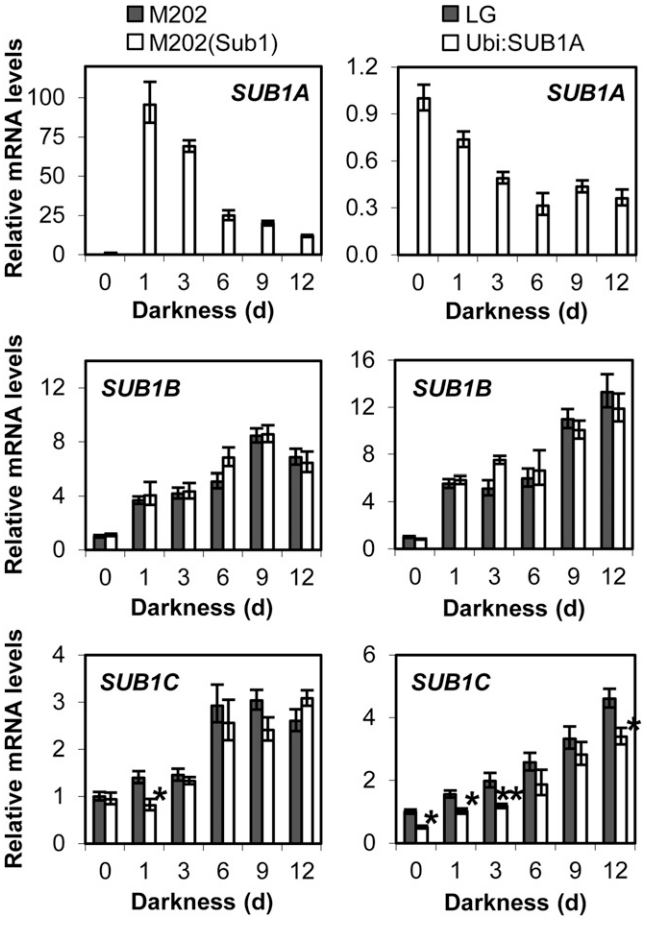

Figure 1. Relative levels of *SUB1* gene transcripts in aerial tissue under prolonged darkness. Developmentally matched 14-d-old [M202, M202(*Sub1*), and LG] and 21-d-old (*Ubi*:*SUB1A*) plants were exposed to complete darkness for up to 12 d, and aerial tissue was analyzed by qRT-PCR using gene-specific primers. The relative level of each mRNA was calculated by comparison with the nonstressed 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 differences between the two genotypes (*$P < 0.05$, **$P < 0.01$).

SUB1A Enhances Survival of Prolonged Darkness through the Maintenance of Chlorophyll and Carbohydrate Reserves in Aerial Tissue

Prolonged darkness discontinues energy conversion by photosynthesis, resulting in an 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 with 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, 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).

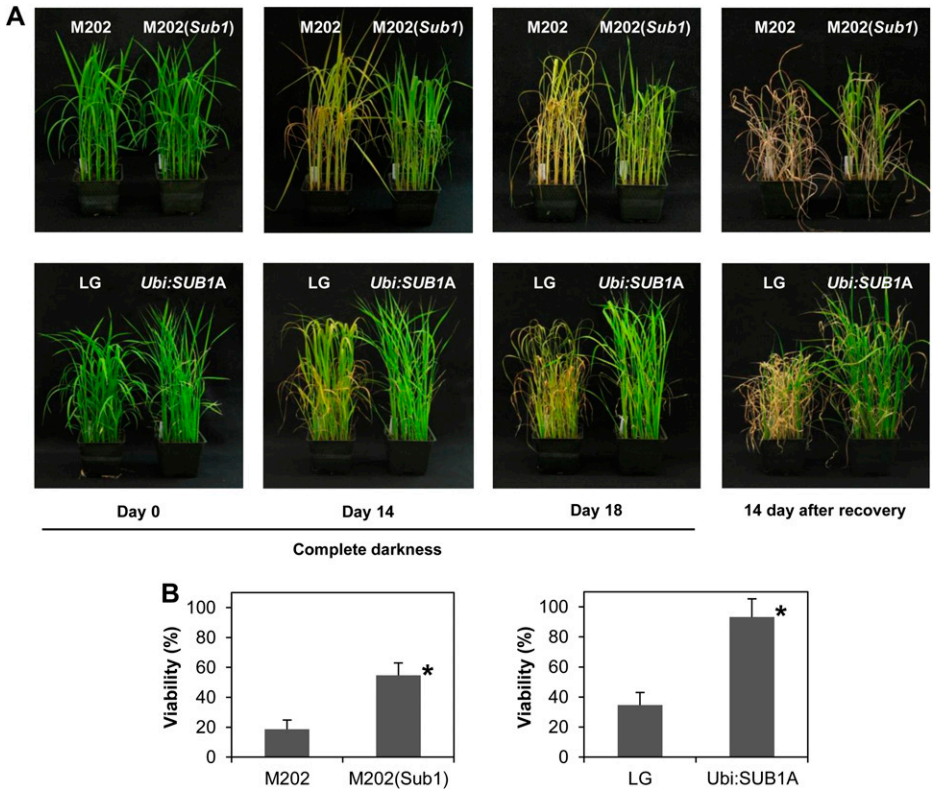

Figure 2. *SUB1A* enhances tolerance to prolonged darkness. A, Photographs of M202, M202(*Sub1*), LG, and *Ubi*:*SUB1A* plants exposed to complete darkness for up to 18 d and recovered under regular growth conditions for 14 d. B, Viability of plants treated with 18 d of complete darkness. The survival 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 differences between the two genotypes ($P < 0.01$).

To determine whether *SUB1A* affects photosynthetic activity after prolonged darkness, CO_2 assimilation by photosynthesis was monitored in dark-treated plants (Fig. 3C). There was no significant difference in photosynthetic activity between M202 versus M202(*Sub1*) and LG versus *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 CO_2 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 a resumption of photosynthesis following a sublethal period of prolonged darkness.

Under conditions in which carbohydrate production through photosynthesis is unavailable, proper management of carbohydrate reserves is key for survival. To evaluate the influence of *SUB1A* in carbohydrate consumption during prolonged darkness, the amount of starch, Glc, Fru, and Suc was monitored in aerial tissue (Fig. 4). Before stress treatment, the levels of starch, Glc, Fru, and Suc 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 Glc, Fru, and Suc under nonstress conditions. Complete darkness rapidly decreased the abundance of carbohydrates in all genotypes, but M202 (*Sub1*) and *Ubi*:*SUB1A* plants maintained more starch, Glc, Fru, and Suc 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 SAGs Is 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 that is necessary for chlorophyll degradation in light-harvesting complex II in Arabidopsis and rice (Jiang et al., 2007; Park et al., 2007; Hörtensteiner, 2009). Red Chlorophyll Catabolite Reductase (RCCR) functions at the last step of

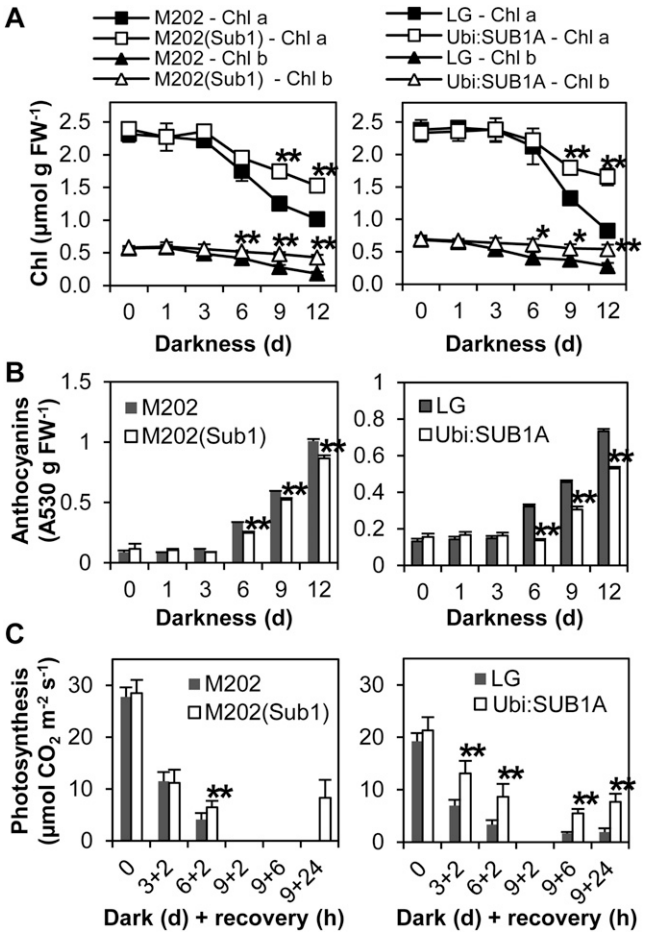

Figure 3. *SUB1A* restricts leaf senescence and maintains photosynthesis capability after prolonged darkness. A, Chlorophyll contents in aerial tissue during prolonged darkness. The levels of chlorophyll *a* and *b* were analyzed in aerial tissue of plants exposed to complete darkness. The data represent means ± SD ($n = 3$). FW, Fresh weight. B, Anthocyanin contents in aerial tissue during prolonged darkness. The level of anthocyanin was monitored in aerial tissue of plants exposed to complete darkness for 12 d. The data represent means ± SD ($n = 3$). C, Photosynthesis capability of plants treated with prolonged darkness. Photosynthetic assimilation of CO_2 was measured in the uppermost leaves of plants exposed to complete darkness. After dark treatment, recovery under light conditions (50 μmol m^{-2} s^{-1}) for more than 2 h was necessary to detect stable CO_2 assimilation. The data represent means ± SD ($n = 8$). Asterisks indicate significant differences between the two genotypes (*$P < 0.05$, **$P < 0.01$).

chlorophyll degradation in chloroplasts (Pruzinská 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 COLORING1* (*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. *Osl85* 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 the 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,

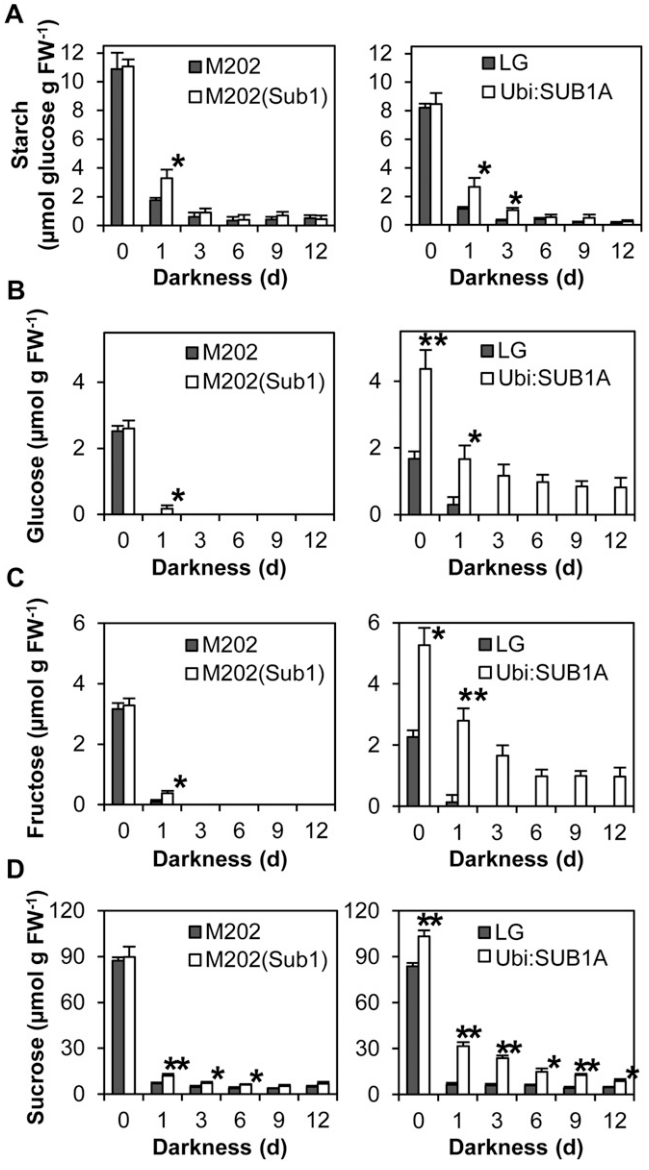

Figure 4. Carbohydrate contents in aerial tissue during prolonged darkness. The levels of starch (A), Glc (B), Fru (C), and Suc (D) were monitored in aerial tissue of plants treated with complete darkness for up to 12 d. The content of starch was quantified after digestion into Glc. The data represent means ± SD ($n = 3$). Asterisks indicate significant differences between the two genotypes (*$P < 0.05$, **$P < 0.01$). FW, Fresh weight.

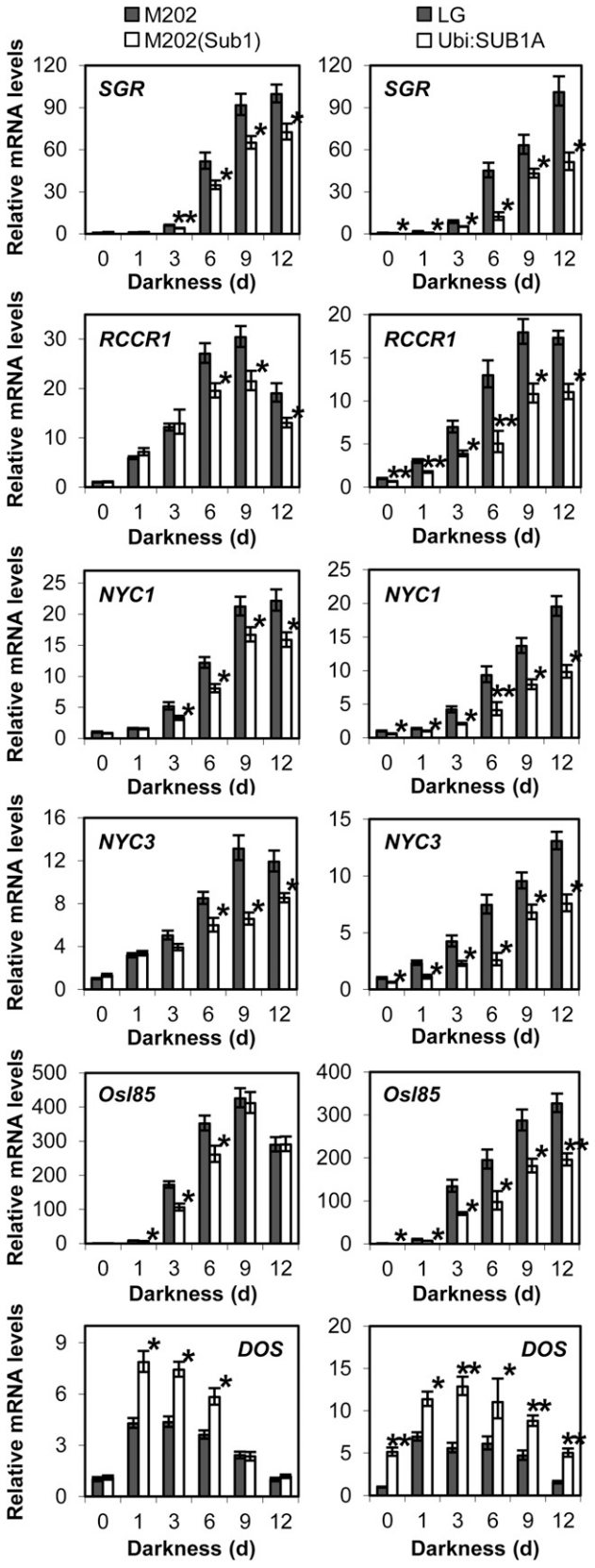

Figure 5. Relative mRNA levels of genes associated with leaf senescence. Transcripts of representative genes were quantified in aerial tissue of plants exposed to complete darkness by qRT-PCR. The relative level of each mRNA was calculated by comparison with nonstressed M202 or LG. The data represent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

the overexpression of *SUB1A* constitutively restricted the 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 nucleus-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*. These data demonstrate that *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, 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 the 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* 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 the 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

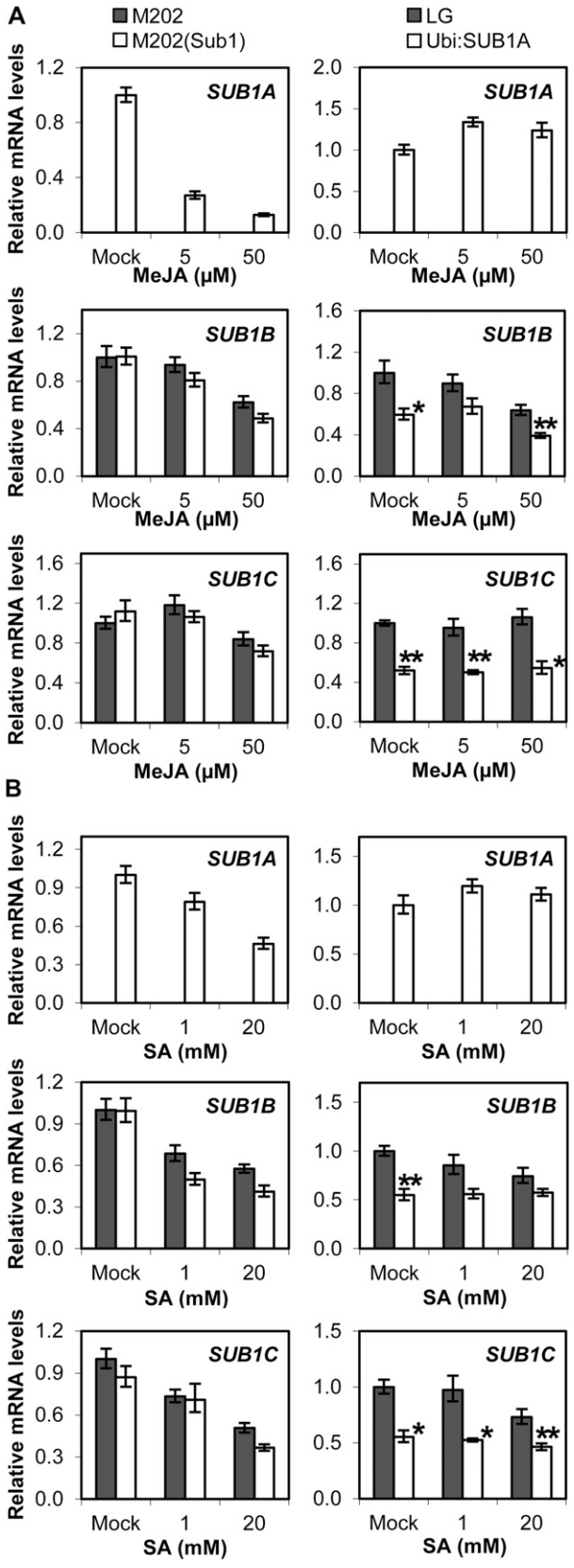

Figure 6. Relative transcript levels of *SUB1* genes following MeJA or SA treatment. The levels of *SUB1A*, *SUB1B*, and *SUB1C* mRNAs were compared between M202 versus M202(Sub1) (A) and LG versus *Ubi*:*SUB1A* (B) plants treated with MeJA or SA by qRT-PCR. The relative level of each mRNA in aerial tissue was calculated by comparison with the nonstressed 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 differences between the two genotypes (*$P < 0.05$, **$P < 0.01$).

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 the 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 the 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 (Bleecker and Kende, 2000; Lim et al., 2007). 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 one-half-strength Murashige and Skoog (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

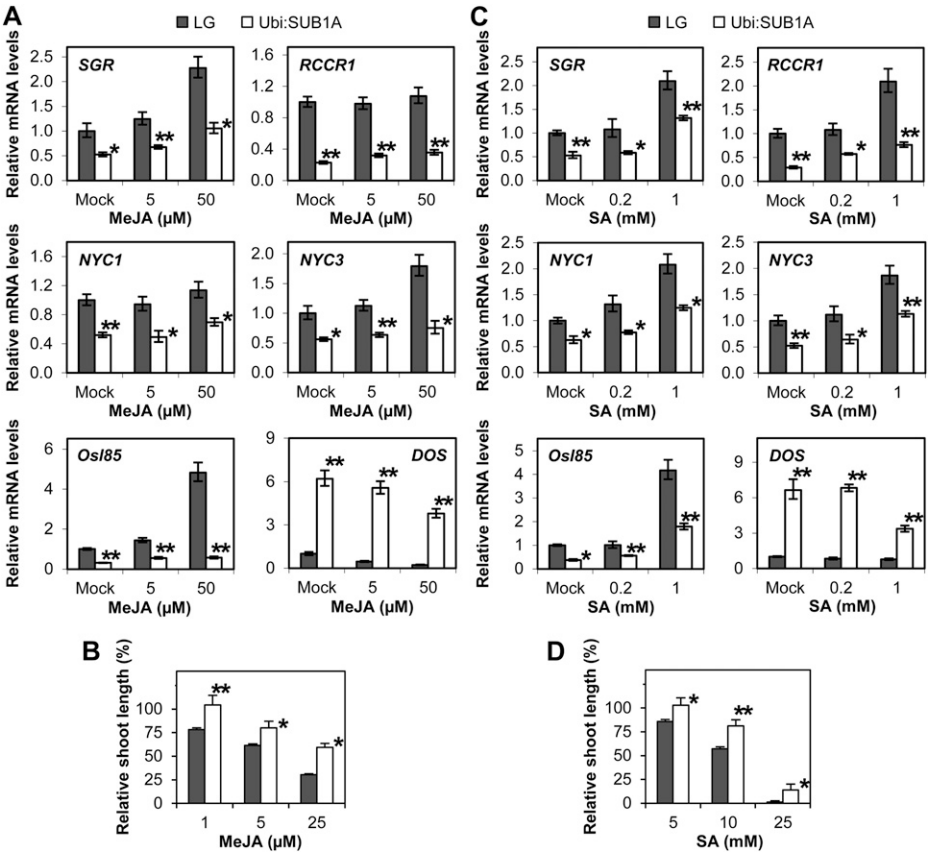

Figure 7. Constitutive expression of *SUB1A* diminishes responsiveness to MeJA and SA. A and C, Relative mRNA levels of senescence-regulated genes following MeJA (A) or SA (C) treatment. The transcript levels of representative genes were monitored in aerial tissue of plants treated with MeJA or SA by qRT-PCR. The relative level of each mRNA was calculated by comparison with nonstressed LG. The data represent means ± SE from three biological replicates. Asterisks indicate significant differences between the two genotypes (*$P < 0.05$, **$P < 0.01$). B and D, MeJA-inhibited (B) or SA-inhibited (D) 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 with the nontreated seeds of individual genotypes. The data represent means ± SE ($n = 25$). Asterisks indicate significant differences between the two genotypes (*$P < 0.05$, **$P < 0.01$).

SUB1A, leaf color was unchanged after 9 d, consistent with the observation at the whole-plant level (Fig. 2A). The 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 the two hormones reduced rather than increased basal *SUB1A* mRNA levels (Fig. 6). 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 the 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 in *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 the 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 oxygen, CO_2, 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 an 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 the 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 that the abundance of starch, Glc, Fru, and Suc 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 with non-*SUB1A* lines (Figs. 3A and 4), consistent with the observations during submergence (Fukao et al., 2006;

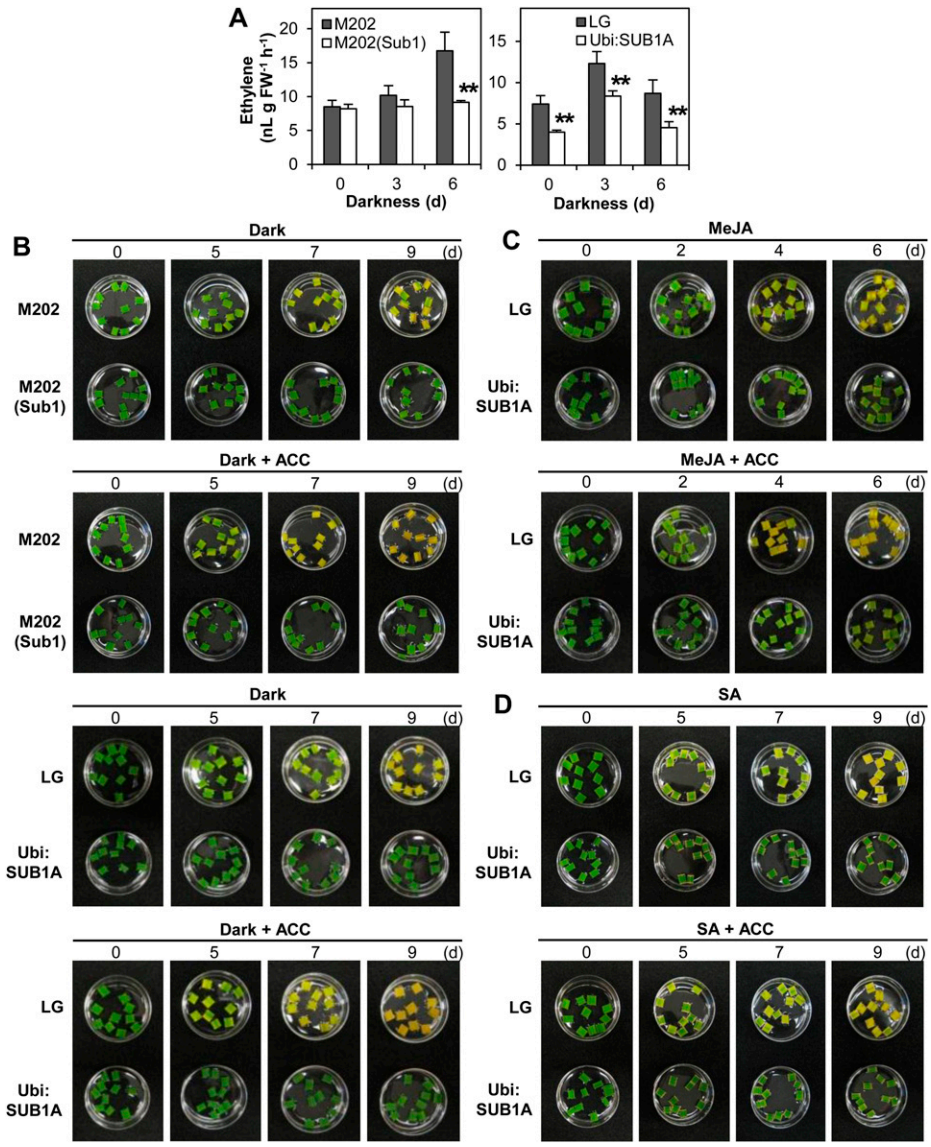

Figure 8. *SUB1A* limits dark- and MeJA-induced senescence promoted by ethylene. A, Ethylene evolution during dark treatment. Developmentally matched 9-day-old [M202, M202(*Sub1*), and LG] or 12-d-old (*Ubi*:*SUB1A*) plants grown in test tubes were exposed to complete darkness for up to 6 d. Following treatment, ethylene accumulated in the test tube was quantified by gas chromatography. The data represent means ± SD ($n = 5$). Asterisks indicate significant differences between the two genotypes (**$P < 0.01$). FW, Fresh weight. B to D, Photographs of leaf segments treated with prolonged darkness (B), MeJA (C), or SA (D) with and without 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 one-half-strength MS medium containing hormone solution with and without ACC (100 μM) for up to 9 d ($n = 10$).

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 CO_2 assimilation by photosynthesis was not altered by the overexpression of *SUB1A* (Figs. 3C and 4). Photosynthesis and carbohydrate catabolism are regulated by light/dark transitions and the circadian clock (Lu et al., 2005; Farré and Weise, 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 (*CIRCADIAN CLOCK ASSOCIATED1/LATE ELONGATED HYPOCOTYL4*) is disrupted, starch is consumed more rapidly during the night, to the detriment of the 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 (more than 90-fold) by 24 h of darkness initiated at midday, correlating with a rapid decline in carbohydrate reserves (Figs. 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 the 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 part of the 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 later 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 et al., 2012). It seems that *SUB1A*-dependent regulation of the key regulators for chlorophyll degradation limits the catabolism of chlorophyll and chloroplast proteins under stress conditions. Consistent with this, our previous ^{1}H-NMR spectrometry study of metabolites demonstrated that *SUB1A* represses the 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 reillumination (Fig. 3C). Despite the impediment of gas exchange and light availability, the degree of underwater photosynthesis influences the survival of terrestrial wetland plants, including rice (Colmer et al., 2011). It follows that the 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 a higher level in M202(*Sub1*) than in M202 (Jung et al., 2010; Mustroph et al., 2010). *DOS* encodes a nucleus-localized CCCH-type zinc finger protein that regulates the 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 (Figs. 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 the 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 the 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 seed maturation, a developmental senescence process.

Previously, we reported that submergence-induced *SUB1A* down-regulates underwater ethylene production and the accumulation of ethylene-inducible genes that are associated with elongation and anaerobic metabolism (Fukao et al., 2006). We found that *SUB1A* also diminishes the induction of ethylene production during constant darkness in both M202(*Sub1*) and *Ubi*:*SUB1A* (Fig. 8A). Overexpression of *SUB1A* also repressed dark- and JA-induced senescence promoted by the application of ACC (Fig. 8, B and C). Thus, ethylene modulates GA-mediated processes during submergence (Fukao and Bailey-Serres, 2008), and ethylene stimulates JA-mediated processes that promote stress-induced senescence. Both of these ethylene-induced responses promote the consumption of energy reserves and are inhibited by SUB1A. Identification of direct targets of SUB1A and functional characterization of the downstream ramifications will aid elucidation of the integrated molecular mechanisms underlying *SUB1A*-mediated hormonal regulation conferring multiple stress tolerance to rice.

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 conditions where energy production through photosynthesis is limited or not available. However, prolonged exposure to the stress eventually exhausts energy resources and causes death. This study demonstrates that the key regulator of submergence tolerance, *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 the 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 *SUB1A*, ethylene induces *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 the 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 the influence of *SUB1A* on JA and SA responsiveness associated with senescence may conditionally regulate 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 near-isogenic non-Sub1 control lines (D. Mackill, personal communication).

MATERIALS AND METHODS

Plant Materials

Rice (*Oryza sativa*) lines cv M202 (*SUB1B-2*, *SUB1C-2*), cv 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 the 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 (10 × 10 × 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 semidwarf 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] dimethyl sulfoxide) or MeJA (5 or 50 μM in 1% [v/v] dimethyl sulfoxide) solution in a 250-mL glass beaker for 24 h in the light (50 μmol m^{-2} s^{-1}). For SA treatment, deionized water and SA (1 or 20 mM) were used as mock and hormone solution, respectively. After each treatment, collected tissue was immediately frozen in liquid nitrogen and stored at −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 in length), and the leaf segments were floated on one-half-strength MS medium in the dark or containing hormone solution in the light (50 μmol m^{-2} s^{-1}) at 25°C for up to 9 d. To monitor hormonal effects on seedling growth, sterilized seeds were incubated on wet filter paper containing MeJA (1, 5, and 25 μM) or SA (5, 10, and 25 mM) at 25°C in the light (50 μmol m^{-2} 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 by 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 of water and 400 μL of chloroform. Following centrifugation at 4°C for 2 min at 16,800*g*, A_{530} and A_{657} were measured.

Carbohydrate Assays

Glc, Fru, and Suc 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 16,800*g*, the supernatant was collected and the extraction process was repeated twice, with the three tissue extracts combined and dried under a vacuum. After rehydration in 0.5 mL of water, the samples were subjected to coupled enzymatic methods with a spectrophotometer. Glc was assayed in a reaction mixture (1 mL) containing 50 μL of extract, 100 mM Tris-HCl (pH 7.6), 8 mM $MgCl_2$, 1 mM ATP, 1.5 mM NADP, 1 unit of hexokinase (Sigma-Aldrich), and 1 unit of Glc-6-P dehydrogenase (Sigma-Aldrich). The mixture was incubated at 37°C for 30 min, and the increase in A_{340} was measured. For Fru, 6 units of phosphoglucose isomerase (Sigma-Aldrich) was added to the Glc assay mixture. For Suc, a reaction mixture (100 μL) containing the extract (50 μL), 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 Glc 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, resuspended in 1 mL of 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) was 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 Glc assay as described above. The reaction efficiency of each method was validated by analyzing known amounts of each carbohydrate.

Photosynthetic Activity Measurement

To analyze photosynthetic light response, maximum rates of net CO_2 assimilation were 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 by Santiago (2007). Uppermost expanded leaves of eight plants were measured at 370 μmol mol^{-1} CO_2 and 1,200 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 CO_2, plants were placed in the light (50 μmol m^{-2} s^{-1}) at 25°C for 2 to 24 h. Following photosynthetic measurements, leaf area was recorded and used for standardization.

qRT-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 manufacturer's protocol. complementaryDNA was synthesized from 2 μg of 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 the CFX96 real-time PCR detection system (Bio-Rad). PCR efficiency (95%–105%) was verified as described by Schmittgen and Livak (2008). Amplification specificity was validated by melt-curve analysis at the end of each PCR cycle. Relative transcript abundance was calculated using the comparative cycle threshold 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 S1.

Ethylene Measurement

The rate of ethylene production was quantified as described by 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 one-half-strength MS medium in a test tube for 9 d [M202, M202(*Sub1*), and LG] or 12 d (*Ubi*:*SUB1A*; 16 h of light/8 h of 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).

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 [GenBank/EMBL accession number; this gene is absent from Nipponbare and therefore has no LOC number]), α-TUBULIN (LOC_Os07g38730) SUB1B (LOC_Os09g11480), SUB1C (LOC_Os09g11460), SGR (LOC_Os09g36200), RCCR1 (LOC_Os10g25030), NYC1 (LOC_Os01g12710), NYC3 (LOC_Os06g24730), Osl85 (LOC_Os07g34520), and DOS (LOC_Os01g09620). To obtain GenBank sequences, which are linked to Rice Annotation Project identification numbers, see http://rapdb.dna.affrc.go.jp/tools/converter to convert from Michigan State University identification numbers (LOC_Os00g00000) to Rice Annotation Project identification numbers (Os00g0000000).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table S1. Primers and PCR conditions used for quantitative RT-PCR.

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