Elevated Levels of CYP94 Family Gene Expression Alleviate the Jasmonate Response and Enhance Salt Tolerance in Rice

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The plant hormone jasmonate and its conjugates (JAs) have important roles in growth control, leaf senescence and defense responses against insects and microbial attacks. JA biosynthesis is induced by several stresses, including mechanical wounding, pathogen attacks, drought, and salinity stresses. However, the roles of JAs under abiotic stress conditions are unclear. Here we report that increased expression of the Cyt P450 family gene CYP94C2b enhanced viability of rice plants under salinity conditions. This gene encodes an enzyme closely related to CYP94C1 that catalyzes conversion of bioactive jasmonate-isoleucine (JA-Ile) into 12OH-JA-Ile and 12COOH-JA-Ile. Inactivation of JA was facilitated in a rice line with enhanced CYP94C2b expression, and responses to exogenous JA and wounding were alleviated. Moreover, salt stress-induced leaf senescence but not natural senescence was delayed in the transgenic rice. These results suggest that bioactive JAs have a negative effect on viability under salt stress conditions and demonstrate that manipulating JA metabolism confers enhanced salt tolerance in rice.

Keywords: Cytochrome P450 • Jasmonate • Rice • Salt tolerance • Senescence.

Abbreviations: bHLH, basic helix–loop–helix; CK, cytokinin; CYP, cytochrome P450; EC, electrical conductivity; FOX, full-length cDNA overexpression; JA, jasmonate; JAZ, jasmonate ZIM domain; MS, Murashige and Skoog; qRT-PCR, quantitative real-time reverse transcription–PCR; SCF, Skp–Cullin–F-box complex; SAM, shoot apical meristem; SGR, STAYGREEN; UBC, ubiquitin conjugating enzyme E2; WT, wild type.

Introduction

Plants have developed various environmental adaptations because of their sessile nature. In general, intrinsic developmental programs in plants are linked to signaling from external factors, allowing growth in accordance with environmental conditions. Plant growth is sustained by continuous cell division, which occurs primarily at meristems, and subsequent cell differentiation, along with organization of tissues or organs. Both cell division and differentiation are controlled depending on environmental conditions. When plants are exposed to mild stress, cell cycles are down-regulated but sustained in co-ordination with cell differentiation (Inze and De Veylder 2006, De Veylder et al. 2007, Ogawa et al. 2011).

Developmental control in plants is also linked to sink-source balance of organs. Senescence is often induced in source tissues in response to various stresses, as exemplified by leaves, where cellular components and temporarily stored materials are degraded and serve as nutrients that are transported to sink tissues such as meristems, young developing tissues, and reproductive organs (Lim et al. 2007). Wounding and pathogen attacks induce JA biosynthesis, leading to activation of defense genes. JA production is also induced under several abiotic stress conditions, including drought, salinity and low temperature (Takeuchi et al. 2011, Hu et al. 2013). However, the roles of JAs in mechanisms that confer tolerance to environmental stresses are contentious. Exogenous application of JA suppresses salinity-dependent growth inhibition (Kang et al. 2005). Moreover, Os-bHLH148, a transcription factor induced by JA, ABA and abiotic stresses such as drought and salinity, confers drought tolerance when overexpressed (See et al. 2011). These studies suggest that JA signaling is involved in adaptation to stressful environmental conditions. In contrast, JA negatively affects plant growth by repressing cell division and cell elongation activities (Chen et al. 2011, Toda et al. 2013a). In cultured Arabidopsis cells, JA mediates transcriptional reprogramming of genes, as reflected in repression of the cell cycle, as well as the biosynthesis of phenylpropanoids (Pauwels et al. 2008). In addition, overexpression of a factor that represses the JA response confers stress tolerance. Enhanced expression of JAZ9 (jasmonate ZIM domain 9)
in rice under control of a stress-inducible promoter alleviates growth inhibition, resulting from salt and dehydration stress (Ye et al. 2009). JA response mutants of Arabidopsis, such as coi1 and jin1, also exhibit tolerance to moderate drought stress (Harb et al. 2010).

JA is converted to bioactive jasmonate-isoleucine (JA-Ile) in plant cells, which activates the JA signal transduction response pathway (Staswick and Tiryaki 2004). JA-Ile binds to a specific SCF$^\text{COI1}$, together with JAZ proteins (Katsir et al. 2008, Sheard et al. 2010). This leads to ubiquitination and subsequent degradation of the JAZ factors by the 26S proteasome, resulting in release of repression of basic helix–loop–helix (bHLH) transcription factors by JAZ proteins (Chini et al. 2007, Thines et al. 2007, Cheng et al. 2011, Fernández-Calvo et al. 2011).

JA-Ile is deactivated in Arabidopsis by conversion into 12OH-JA-Ile and 12COOH-JA-Ile, mediated by two Cyt P450 enzymes, CYP94B3 and CYP94C1 (Kitaoka et al. 2011, Koo et al. 2011, Heitz et al. 2012). 12OH-JA-Ile has lower binding activity to the COI1 receptor than that of JA-Ile (Koo et al. 2011). Levels of JA-Ile transiently increase after mechanical wounding, followed by accumulation of 12OH-JA-Ile and 12COOH-JA-Ile. JA-Ile induced after wounding in cyc94b3 cyc94c1 double mutants is maintained at high levels, concomitant with less accumulation of 12OH-JA-Ile and 12COOH-JA-Ile (Heitz et al. 2012). The CYP94B3 and CYP94C1 genes are also induced by wounding, suggesting that both enzymes are involved in negative feedback regulation of the JA signaling (Koo et al. 2011, Heitz et al. 2012).

Soil salinity and drought are stresses that threaten worldwide crop production (Zhu et al. 2002). Approximately 20% of irrigated land is affected by increased salt content (Rozema and Flowers 2008). Improving crops to create novel varieties that are more tolerant to salinity is an important issue (Yamaguchi and Blumwald 2005, Takeda and Matsuoka 2005, Qin et al. 2011). In agriculture, use of seawater has been proposed for fields with low water availability (Glenn et al. 1991, Rozema and Flowers 2008). Thus, numerous studies on salt tolerance mechanisms in plants have been conducted, including those involved in Na$^+$ exclusion from the cytosol, accumulation of osmoprotectants and detoxification of oxidative stresses (Zhu et al. 2001, Zhu et al. 2002, Yamaguchi and Blumwald 2005, Qin et al. 2011). The QTL gene, which is responsible for salt stress tolerance, was identified in the monocot rice crop, which supplies the principal food for the world population (Ren et al. 2005). This gene encodes a HKT transporter (HKT1;5) that is proposed to unload Na$^+$ from xylem vessels (Ren et al. 2005). Furthermore, Na$^+$ exclusion by HKT1;5 contributes to salt tolerance in wheat (Munns et al. 2012). In addition to the mechanisms in developed tissues, the importance of meristematic activity in salt tolerance has also been suggested in rice (Ogawa et al. 2011). However, it remains a challenge to confer salt tolerance to crops partly because of the complicated nature of salinity stress (Takeda and Matsuoka 2008).

In this study, we report that enhanced expression of the Cyt P450 family gene CYP94C2b confers salt stress tolerance in rice. Extended survivability under saline conditions was associated with preservation of meristem function and the ability to produce new leaves. This occurred concomitantly with changes in JA metabolism, resulting in reduced responses to JA, but not to coronatine, and repression of stress-induced senescence. These results suggest that repression of exaggerated JA responses is important for rice plant survival under harsh environmental conditions.

### Results

**The FE047 rice line shows enhanced viability under salt stress conditions**

We identified the FE047 rice line, which showed extended survivability under saline conditions, while screening rice FOX (full-length cDNA overexpression) lines to identify a gene conferring salt tolerance (Nakamura et al. 2007, Hakata et al. 2010, Tsuchida-Mayama et al. 2010) (Fig. 1A). When shoot explants of this salt-tolerant line and of the non-transgenic wild type (WT) on medium were immersed in a high concentration (600 mM) of NaCl solution, their growth was severely inhibited. However, some leaves in the FE047 shoots remained green after 5 weeks of salt stress treatment, whereas most leaves of the WT shoots turned brown. When the salt-stressed shoots were transferred to a condition without salinity, outgrowth of green leaves and adventitious crown roots appeared from the basal shoots in the tolerant lines within 1 week (Supplementary Fig. S1). At this stage, 80% of the FE047 shoots were viable, and only 10% of the WT shoots survived (Fig. 1B). The surviving FE047 plants further recovered in soil sufficiently to become fertile. Enhanced viability of FE047 was observed when the line was grown under various saline conditions, including hydroponic culture and soil cultivation (Fig. 1C–F). Hydroponically cultured WT and FE047 seedlings were treated with salinity stress in the greenhouse, and symptoms caused by salinity were scored as described by Thomson et al. (2010). As shown in Fig. 1C, there was an apparent difference in score distribution between FE047 and the WT, indicating that FE047 had a tendency to be less damaged than the WT. When grown on salinized soil with 100 and 150 mM NaCl, survivability of WT plants, as well as seed setting, was decreased more drastically than those of FE047 (Fig. 1D–F).

**Expression of the CYP94C family gene CYP94C2b increases in the FE047 line**

We found that an overexpressed cDNA in FE047 corresponded to Os12g0150200, which encodes a protein that is closely related to the Cyt P450 CYP94C2 (Os12g0151400) (Supplementary Fig. S2). We designated the original CYP94C2 as CYP94C2a, and the protein encoded by Os12g0150200 as CYP94C2b. Enhanced viability under salt stress conditions conferred by overexpression of Os12g0150200 was observed in independently prepared transgenic lines (Fig. 1B). We examined the relationship between the expression levels of CYP94C2b and the degree of viability under saline conditions among the transgenic lines and the WT. An increase in CYP94C2b expression levels (approximately 5- to 150-fold higher than WT levels) was well correlated with
enhanced viability under saline conditions (Supplementary Fig. S3). However, some plants that expressed extraordinarily high levels of CYP94C2b (>150-fold higher than WT levels) showed only limited enhanced viability (Supplementary Fig. S3). These results suggest that an increase in CYP94C2b expression levels is responsible for enhanced viability in FE047, although too high a level of enzyme activity had detrimental effects, at least under saline conditions.

CYP94C2b is involved in inactivating the JA response

Both CYP94C2a and CYP94C2b belong to the same clade as Arabidopsis CYP94C1. CYP94C1 catalyzes the conversion of JA-Ile to 12OH-JA-Ile, and subsequently to 12COOH-JA-Ile, and thereby has been proposed to inactivate JA-Ile (Heitz et al. 2012). The induction levels of JA and JA-Ile in the FE047 line

Fig. 1 Enhanced salt tolerance of the FE047 line. (A) Shoot explants excised from 7-day-old seedlings were grown in MS medium supplemented with an equal volume of 600 mM NaCl for 5 weeks. Representative shoots of the WT and T2 lines of FE047 are shown. Scale bar = 1 cm. (B) Survivability of the shoots grown in the salinized medium as in (A). Survivability was scored after naturalization in the absence of saline stress for 1 week. The three BBC105 lines indicate independently prepared transgenic lines that carry the same gene (Os12g0150200) under the constitutive promoter. n = 10 (for WT and FE047), 4 (for BBC105_2) and 5 (for BBC105_6 and 9). (C) Salinity tolerance of hydroponically cultured seedlings in the greenhouse. Symptoms caused by salt stress were scored as described by Thomson et al. (2010). Box plots indicate the interquartile range (IQR). Outliers indicate values outside the range of 1.5 × IQR above the third quartile and below the first quartile. Whiskers represent the range of maximum and minimum values. Median values are represented by bold lines. n = 38 for the WT and 20 for FE047. (D–F) Survivability (D) and fertility (E) of WT and FE047 plants grown in soil salinized with the indicated concentrations of NaCl. n = 17 (for WT, 100 mM), 17 (for WT, 150 mM), 4 (for FE047, 100 mM) and 3 (for FE047, 150 mM). For fertility, the average score obtained by two experimental repeats using different tanks is shown. (F) Representative WT and FE047 plants grown in soil salinized with 150 mM NaCl. Photos were taken 40 d after salt treatment.
after wounding (within 1 h) decreased significantly compared with those in the WT, concomitant with increased induction levels of 12OH-JA-Ile (Fig. 2). This result suggests that conversion and inactivation of JA-Ile is facilitated by enhanced expression of CYP94C2b. Decreased levels of JA-Ile have been observed in the wounded leaves of CYP94C1-overexpressing Arabidopsis (Heitz et al. 2012). CYP94C2b expression is induced by JA and wounding in rice, as reported in the public expression profile database and in the case of the Arabidopsis CYP94C1 gene (Heitz et al. 2012, Sato et al. 2013).

We compared the effects of exogenous JA on growth of the WT and FE047 lines. Without JA treatment, the shoot and root of FE047 elongated more than those of the WT but only slightly, probably reflecting a difference in metabolism of endogenous JAs (Fig. 3A, B; Supplementary Table S1). FE047 showed impaired sensitivity to exogenously supplied JA at 1–20 μM, in terms of inhibiting shoot and root growth (Fig. 3A, B; Supplementary Table S1). Moreover, the levels of expression of JA-responsive genes, JAZ11 and JAmyb, after wounding decreased significantly in FE047 leaves (Fig. 3E, F). These results are consistent with the finding that the JA inactivation pathway is facilitated in the FE047 line. In contrast, sensitivity to coronatine, an agonist of the JA-Ile receptor (Uppalapati et al. 2005, Katsir et al. 2008, Sheard et al. 2010), did not decrease in FE047 compared with that in the WT (Fig. 3C, D; Supplementary Table S1), indicating that the COI1-mediated JA signaling pathway is not affected. Therefore, coronatine appears not to be catalyzed by CYP94C2b, as in the case of CYP94C1 and CYP94B3 (Koo et al. 2011; Heitz et al. 2012).

Leaf senescence is delayed in the lines that overexpress CYP94C2b under saline conditions

Because JA promotes leaf senescence, we examined whether progression of leaf senescence is altered in the FE047 line. When grown under normal conditions, no striking difference in progression of leaf senescence was observed between FE047 and the WT (Supplementary Fig. S4). In contrast, the number of de-greening senescent leaves decreased in FE047 compared with that in the WT grown in medium with 275–300 mM NaCl (Fig. 4A, B). To examine whether leaf senescence is delayed in FE047 with a lower concentration of NaCl, we compared expression levels of the senescence marker gene, STAYGREEN (SGR), which has a pivotal role in Chl degradation (Park et al. 2007). SGR expression was much lower in old leaves (leaves 1–4) of FE047 than in those of the WT grown in medium with 250 mM NaCl (Fig. 4C; Supplementary Fig. S5A, B). These results indicate that initiating stress-induced senescence is avoided in old FE047 leaves. We also observed delayed leaf senescence in the three independent CYP94C2b-overexpressing lines (Supplementary Fig. S5C, D).

Because senescence progression eventually leads to cell death, it raises the question of whether enhanced viability under saline conditions is linked to an increase in anti-senescence activity. When kinetin, a cytokinin (CK) that has an anti-senescence effect (Gan and Amashino 1995, Lim et al. 2007), was applied, viability of the WT shoot explants under the salt stress condition increased (Supplementary Fig. S6). After subsequent naturalization under a non-stress condition, new leaves expanded in the surviving shoots in the presence of kinetin (Supplementary Fig. S6). Thus, the inhibited senescence may have been caused by enhanced viability in FE047. We further examined the relationship between CK action and JA inactivation in terms of anti-senescence activities by monitoring the expression of the marker gene OsRR10, which is responsive to CK (Ito and Kurata 2006). OsRR10 was expressed at higher levels in young leaves than in old leaves of the WT (Fig. 4C; Supplementary Fig. S5B). In contrast to the strong correlation between SGR expression levels and progression of leaf senescence, OsRR10 expression levels were not tightly associated with delayed leaf senescence (Fig. 4C; Supplementary Fig. S5B). This finding suggests that delay of salinity stress-induced senescence in FE047 leaves is not mediated by CK action.
Expression of JA-responsive genes induced by salt treatment is partially suppressed in the FE047 line

We examined whether the JA response is altered in the FE047 line during salinity stress treatment. Expression levels of the JA-responsive genes, JAmyb and JAZ11, increased in WT explants by immersion in a 600 mM NaCl solution for 24 h (Supplementary Fig. S7), indicating that the JA response is induced by salinity stress under our experimental conditions. However, we did not find a difference in expression levels between the WT and FE047. Increased JAmyb and JAZ11 expression levels were also observed in the WT at 7 and 14 d after NaCl immersion. The expression levels of both genes were significantly lower in FE047 than in the WT on the seventh day but not on 14th day (Fig. 5A). Thus, the JA response was partially suppressed in FE047 during salinity stress.

To investigate whether the JA signal has negative effects on salt tolerance, we examined the viability of rice explants in the presence of JA under saline conditions. As shown in Fig. 6, WT rice explants showed diminished viability within 4–5 weeks after exposure to 20–50 μM JA and immersion in NaCl solution.
This result suggests that JA negatively affects shoot viability under saline conditions.

**General cell proliferation activity is not enhanced in the FE047 line**

JA inhibits cell division (Chen et al. 2011); thus, we examined whether the FE047 line could enhance shoot cell proliferation activity. We monitored expression of the cell cycle marker genes PCNA and CycB2;1, as S phase- and G2–M phase-specific genes, respectively. The expression levels of both genes in FE047 plants grown under non-stressed conditions were almost the same as those in the WT (Fig. 5B). The expression levels of both genes in the WT decreased 7–14 d after the onset of salt stress treatment, indicating that cell division was inhibited by the salt stress treatment (Fig. 5B). PCNA and CycB2;1 expression levels were nearly the same between the WT and FE047 (Fig. 5B). Therefore, enhanced viability of FE047 was not primarily due to a difference in cell division activity under non-stress conditions or alleviated inhibition of cell division by salinity at this stage. In addition, cell proliferation between WT and FE047 calli was comparable, regardless of salt stress (Supplementary Fig. S8). This result indicates that the proliferation activity of dividing cells and general cellular activity is not enhanced in the FE047 line.

**Morphological aberrations caused by salinity are alleviated in FE047 shoots**

We histologically analyzed the shoot apical meristem (SAM) and leaf primordia in the WT and FE047. No obvious difference in SAM size or organization was observed between 7-day-old seedlings of the WT and FE047 grown without salinity stress (Supplementary Figs. S9A, B, S10). Similarly, in the explants treated with salinity for a week, we did not observe any difference in SAM size or organization between the WT and FE047 (Supplementary Figs. S9C, D, S10). Some WT leaf primordial and SAM cells were enlarged and contained large vacuoles after salt treatment for 3 weeks, but not those of FE047 (Supplementary Fig. S9E, F). Similar changes appeared in some FE047 shoot cells 5 weeks after salinity treatment (Supplementary Fig. S9G). Such aberrant morphology is not usually observed in plants grown under non-stress conditions, suggesting that division activity is lost in those cells. These results suggest that a qualitative shift of cells in the leaf primordia and SAM occurs in both the WT and FE047, but it is delayed in FE047.

**Discussion**

JA plays versatile roles in plant development (Creelman and Mullet 1997, Wasternack 2007), including repression of cell division and elongation and promotion of leaf senescence. We previously showed that repression of an exaggerated JA response is important for rice root elongation under saline conditions (Toda et al. 2013a, Toda et al. 2013b). This repression is mediated by the RSS3 protein, which binds to JAZ and bHLH factors (Toda et al. 2013a). In the rss3 mutant, the JA-responsive
genes were de-repressed, and cell elongation was drastically inhibited in the presence of salt stress. Thus, it has been suggested that JA negatively affects a plant’s adaptation to a salinity stress condition. In this study, we showed that increased expression levels of \( \text{CYP94C2b} \), a rice homolog of \( \text{CYP94C1} \), caused reduced JA responses and enhanced shoot viability under salinity stress conditions. In Arabidopsis, \( \text{CYP94C1} \) hydroxylates and inactivates JA-Ile, an active form of JA (Heitz et al. 2012). \( \text{CYP94C1} \) expression is induced by JA, and accumulation of JA-Ile after wounding is followed by that of 12OH-JA-Ile and 12COOH-JA-Ile (Heitz et al. 2012). In addition, overexpression of \( \text{CYP94B3} \) and \( \text{CYP94C1} \) causes a reduced response to JA (Koo et al. 2011, Heitz et al. 2012). Similarly, the levels of JA-Ile after wounding and responses to exogenous JA and wounding decreased in the FE047 line, in which \( \text{CYP94C2b} \) is overexpressed. Our findings suggest that repressing JA action is responsible for enhanced salt tolerance in FE047 plants. It is currently unclear, however, whether JA signaling interacts with the salt-responsive pathway and that such an interaction affects the salinity tolerance, or whether JA and salinity have additive but independent effects on leaf senescence and viability of plants.

Notably, the levels of 12OH-JA-Ile after wounding were increased in FE047 (Fig. 2). This is in contrast to the finding that overexpression of \( \text{CYP94C1} \) results in decreased levels of 12OH-JA-Ile after wounding in Arabidopsis (Heitz et al. 2012).

**Fig. 5** Expression of the JA-responsive genes (A) and cell cycle-related genes (B) during salt stress treatment. Shoot explants excised from 7-day-old WT seedlings were grown in MS medium supplemented with an equal volume of 600 mM NaCl for the indicated periods. Expression levels of the indicated genes were determined by qRT-PCR and normalized to the levels of \( \text{UBC} \). Means ± SD, \( n = 5 \) (biological repeats). Asterisks indicate significant differences (**P-value of < 0.01; *P-value of < 0.05, Student’s t-test).
non-stress conditions and at the early stages (0–14 d) of salinity treatment. The phenotypic differences between FE047 and WT were rather pronounced after long exposure to salt stress, both in recovery after removing the salinity stress and in stress-induced senescence. Because leaves were continuously generated in surviving plants after transfer to non-stress conditions, some sort of competency linked to leaf primordia and shoot meristem functions may be preserved more in FE047 than that in the WT under saline conditions.

The progression of leaf senescence under saline conditions was delayed in the FE047 line, indicating that JA has a pivotal role in the progression of stress-induced leaf senescence. Exogenous application of CK, which plays roles in meristem maintenance and repression of leaf senescence, also enhanced shoot viability under saline conditions. Similar effects of CK in conferring drought tolerance have been reported in several plants (Rivero et al. 2007, Zhang et al. 2010, Peleg et al. 2011). Applying CK to shoots also reduces salt sensitivity of the rss1 mutant (Ogawa et al. 2011). Thus, under saline conditions, anti-senescent activity in the FE047 line may be linked to the maintenance of leaf primordial and shoot meristem functions, which affect viability of individual plants. However, CK does not mediate the delay of senescence observed in FE047 because OsRR10 expression, which is responsive to CK, was not correspondingly enhanced. Thus, other factors that are negatively controlled by JA may be responsible for shoot meristem vigor. It is also plausible that delay of senescence, which is associated with retarded Chl degradation and reduction of photosynthesis, per se contributes to the enhanced viability of FE047. If this is the case, FE047 might exhibit enhanced tolerance to abiotic stresses other than salinity. This possibility is currently being tested with several stress conditions in our laboratory. Future studies are needed to understand how anti-senescent activity is linked to meristem and leaf primordial functions under stressful conditions.

In contrast, the number of senescent leaves remained unchanged in FE047 under normal growth conditions, suggesting that endogenous JA may play only a limited role in senescence without salinity stress. This is in accordance with the finding that JA biosynthesis mutants and coi1 signaling mutants do not show a defect in natural senescence in Arabidopsis (Schommer et al. 2008, Selte et al. 2010a, Selte et al. 2010b). Moreover, sorbitol stress-induced senescence but not natural senescence is delayed in Arabidopsis LOX2 RNAi (RNA interference) lines, in which JA biosynthesis is disrupted (Selte et al. 2010a, Selte et al. 2010b). In contrast, leaf senescence is delayed in the maize opr7opr8 double mutant, which is deficient in JA production (Yan et al. 2012). Thus, together with the JA signal, unknown factors may promote stress-induced senescence in rice. Several studies in Arabidopsis have suggested molecular links between natural and stress-induced senescence. In particular, the roles of ethylene signaling factors, such as EIN2 and EIN3, and NAC transcription factors in natural senescence are well documented (Kim et al. 2009, Li et al. 2013). It would be interesting to test the possibility that both the ethylene and JA signals are involved in salt-induced senescence in rice.
It remains to be elucidated which factors acting downstream of JA signaling are responsible for salt-induced senescence. Interestingly, a NAC transcriptional regulator, VNI2, has been suggested to repress leaf senescence and facilitate stress resistance in Arabidopsis (Yang et al. 2011). Expression of the JA-inducible RNA-binding zinc-finger protein OsTZF1 confers delayed senescence and stress tolerance in rice (Jan et al. 2013). Therefore, stress-induced senescence may also be under control of negative regulation by OsTZF1, in addition to positive regulation by unknown factors acting downstream of JA signaling, which would allow more elaborate control of senescence.

Although an increase in CYP94C2b expression levels enhanced viability under saline conditions, extremely high levels of CYP94C2b expression appeared to have detrimental effects. This may reflect the fact that a decrease in JA-Ile levels is partly responsible for enhanced viability under saline conditions, but that a further reduction in JA-Ile levels may negatively affect survivability. It is possible that some portion of the JA response is involved in adaptation to an abiotic stress condition, as described previously (Kang et al. 2005, Seo et al. 2011). However, an exaggerated JA response should be repressed to survive under saline conditions. Taken together, our results suggest that optimal regulation of JA metabolism is required depending on environmental conditions.

In conclusion, we identified a gene that represses the JA response and confers salinity tolerance in rice. The enhanced viability observed in FE047 shoots under saline conditions was associated with promoting JA inactivation and delayed senescence. Further studies on how plant viability is maintained under stress conditions will lead to understanding of the mechanisms underlying the environmental adaptations of plants and thus improving stress tolerance in crops.

### Materials and Methods

#### Plant materials and growth conditions

Seeds of WT (Oryza sativa cv. Nipponbare) and transgenic rice were surface sterilized and germinated on solid Murashige and Skoog (MS) basal medium (1% sucrose, 0.25% gellan gum and 0.05% MES-KOH, pH 5.8) in 13 cm high capped bottles. Seedlings were grown at 25°C under a photoperiod of 14 h light (4,000 lux, white light) and 10 h dark. A population of FOX lines of rice (2,229 T1 lines) were naturalized with tap water to evaluate viability. Surviving plants were immersed in an equal volume of 600 mM NaCl after culturing in the capped bottles for 5 weeks, the plants were placed on new medium and immersed in an equal volume of 300 mM NaCl to evaluate leaf senescence under saline conditions. A diluted solution of JA in ethanol (Cayman Chemical; #88330) was added to the medium. Coronatine (Sigma-Aldrich, C8115) was dissolved in dimethylsulfoxide, and a 10 mM solution was stored at −20°C. Coronatine or mock solution was applied to the solid MS-based medium. Treatment of rice shoots with CK was performed as described previously (Ogawa et al. 2011).

#### Rice transformation

The corresponding rice cDNA (accession No. AK066287) was amplified from the NIAS cDNA clone (002-105-H01) to prepare independent overexpressing lines of CYP94C2b (line BBC105s). The PCR product subcloned in the pENTR/D-TOPO vector (Invitrogen) was transformed into Agrobacterium-mediated transformation. Transformed calli were selected with 50 ng l⁻¹ hygromycin. Primer sets used for cDNA amplification are listed in Supplementary Table S2.

#### RNA expression analysis

Total RNA was extracted and purified using the RNeasy Mini kit (Qiagen). Reverse transcription was performed using the QuantiTect Rev Transcription kit (Qiagen). First-strand cDNA was amplified by PCR PrimeStar HS DNA polymerase (TAKARA) using the GeneAmp PCR System 9700 (Applied Biosystems) or by real-time PCR with Power SYBR Green PCR Master Mix (Applied Biosystems) using an Mx3000P (Agilent), and the primer sets listed in Supplementary Table S2. The marker genes JAZ1 and R1R10, which are responsive to JA and CK, respectively, were selected from the expression profile published by Sato et al. (2013). JAmyb was selected on the basis of its responsiveness to JA as reported by Yokotani et al. (2013). The quantified expression levels of the tested genes were normalized with those of UBC and 25S rRNA (Jain et al. 2006).

Leaves of 7-day-old WT and FE047 seedlings were mechanically wounded 20 times with forceps to induce wounded gene expression. The seedlings were incubated in a moist chamber for 16 h. Leaf tissues were frozen in liquid N₂ and stored at −80°C until RNA extraction.

#### Measurement of JA, JA-Ile and 12OH-JA-Ile

Halves of leaf blades of the third leaves of rice seedlings were excised with a surgical knife and immediately frozen in liquid N₂. The other halves were wounded by pinching with forceps 20 times each, and the entire seedling with injured leaves was incubated in a moist chamber at room temperature. Frozen leaf tissues were crushed in a microtube with stainless steel beads using a Mini-BeadBeater-8 (BioSpec). JA and its conjugates were then extracted from the frozen tissues with 99.5% ethanol at 4°C overnight in the dark. After removing debris by centrifugation (20,000 g) at room temperature for 5 min, the ethanol supernatant was recovered and stored at 4°C. The ethanol solution was dried with N₂ gas, and then dissolved in water. After removing debris by centrifugation (15,000 g) at room temperature for 20 min, the supernatant was used for measurement.

Ultra-performance liquid chromatography/time of flight mass spectrometry (UPLC/TOFMS) analysis was performed with 10 µl samples from leaf extracts using an Agilent 1290 Infinity coupled to a Bruker microTOF II. A ZORBAX Eclipse Plus C18 (1.8 µm, 2.1 x 50 mm) column was used to separate the substrates. The mobile phases were: A 20% (v/v) aqueous MeOH with 0.05% (v/v) AcOH and B, MeOH with 0.05% (v/v) AcOH. The gradient program was: 0–3.5 min, isocratic 90% A; 3.5–6 min, linear gradient 90–0% A; 6.1–9 min, isocratic 90% A, and the flow rate was 0.15 ml min⁻¹. The mass spectrometer
solution of tuberonic acid in CH$_2$Cl$_2$ (Wako Pure Chemical) was added to for 4 h at room temperature under an argon atmosphere. The reaction mixture was purified to 12-OH-JA-Ile methyl ester as a colorless oil by aqueous LiOH. After filtration, this reaction mixture was neutralized with Amberlite IR120B. After filtration, this solution was concentrated and the residue was purified to 12-OH-JA-Ile methyl ester as a colorless oil by silica gel column chromatography (CHCl$_3$:MeOH = 100:1). Aqueous LiOH (1 M) was added to a solution of 12-OH-JA-Ile methyl ester in MeOH (Wako Pure Chemical), 4-dimethylamino tourists and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (Tokyo Chemical) at 0°C. The reaction mixture was diluted with EtOAc and washed with 1 M HCl, saturated aqueous NaHCO$_3$ and brine. The organic layer was dried over Na$_2$SO$_4$ and filtered. After evaporation, the residue was purified to 12-OH-JA-Ile methyl ester as a colorless oil by silica gel column chromatography (CHCl$_3$:MeOH = 100:1). Aqueous LiOH (1 M) was added to a solution of 12-OH-JA-Ile methyl ester in MeOH (Wako Pure Chemical) at 0°C and stirred for 2 h at room temperature. Then, the reaction mixture was neutralized with Amberlite IR120B. After filtration, the filtrate was concentrated in vacuo. The residue was purified by HPLC (ODS-HG (ø 20 × 250 mm): 35% aqueous MeOH 10 ml min$^{-1}$, retention time: UV 230 nm) to 12-OH-JA-Ile as a colorless oil.

**Histological analysis of rice shoots**

Basal shoot tissues were fixed in solution containing 5% formaldehyde, 5% acetic acid and 45% ethanol (FAA) at room temperature overnight, dehydrated through an ethanol series, embedded in Technovit 7100 resin (Heraeus Kulzer GmbH) and cut into 5 µm thick sections. The tissue sections were stained with 1% toluidine blue and observed under a microscope (Axioscop2, Zeiss).

**Supplementary data**

Supplementary data are available at PCP online.

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**Disclosures**

The authors have no conflicts of interest to declare.

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