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ONION2 Fatty Acid Elongase is Required for Shoot Development in Rice

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Regular Paper

A plant's surface is covered with epicuticular wax, which protects plants from inappropriate environmental conditions such as drought and pathogen attack. Very-long-chain fatty acids (VLCFAs) are the main component of epicuticular wax on the surface of above-ground organs. Here we show that a fatty acid elongase catalyzing an elongation reaction of VLCFAs is required for shoot development in rice. *onion2* (*oni2*) mutants produced very small shoots in which leaves were fused to each other, and ceased growing after germination. The midrib of *oni2* leaf blades was not developed correctly. Molecular cloning showed that *ONI2* encodes a fatty acid elongase, which catalyzes the first step of elongation reactions of a carbon chain of VLCFAs, and *oni2* had a reduced amount of VLCFAs. Expression analysis showed that *ONI2* is specifically expressed in the outermost cell layer of young lateral organs. These results suggest that *ONI2* is a layer 1-specific gene required for development of the entire shoot and that VLCFAs play an essential role in normal shoot development in rice.

Keywords: Fatty acid elongase • L1 • Rice • Shoot • Very-long-chain fatty acid.

Abbreviations: DIG,digoxigenin, *FDH*; *FIDDLEHEAD*, L1; layer 1, *ONION2*; *ONI2*, RT–PCR; reverse transcription–PCR, SAM; shoot apical meristem, SEM; scanning electron microscopy, VLCFA; very-long-chain fatty acid.

Introduction

Epicuticular wax, which is present on the surface of the epidermis, protects plants from inappropriate environmental conditions producing biological and non-biological stresses. Very-long-chain fatty acids (VLCFAs) are the main component of the wax and are synthesized from C18 carbon chains by four-step sequential elongation reactions. The first step is a condensation reaction of a C2 unit to an acyl-CoA that is catalyzed by fatty acid elongase (β-ketoacyl-CoA synthase), the second step is a reduction reaction catalyzed by β-ketoacyl-CoA reductase, the third step is a dehydration reaction catalyzed by β-hydroxyl-CoA dehydratase and the last step is a reduction reaction catalyzed by enoyl-CoA reductase (Kunst and Samuels 2009). Several genes that encode fatty acid elongase have been identified, and their analyses showed that fatty acid elongase is necessary for the proper composition of VLCFAs and for avoiding ectopic organ fusions. For example, *fiddlehead* (*fdh*) mutants in Arabidopsis showed defective epidermal function, which brings about altered floral architecture due to abnormal floral organ formation, resulting in fusion of organs to one another (Yephremov et al. 1999, Pruitt et al. 2000). The *fdh* mutants also showed organ fusions in leaves, although vegetative shoot development appeared rather normal (Yephremov et al. 1999, Pruitt et al. 2000). *fdh* showed an altered composition, but not a reduced amount of VLCFAs (Voisin et al. 2009). In contrast to *fdh* mutants, mutations of the *ONION1* (*ONI1*) gene in rice, which is an ortholog of *FDH*, showed severe morphological defects leading to seedling lethality (Ito et al. 2011). Although *oni1* also showed organ fusions as is the case in Arabidopsis *fdh*, the *oni1* mutants lacked a normal outermost cell layer (layer 1 or L1) and failed to maintain the shoot apical meristem (SAM). Along with the expression of *ONI1* in embryogenesis, defects in the SAM were also observed even at this stage. In addition, *oni1* had a reduced amount of VLCFAs. Consistent with these phenotypic

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differences, *FDH* failed to complement the *oni1* mutation. These results suggest that rice fatty acid elongase ONI1 has a more critical function than Arabidopsis FDH in plant development, or *ONI1* has an additional function to that of *FDH*.

To better understand the function of fatty acid elongase in plant development, we analyzed *oni2* mutants, which were identified as mutants resembling the morphology of a *KNOX* gene-overexpressing plant and were morphologically similar to *oni1* (Tsuda et al. 2009). The *KNOX* gene, which is specifically expressed in the SAM, is known to play an essential role in SAM formation and maintenance, and its ectopic expression brought about abnormal shoot morphologies (Ito et al. 2001, Tsuda et al. 2011). We previously reported that *oni2* showed ectopic expression of *KNOX* genes in leaves, and *ONI2* was mapped on the long arm of chromosome 10 (Tsuda et al. 2009). In this study, we carried out a detailed analysis of *oni2* mutants and molecular characterization of *ONI2*. *oni2* shoots showed various defects such as organ fusions between leaves and incorrect development of the midrib in the leaf blade. Molecular cloning showed that *ONI2* encodes a fatty acid elongase similar to ONI1 and FDH, and is expressed specifically in L1 of young lateral organs throughout its life cycle. *oni2* had a reduced amount of VLCFAs. Our results suggest that the fatty acid elongase and VLCFAs play an essential role in shoot development in rice.

Results

Morphological analysis of *oni2*

We previously reported identification of *onion* mutants (Tsuda et al. 2009). In this study we examined the morphology of *oni2* seedlings. Since *oni2* mutants were seedling lethal (Tsuda et al. 2009), we could not examine phenotypes at later developmental stages including reproductive stages, although *ONI2* was expressed throughout its life cycle (see 'Expression of *ONI2*').

The severity of *oni2* phenotypes was very different among individuals even in the same allele (**Fig. 1**). In the most severe case, *oni2-1* mutant seeds produced dark green and very small shoots, and ceased growing soon after germination (**Fig. 1B**). In milder cases, *oni2-1* mutant shoots could elongate, and their leaf blade and leaf sheath could be clearly distinguished from each other, albeit that *oni2* mutants were still shorter than wild-type shoots and did not reach maturity (**Fig. 1A, B**).

Longitudinal and transverse sections of *oni2-1* showed organ fusions between neighboring leaves, which were observed in both severe and mild mutant shoots (**Fig. 2A–F**). Organ fusions were observed in continuous regions along the proximo-distal and lateral axes of the leaf, but not in an entire region of the leaf (**Fig. 2B–D, F**). In contrast, no organ fusion was observed in the wild type, and instead space was clearly observed between leaves (**Fig. 2A, E**). Transverse sections of leaf blades of mild mutant shoots showed abnormal midribs (**Fig. 2G–J**). In the wild type, two lacunae were observed in the midrib, whereas in the *oni2-1* midrib no lacuna was observed. The overall leaf architecture was quite discordant in *oni2-1*, and the *oni2-1* leaf was thick, except for the midribs (**Fig. 2I, J**).

Since organ fusions suggested a defect in L1, we examined expression of *ROC1* in *oni2-1*. *ROC1* is an ortholog of an Arabidopsis L1-specific gene *ATML1* required for L1 identification and is specifically expressed in L1 in rice (Ito et al. 2002, Abe et al. 2003). In situ hybridization detected *ROC1* expression in L1 of both the abaxial and adaxial sides in the wild-type leaf, but in the *oni2-1* mutant leaf *ROC1* expression in the abaxial side was hardly detected, and in the adaxial side it was reduced (**Fig. 3**). Perturbed expression of *ROC1* suggested that the differentiation and/or functionality of L1 was compromised in *oni2*. Thus, *oni2* seemed to lack normal L1 in the leaf.

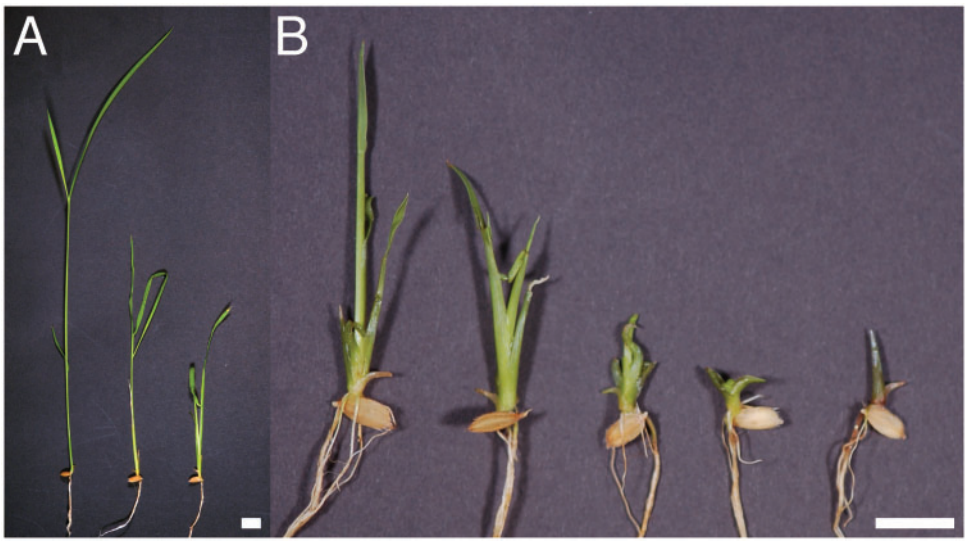

Fig. 1 Various gross morphologies of *oni2* shoots. (A) The wild-type (left) and mild *oni2-1* (middle and right) mutant shoots. (B) Moderate (left) to severe (right) mutant *oni2-1* shoots. Bars = 1 cm.

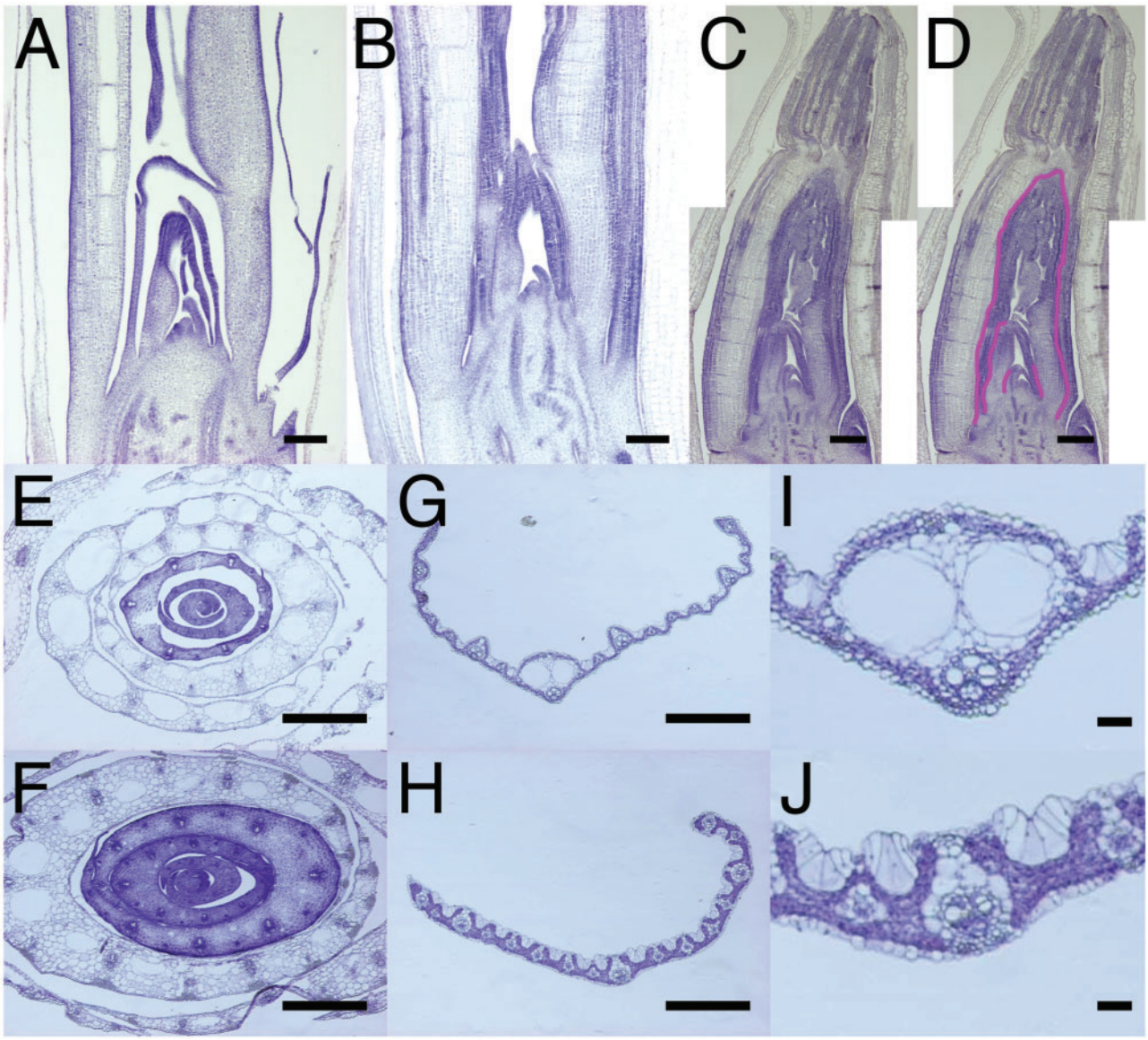

Fig. 2 Histological observation of *oni2* shoots. (A–D) Longitudinal sections of shoots of the wild type (A), and mild *oni2-1* (B) and severe *oni2-1* mutants (C and D). Organ fusions between neighboring leaves were observed in *oni2-1*. Fused regions are labeled in magenta (D). (E and F) Transverse sections of wild-type (E) and *oni2-1* (F) shoots. Organ fusions between neighboring leaves were observed. (G and H) Transverse sections of the third leaf of the wild type (G) and *oni2-1* (H). The midrib was not well developed in *oni2-1*. (I and J) Enlargement of the midribs of the wild type (I) and *oni2-1* (J). Lacunae were not observed in *oni2-1*. Bars = 200 μm in (A–D), 1 mm in (E–H) and 100 μm in (I) and (J).

Molecular cloning of *ONI2*

We previously mapped *ONI2* on the long arm of chromosome 10 (Tsuda et al. 2009). Fine mapping using 233 F_2 plants derived from a crossing of an *oni2* heterozygous plant and the indica cultivar Kasalath narrowed down the candidate region within 94 kb, and in this region 15 genes were predicted (**Fig. 4A**). Among them, Os10 g0416200 was predicted to encode fatty acid elongase. Since a fatty acid elongase mutant *oni1* showed a very similar morphology to that of severe *oni2* mutants (Tsuda et al. 2009, Ito et al. 2011), we analyzed the sequence of Os10 g0416200 in *oni2*. PCR amplification and sequence analysis showed that Os10 g0416200 has a 16 bp deletion in the second exon in *oni2-1* (**Fig. 4A**). In addition, we failed to amplify the first exon of Os10 g0416200 by PCR in *oni2-2*, probably due to deletion of the exon, a large insertion in the exon or an inversion (**Fig. 4A**). These results indicated that Os10 g0416200 is a strong candidate for *ONI2*. To examine this possibility, a complementation test was carried out. When *oni2-1* mutant calli were transformed with a vector containing a wild-type *ONI2* genome, normal shoots were regenerated. They reached maturity and set seeds. On the other hand, when *oni2-1* mutant calli were transformed with an empty vector, mutant shoots were regenerated and stopped growing

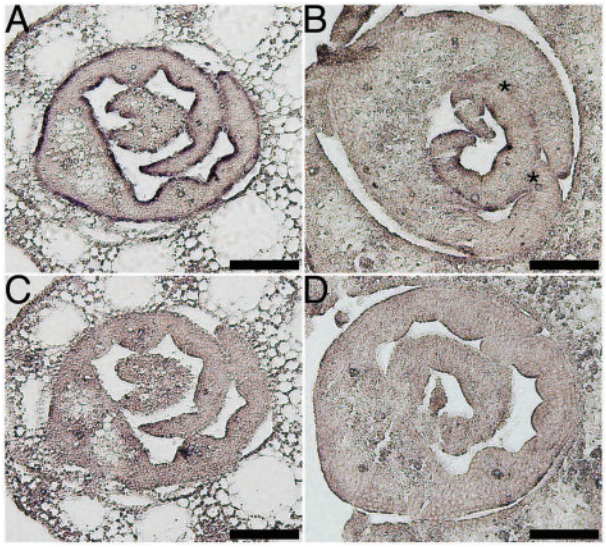

Fig. 3 Expression of *ROC1* in *oni2*. Transverse sections of the wild type (A and C) or *oni2* (B and D) were hybridized with the antisense probe (A and B) or sense probe (C and D) of *ROC1*. Asterisks in (B) indicate the sites of organ fusions. Bars = 100 μm.

before maturity (**Fig. 4B**). From these results, we concluded that Os10g0416200 is *ONI2*.

Structural characteristics of ONI2

ONI2 consists of two exons separated by an intron and encodes a protein with 523 amino acid residues. A similarity search and phylogenetic analysis showed that ONI2 is homologous to fatty acid elongase (β-ketoacyl-CoA synthase) that catalyzes the first step of elongation reactions of the carbon chain of VLCFAs (**Fig. 4C**). We previously identified *ONI1*, which also encodes fatty acid elongase and is required for proper shoot development (Ito et al. 2011). ONI2 was 44% identical to ONI1 in their entire amino acid sequences. No close homolog of *ONI2* was identified in Arabidopsis (**Fig. 4C**).

Expression of *ONI2*

Reverse transcription–PCR (RT–PCR) analysis showed that *ONI2* is expressed in the shoot apex, panicle and pistil, and weakly in the leaf sheath, glume and callus, but not in the leaf blade, anther or root (**Fig. 5A**). RNA in situ hybridization showed that *ONI2* was expressed in L1 of the embryo, young leaf and flower. In the embryo, *ONI2* expression was detected in L1 of developing organs such as leaves and coleoptile (**Fig. 5B**). The expression was hardly detected in the SAM, and no signal was detected in the radicle. In the vegetative growth stage, *ONI2* expression was specifically detected in L1 of young developing leaves, but no signal was detected in the SAM (**Fig. 5C**). In the reproductive growth phase, *ONI2* expression was detected in L1 of the developing panicle and floral organs (**Fig. 5D, E**). No signal was detected in the inner cells in any organs examined. A sense probe also produced no signal above the background (**Fig. 5F**). These expression analyses revealed that *ONI2* is an L1-specific gene of the young above-ground lateral organs.

Fatty acid composition of *oni2*

To examine whether *ONI2* encodes a functional fatty acid elongase, we examined the composition of VLCFAs in a 2-week-old *oni2* shoot. In the *oni2-1* shoot, the amount of free saturated VLCFAs with a carbon number of 20 (C = 20) or more was reduced compared with that of the wild-type shoot. In particular, VLCFAs of C = 32 and C = 34 were barely detected (**Fig. 6A**).

We further examined the composition of VLCFAs in the alkyl ester fraction of the epicuticular wax of a 2-week-old *oni2-1* shoot. We found that the amount of saturated VLCFAs of C = 20, 24, 26, 28 and 30 was reduced in the *oni2-1* shoot compared with the wild type shoot. In particular, VLCFAs of C = 26 and longer were not detected (**Fig. 6B**). These results indicate that VLCFAs in the alkyl ester fraction of epicuticular wax were reduced in the *oni2-1* shoot.

Since VLCFAs are the main components of cuticle wax, we examined protrusions of cuticle on the abaxial surface of the leaf sheath. Scanning electron microscopy (SEM) observation showed that the cuticular architecture with protrusions was well developed on the surface of the leaf epidermis in the wild type, but such protrusions were not observed on the surface of *oni2-1* epidermis (**Fig. 7**). These results indicate that *oni2* had a reduced amount of VLCFAs, which is consistent with the idea that *ONI2* encodes functional fatty acid elongase.

Expression of auxin-related genes

Since expression of *KNOX* genes, which are ectopically expressed in *oni2*, is known to be negatively regulated by auxin (Hay et al. 2006, Tsuda et al. 2009, Perez-Perez 2010, Tabata et al. 2010), and *oni1* showed altered expression of auxin-inducible *IAA* genes, suggesting altered distribution of auxin in its shoot apex (Takasugi and Ito 2011), we examined their expression in *oni2*. Quantitiative real-time PCR analysis showed that *OsIAA7* was up-regulated in *oni2-1*, whereas *OsIAA14* was down-regulated (**Fig. 8**). These results suggest that the expression of auxin-inducible genes is perturbed in *oni2* shoots.

Discussion

In this study, we showed that *ONI2* encodes a fatty acid elongase catalyzing an elongation reaction of VLCFAs and is specifically expressed in L1 in the shoot. In spite of the L1-specific expression of *ONI2*, the effects of *oni2* mutations were not restricted to L1, but were expanded over the entire shoot. From these results, we suggest that *ONI2* and VLCFAs or VLCFA-containing compound(s) are required for L1 development, and L1 supports the development of the entire shoot possibly by producing or transmitting a signal required for inner cell development, in agreement with the previous suggestion that L1 both promotes and restricts shoot growth by providing a non-autonomous signal to inner cells (Savaldi-Goldstein et al. 2007).

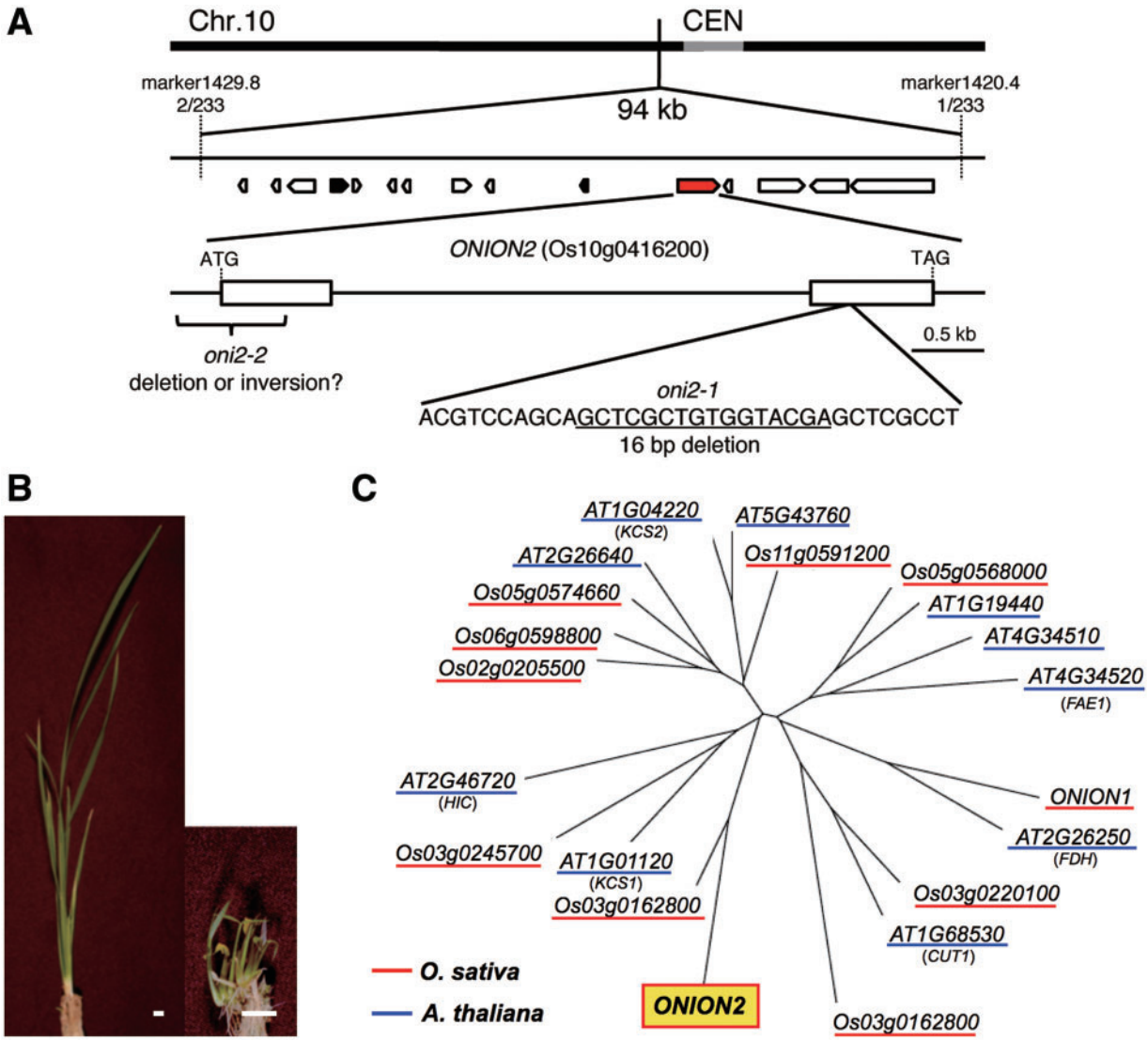

Fig. 4 Cloning of *ONI2*. (A) The *ONI2* locus on chromosome 10. Mapping with 233 F_2 plants narrowed down the *ONI2* locus to within a 94 kb region between marker 1429.8 and marker1420.4 near the centromere. In this region, 15 genes were predicted, one of which, Os10g0416200 marked in red, encodes a protein similar to fatty acid elongase. Os10g0416200 had mutations in two *oni2* mutant alleles examined. (B) Complementation of *oni2* by the fatty acid elongase gene Os10g0416200. The *oni2-1* mutant calli transformed with the wild-type genomic fragment (left) showed regeneration of wild-type shoots, whereas the mutant calli transformed with an empty vector showed regeneration of mutant shoots (right). Bars = 1 cm. (C) A phylogenetic tree of fatty acid elongases of rice and Arabidopsis drawn on the basis of the entire amino acid sequences. Rice and Arabidopsis proteins are underlined in red and blue, respectively. ONI2 is shaded in yellow. AT1G01120, *KCS1*; AT1G04220, *KCS2*; AT1G68530, *CUT1*; AT2G26250, *FDH*; AT4G34520, *FAE1*.

VLCFA biosynthesis genes and shoot development

In addition to *oni2*, several mutants involved in the biosynthesis of VLCFAs have been analyzed thus far. For example, *oni1*, another fatty acid elongase mutant of rice, showed very similar phenotypes to *oni2*, including organ fusions, lack of normal L1, a reduced amount of VLCFAs and perturbed expression of auxin-related genes and *KNOX* genes (Tsuda et al. 2009, Ito et al. 2011, Takasugi and Ito 2011). In Arabidopsis, *fdh* showed organ fusions mainly in the reproductive phase and some defects of epidermal cell differentiation (Yephremov et al. 1999, Pruitt et al. 2000). In addition to genes encoding fatty acid elongase, which catalyzes the first step of the elongation reactions of VLCFAs, some genes involved in other steps of elongation reactions have also been shown to play an essential role in plant development. A double mutant of maize *GL8a* and *GL8b*, both of which encode 3-ketoacyl reductase catalyzing the second step of the elongation reactions, has been shown to result in embryonic lethality (Dietrich et al. 2005). A null mutation in Arabidopsis *PASTICCINO2* (*PAS2*), which encodes a hydroxy fatty acyl-CoA dehydratase catalyzing the third step of the elongation reactions, has also been shown to result

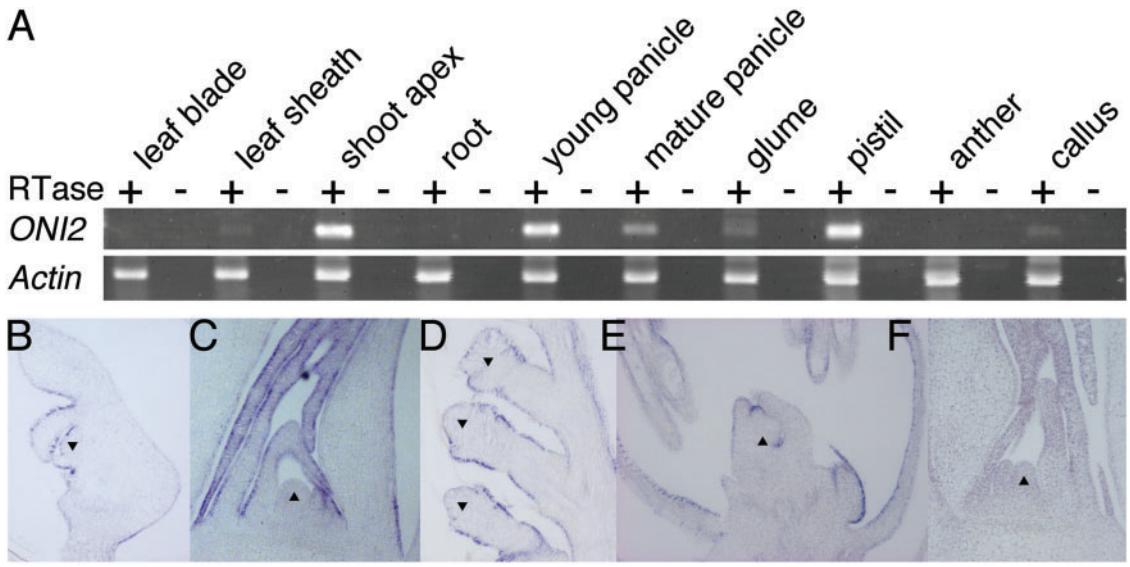

Fig. 5 Expression pattern of *ONI2*. (A) RT–PCR. RNAs isolated from the indicated organs were reverse transcribed with the oligo(dT) primer and amplified by *ONI2* or actin-specific primers (**Supplementary Table S1**). + and – indicate whether reverse transcriptase was added to or omitted from the reaction mixture, respectively. (B–F) RNA in situ hybridization. Longitudinal sections were hybridized with the antisense probe of *ONI2* (B–E). (B) Embryo. (C) Vegetative shoot apex. (D) Panicle branches. (E) Developing flower. (F) Vegetative shoot apex hybridized with the sense probe of *ONI2* as a negative control. Triangles indicate the SAM (B, C and F), rachis branch meristem (D) and developing pistil (E).

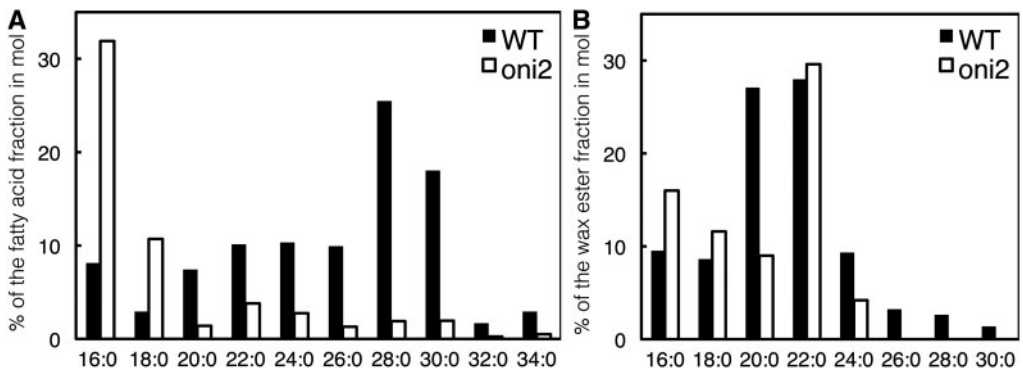

Fig. 6 Amount of saturated VLCFAs in *oni2*. (A) Amount of free saturated VLCFAs. The percentages of saturated VLCFAs per total fatty acids in a molar ratio are shown. The values are the average of two independent experiments. (B) Amount of saturated VLCFAs in the alkyl ester fraction of epicuticular wax. The percentages of methyl esters of saturated VLCFAs per total fatty acid methyl esters in a molar ratio are shown.

in embryonic lethality (Bach et al. 2008). *pas2* mutants also showed defects in plastid division (Nobusawa and Umeda 2012). Since several genes necessary for different steps of VLCFA biosynthesis are required for normal shoot development in different plant species, it is suggested that VLCFAs or VLCFA-containing compounds and their biosynthesis genes play indispensable roles in plant growth and development.

How do VLCFAs affect shoot development? One of the simple explanations is that VLCFAs or VLCFA-containing compounds have a physiological role required for proper L1 development. In this case, altered L1 development is directly affected by a change in the amount or composition of VLCFAs, and a lack of normal L1 consequently affects development of the entire shoot. However, one could argue that perturbing the amount or composition of VLCFAs removes natural barriers that normally block inappropriate information exchange between cells or between a cell and the environment. If signaling is abnormal, downstream developmental events may be abnormal, and this cascade of signaling events results in the evident phenotype. In this case, the primary defect can bring about downstream consequences, and those consequences manifest as a defect in shoot development.

Importance of L1 for shoot development

oni2 showed organ fusions, lack of cuticle protrusions on the surface of the epidermis and perturbed expression of an

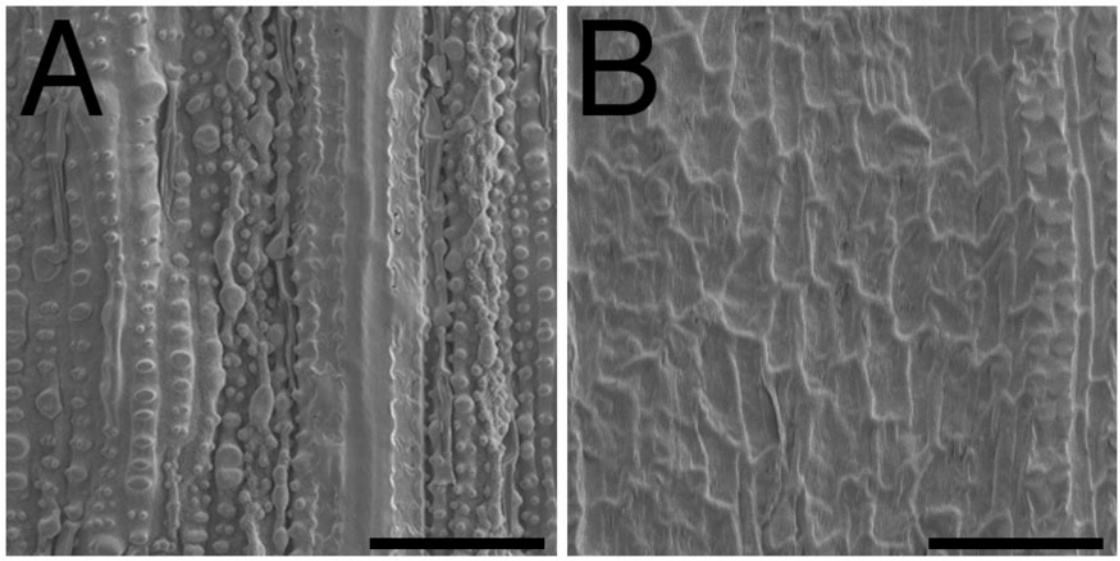

Fig. 7 SEM observation of the leaf surface. (A) Wild type. (B) *oni2*. The surface was rough in the wild type due to cuticle protrusions, but it was rather smooth in *oni2*. Bars = 50 μm.

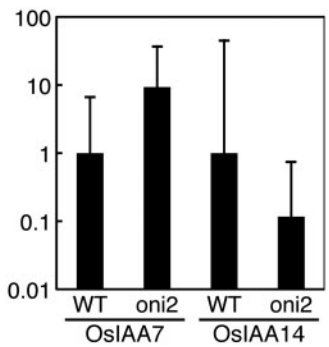

Fig. 8 Expression of auxin-related genes in *oni2* shoots. Expression of *OsIAA7* and *OsIAA14*. RNAs were isolated from shoots of 1-week-old seedlings grown in a growth chamber at 30°C with continuous light, and subjected to quantitative real-time PCR. Actin was used as a reference, and the relative expression level is shown.

L1-specific gene *ROC1*. These phenotypes suggest that the differentiation and/or functionality of L1 is impaired in *oni2*. However, in spite of L1-specific expression of *ONI2*, the effects of the *oni2* mutations were not restricted to L1 as *oni2* did not show simply an epidermis-less or abnormal epidermis phenotype, but expanded to an entire shoot to produce a small shoot and abnormal development of the midrib. Thus, *ONI2* function could be non-cell autonomous. Another fatty acid elongase gene *ONI1* also showed L1-specific expression, and *oni1* mutants had abnormal L1, which brought about abnormal development of the entire shoot. These mutant analyses suggest that L1 is necessary for normal development of inner cells and supports development of the entire shoot. A non-autonomous signal provided from L1 to the inner cells has been suggested in this process in Arabidopsis (Savaldi-Goldstein et al. 2007).

Difference in phenotypic severity between *oni1* and *oni2*

oni2 showed various degrees of phenotypic severity even in the same allele, from severe phenotypes with very small shoots to mild phenotypes with more or less elongated shoots. A simple explanation for the variation in morphological severity is developmental timing and the environment that induce organ fusions. The fusion events may occur only if sustained surface contact is achieved. Otherwise, epidermal cells do not adhere, and growth continues normally.

In contrast to *oni2*, which showed various degrees of phenotypic severity, *oni1* showed only severe phenotypes with very small shoots (Tsuda et al. 2009, Ito et al. 2011). Two possible reasons may be able to account for this difference. One is the difference in substrate specificities between ONI1 and ONI2, and the other is the difference in expression patterns of these two genes.

Analysis of the fatty acid composition showed that *oni2* had a reduced amount of VLCFAs with a carbon number of $\geq$20. This suggests that ONI2 catalyzes an elongation reaction of VLCFAs with a carbon number of $\geq$20. ONI1 was also suggested to catalyze an elongation reaction of VLCFAs with a carbon number of $\geq$20 (Ito et al. 2011). Thus, ONI and ONI2 seemed to have similar substrate specificities, at least when focusing on saturated VLCFAs. Therefore, substrate specificities for saturated VLCFAs may not be the cause of the difference in the phenotypic severity between *oni1* and *oni2*, although the difference in the composition of the non-saturated VLCFAs between these two mutants cannot be ruled out.

In contrast, the difference in expression patterns may be able to explain the difference of phenotypic severity. *ONI1* is strongly expressed in the entire L1 of the SAM and young lateral organs

in the shoot (Ito et al. 2011). In contrast, *ONI2* was expressed in young lateral organs in the shoot, but not in the SAM. The differences in expression patterns may be a possible cause of the difference in the phenotypic severities between *oni1* and *oni2*. Analysis of the *oni1 oni2* double mutant would be a useful approach to understand the functional differences, if any, between *ONI1* and *ONI2*.

Perturbed expression of auxin-inducible genes

Auxin is known to play indispensable roles in various aspects of plant growth and development (Shani et al. 2006). *oni2* lacked normal L1, which is a preferential pathway of polar auxin transport in the rice shoot (Miyashita et al. 2010). This may result in altered auxin distribution in *oni2*. Consistent with this, *oni2* showed altered expression of *IAA* genes whose expression is generally induced by auxin (Jain et al. 2006). *KNOX* genes, which are specifically expressed in the SAM and bring about abnormal shoot morphologies when overexpressed, are ectopically expressed in *oni2* leaves (Tsuda et al. 2009). It is also known that auxin negatively regulates expression of *KNOX* genes, and enhanced auxin response is reported to change leaf morphologies by interacting with a *KNOX* repressing pathway (Hay et al. 2006, Perez-Perez 2010, Tabata et al. 2010). The altered expression of *IAA* and *KNOX* genes is consistent with the idea that auxin distribution might be altered in *oni2* shoots. If this is the case, this might be one of the possible causes of the abnormal shoot morphology of *oni2*. Analysis of auxin distribution in the *oni2* shoot will clarify this notion.

Materials and Methods

Plant materials

oni2 mutants were identified from *Tos17*-mutant populations derived from *Oryza sativa* L. cultivar Nipponbare (Tsuda et al. 2009). Nipponbare was used as the wild type.

Histological analysis

Samples were fixed in FAA [formaldehyde : glacial acetic acid : 50% ethanol (1 : 1 : 18)] overnight at 4°C and then dehydrated in a graded ethanol series. The absolute ethanol in dehydrated samples was replaced with xylene and the samples were embedded in Paraplast plus (McCormick Scientific). Microtome sections (10 μm thick) were stained with Delafield's hematoxylin and observed with a light microscope.

SEM analysis

The leaf sheaths of 10-day-old seedlings grown in an incubator at 30°C under 24 h light conditions were used for SEM analysis. The samples were freeze-dried, coated with platinum–palladium and observed using a Hitachi SU8000.

Cloning of *ONI2*

ONI2 was mapped on the long arm of chromosome 10 (Tsuda et al. 2009). Fine mapping was carried out using an F_2 population of 233 individuals derived from a crossing of an *oni2* heterozygous plant and the indica cultivar Kasalath. Coding regions of a candidate gene were amplified by PCR with the primer combinations e1-2f and e1-2r, and e2-2f and e2-2r, and then sequenced directly. Primer sequences are shown in **Supplementary Table S1**.

Complementation of *oni2*

Selfed seeds of an *oni2* heterozygous plant were used for callus induction on an N6CI medium, and a small part of each callus was used for DNA isolation and subsequently for genotyping. For genotyping, PCR was carried out with the primer combination of ONI2-3 and ONI2-4 (**Supplementary Table S1**). The PCR product amplified from the mutant allele was 16 bp shorter than that from the wild-type allele due to deletion. Homozygous mutant calli were used for *Agrobacterium*-mediated transformation (Hiei et al. 1994). A 10.1 kb *Nco*I fragment, which covers *ONI2* with 5.0 kb upstream and 0.2 kb downstream regions, was cloned into the *Nco*I site of a derivative of pENTR4 (Invitrogen) in which *ccdB* was removed by cutting with *Eco*RI followed by self-ligation, and then cloned into a binary vector plasmid pGWB by LR reaction (Nakagawa et al. 2007). The resultant vector was used for the transformation of the mutant calli. An empty vector was used as a control.

Expression analysis

RT–PCR and quantitative real-time PCR analyses were carried out as described previously using RNAs isolated from the indicated organs and calli (Ito et al. 2001, Ito et al. 2011). The expression level of actin was used as the internal control. Specific primers used for each gene are listed in **Supplementary Table S1**.

RNA in situ hybridization was carried out using paraffin sections prepared as described above (Ito et al. 2001). An RT–PCR product amplified by the primer combination of ONI2-19 and ONI2-20, which covers a complete coding region, was cloned into pCR-BluntII-TOPO and used as a template for transcription of digoxigenin (DIG)-labeled *ONI2* RNA probes. A full-length cDNA of *ROC1* (AK120496) was used for preparation of DIG-labeled *ROC1* RNA probes, which were hybridized with 10-day-old shoot sections.

VLCFA analysis

Analysis of fatty acids was carried out as described previously using shoots of 2-week-old seedlings grown in an incubator at 30°C under constant light (Ito et al. 2011). For analysis of free fatty acids, the sample was ground in methanol and then methanolysed at 80°C for 1.5 h, followed by extraction with *n*-hexane. The *n*-hexane-soluble fraction was subjected to gas chromatography.

For analysis of fatty acids in epicuticular wax, free VLCFAs and alkyl VLCFAs of chloroform-extracted wax were separated by thin-layer chromatography (hexane : ethyl ether : acetic acid, 9 : 1 : 0.1), and derivatized to the methyl ester by an HCl/methanol method (Lepage and Roy 1986). The fatty acid methyl ester composition was analyzed by gas chromatography with a flame ionization detector (GC-380, GL Sciences) and column (ZB-5 ms, 30 m × 0.25 mm internal diameter, 0.2 μm film thickness, Phenomenex). The column temperature program was as follows; 170°C for 1 min, to 200°C at 2°C min^{-1}, to 320°C at 10°C min^{-1} and a hold at 320°C for 10 min.

Supplementary data

Supplementary data are available at PCP online.

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