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Expression and functional analysis of the barley *Nud* gene using transgenic rice

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Most cereal crops have hulless grains (naked caryopses) with a free-threshing trait, whereas the majority of barley cultivars show hulled (covered) caryopses. The naked caryopsis in barley is genetically controlled by a single locus, *nud*. The *Nud* gene (the covered caryopsis allele) encodes an ethylene response factor (ERF) family transcription factor that regulates a lipid biosynthetic pathway. For functional analysis of the barley *Nud* gene, we produced transgenic rice expressing *Nud* in the developing caryopses. All transgenic lines had caryopses that were easily dehulled at maturity, indicating that the naked caryopsis phenotype remained in spite of expression of the *Nud* transgene. Histochemical and lipid analyses of the transgenic rice caryopses did not show increased lipid accumulation on the surface of developing caryopses, suggesting that the *Nud*-mediated lipid pathway may not function in rice caryopses. The predicted rice ortholog of *Nud*, *Os06ERF* was expressed specifically in the developing caryopses. However, expression of *Os06ERF* ceased at an earlier developmental stage than that of the native *Nud* gene in barley caryopses, which was also the case for expression of the *Nud* transgene. This raises the alternative hypothesis that the timing of *Nud* expression may be critical for activating the pathway for hull-caryopsis adhesion.

Key Words: *Nud* gene, transformation, covered/naked caryopsis, lipid biosynthesis, ERF/AP2, grass.

Introduction

Barley (*Hordeum vulgare* L.) has a unique feature among cereal crops with regard to grain (caryopsis) type. The majority of barley cultivars have hulled (covered) caryopses (called covered barley), in which the hull firmly adheres to the pericarp epidermis at maturity. In contrast to barley, all other cereal crops, including rice, wheat and maize, generally have hulless (naked) caryopses with a free-threshing trait. This is unequivocally one of the most important characters for domestication as a food crop. A free-threshing variant with naked caryopses in barley (called naked barley) also appeared in ancient times, though its cultivation is currently limited primarily to East Asia. Covered caryopses with adherent hulls are advantageous for protecting seeds from pathogens and physical damage. In addition to cultivated barley, almost all wild *Hordeum* species have covered caryopses. Wild progenitors of other cereal crops also have ways to protect the seeds, such as difficult-to-separate caryopses and hulls, rather than hull-caryopsis adhesion. For instance, the maize progenitor teosinte has a hardened, protective casing that envelops the kernel, whereas maize has naked grain, free of this casing (Dorweiler *et al.* 1993, Wang *et al.* 2005). Divergence of the covered vs. naked caryopsis trait in the grass family is not only interesting from an evolutionary viewpoint but also worth investigating for its agronomical importance.

The naked caryopsis in barley is genetically controlled by a single locus, *nud*, where the covered type is dominant over the naked one (Franckowiack and Konishi 1997). The *Nud* locus was finely mapped on chromosome arm 7HL (Kikuchi *et al.* 2003, Taketa *et al.* 2004, 2006) and has recently been isolated by positional cloning (Taketa *et al.* 2008). Our previous study demonstrated that the *Nud* gene (dominant allele) in the covered barley encodes an ethylene response factor (ERF) family transcription factor, while a 17-kb deletion harboring the ERF gene was found in all 100 naked barley cultivars tested (Taketa *et al.* 2008). The *Nud* gene has homology to the *Arabidopsis WIN1*/*SHN1* (Aharoni *et al.* 2004, Broun *et al.* 2004) transcription factor gene that regulates a lipid biosynthetic pathway and plays a role in tissue separation during development. This observation is consistent with the heavy lipid accumulation on the caryopsis surface observed only in covered barley (Taketa *et al.* 2008). Histological analyses also showed that the covered caryopsis-specific lipid layer appearing on the pericarp epidermis matched the site of adhesion with the hull. Together with the observation that expression of *Nud* is localized to the testa (a layer inside the pericarp), it was speculated that Nud protein might activate production of special lipids in the testa, which are secreted out of the pericarp layer and somehow cause hull-caryopsis adhesion (Taketa *et al.* 2008). However, both the biochemical properties of the lipids and the involvement of Nud transcription factor in the

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biosynthetic pathway remain to be investigated.

It remains unknown whether the mechanism of hull-caryopsis adhesion mediated by the Nud transcription factor is conserved in cereals. In this study, we addressed this question using transgenic rice transformed with the barley *Nud* gene. The genomic sequence of the *Nud* gene including its native promoter was transferred into rice by *Agrobacterium*-mediated transformation. We obtained five transgenic rice lines expressing *Nud* in developing caryopses. Phenotypic and histochemical analyses of transgenic rice caryopses revealed significant differences between barley and rice caryopses expressing *Nud* with regard to hull-caryopsis adhesion and lipid synthesis as well as the timing of expression of *Nud* member genes during the development of caryopses.

Materials and Methods

Plant materials and genetic transformation

A *japonica* rice cultivar, Nipponbare, was used for *Agrobacterium*-mediated transformation. The genomic fragment of *Nud* used for the transformation construct was obtained from a BAC clone (589B20) from the covered barley cultivar Haruna Nijo (Taketa *et al.* 2008). A 1.6-kb *Hin*dIII fragment containing the complete coding region was subcloned from the BAC clone into vector pBluescript SK. Further 5′ flanking sequence was PCR-amplified from the BAC clone, cloned into vector pCR2.1 and checked for the correct sequence. After digestion with *Hin*dIII, the 5′ flanking sequence (987 bp) was ligated to the 5′ end of the 1.6-kb *Hin*dIII fragment. The NOS terminator sequence was also ligated to the *Kpn*I site near the 3′ end of the fragment. Thus, the cloned genomic fragment of *Nud* was 2557 bp, including the promoter sequence of 1555 bp. It was inserted into the *Agrobacterium* binary vector pWBVec.8 (Wang *et al.* 1998) to generate the *Nud* gene construct for transformation (Fig. 1A). The plasmid construct was introduced into *Agrobacterium* strain AGL1 and used for rice transformation according to the protocol of Hiei *et al.* (1994).

For analysis of transgenic rice, five independent primary (T_0) lines (1-3, 2-1, 2-3, 5-2 and 5-4) were mainly used, while individuals selected from their selfed T_1 progeny were also used for some of the experiments. Transgenic rice plants were grown in a glasshouse under natural conditions. For histochemical analyses, covered barley cultivars Bowman and Golden Promise, and a naked isogenic line of Bowman (*nud*-Bowman, Taketa *et al.* 2008) were used.

DNA blot and RT-PCR analyses

DNA blot analysis was performed as described previously (Kakeda *et al.* 2008). Ten micrograms of genomic DNA from each plant was digested with *Hin*dIII, blotted and hybridized to PCR-amplified probe sequences of *Nud* (amplified with primers Nud-F and Nud-R) and *Os06ERF* (amplified with primers Os06ERF-F and Os06ERF-R) (see Table 1 for primer sequences). The filter was finally washed twice with 0.1× SSC at 60°C.

Table 1. Sequences of primers used in this study

Primer	Sequence (5′-3′)
Nud-F	GAGATCAGGCATCCTCTCCTG
Nud-R	CTCTCTTCGTCCTCCATTG
Os06ERF-F	GTGAGATCATGGGACAGTCG
Os06ERF-R	CCTGTTCTTCCGGAATTCAG
Os02ERF-F	AACTCCACCGGTGATCTCGC
Os02ERF-R	ACCCTCACATGGAGAGGGAG
Actin-F	TTGAAGTACCCGATCGAGCATG
Actin-R	CAGGCAGCTCATAGCTCTTCTC

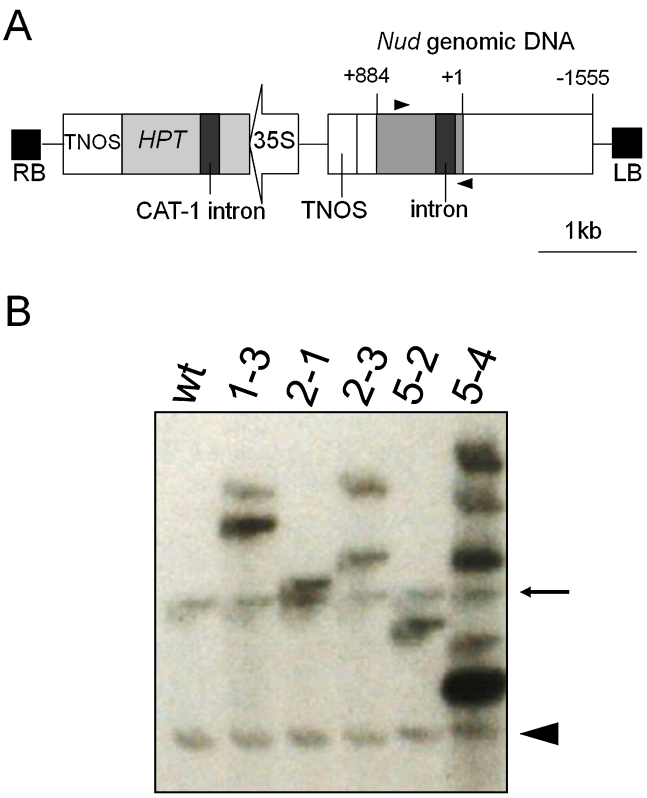

Fig. 1. *Nud* gene construct and its integration in the genome of transgenic rice plants. (A) Binary vector construct used for *Agrobacterium*-mediated transformation. Genomic sequence of the barley *Nud* gene with the nopaline synthase (TNOS) terminator sequence added was introduced into the *Agrobacterium* vector pWBVec.8 (Wang *et al.* 1998) carrying the 35S promoter-driven hygromycin-resistance (*HPT*) gene with a CAT-1 intron. Numbers above the *Nud* gene indicate nucleotide positions from the translation start site (+1) and the coding region is shaded. Arrows indicate positions of primers used for RT-PCR analysis of the *Nud* transgene. RB, right border; LB, left border. (B) DNA blot analysis of transgenic rice plants. Genomic DNA from the wild-type Nipponbare (wt) and five primary transgenic (T_0) lines (1-3, 2-1, 2-3, 5-2 and 5-4) was digested with *Hin*dIII and hybridized with the *Nud* gene probe. Arrow and arrowhead indicate hybridizing bands of rice *Nud* homologs *Os06ERF* and *Os02ERF*, respectively.

For expression analysis, total RNA from each tissue was extracted using an RNeasy Mini Kit (Qiagen) and then treated with DNase I and precipitated with ethanol. First-strand cDNA was synthesized using 5 μg total RNA and used as a

template for reverse transcription (RT)-PCR according to Rahman *et al.* (2007). The conditions for PCR were heating at 95°C for 2 min; 28 cycles of denaturation for 30 sec at 95°C, annealing for 30 sec at 58°C and extension for 1 min at 72°C, with a final cycle at 72°C for 5 min. Actin (GenBank accession no. AJ234400) was used as the control. Primers used for RT-PCR are also shown in Table 1.

Histochemical analyses and microscopy

Caryopses with hulls were fixed with FAA solution (5% formalin, 5% acetic acid, 45% ethanol), dehydrated, and embedded in paraffin (or Technovit only in the case of rice caryopses at 3 weeks after anthesis). Using a microtome, 10-µm-thick grain sections were prepared and observed under a light or fluorescent microscope after staining with 0.1% Toluidine blue (Wako), 0.1% Sudan black B (Wako; dissolved in 70% ethanol) or 0.5% Calcofluor white (Sigma). Dehulled caryopses were also stained with each of these three dyes following the protocols of Tanaka *et al.* (2004), Taketa *et al.* (2008) and Bessire *et al.* (2007), respectively. Chlorophyll leaching and water-loss assays were also done according to Taketa *et al.* (2008).

Scanning electron microscopy analysis was performed to examine the surface structure of caryopses using a Hitachi Miniscope. Unfixed caryopsis samples were directly transferred to the Miniscope and examined under default conditions.

Surface lipid analysis

For extraction of surface lipids, 20 dehulled caryopses collected from 2-week-old grains were immersed in 3 ml of hexane with gentle agitation for 5 min at room temperature. The extracts were dried under a stream of nitrogen and dissolved into 20 µl of chloroform : methanol (2 : 1). A 2-µl aliquot of each sample was applied to an HPTLC plate (Merck), separated with hexane : benzene (1 : 1) and detected with sulfuric acid.

Results

Expression analysis of the Nud transgene and homologous genes in rice

Transformation of rice (Nipponbare) was carried out using a binary vector constructed with the genomic sequence of *Nud* isolated from covered barley (Fig. 1A). *Agrobacterium*-mediated transformation resulted in five independent T_0 plants regenerated from hygromycin-resistant calli. DNA blot analysis confirmed the integration of one to five copies of the *Nud* transgene in the genomes of these T_0 lines (named 1-3, 2-1, 2-3, 5-2 and 5-4) in addition to the presence of homologous rice genes (indicated by an arrow and arrowhead; Fig. 1B). We also confirmed transmission of the transgenes to the next generation (T_1) by DNA blot analysis (data not shown).

RT-PCR analysis clearly showed that the *Nud* transgene was expressed in the caryopses at 7 days after pollination

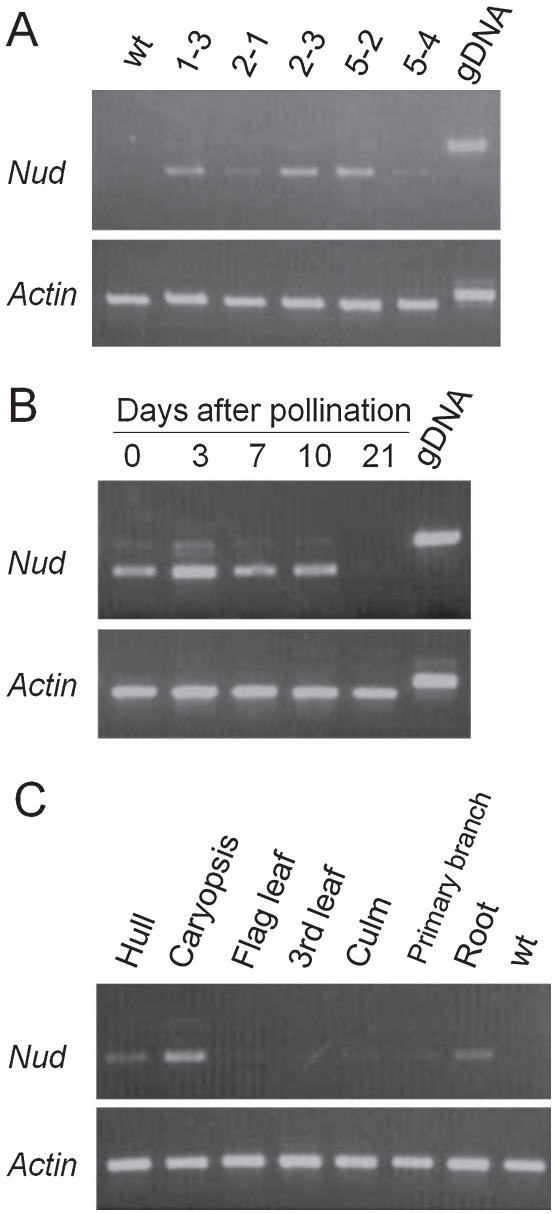

Fig. 2. RT-PCR analysis of the *Nud* transgene in transgenic rice. (A) Expression of *Nud* in the caryopses at 7 days after pollination (7DAP) in five transgenic lines. wt, wild-type (Nipponbare); gDNA, genomic DNA from transgenic line 2-3. (B) Temporal expression pattern of *Nud* in the caryopses at different stages (0-21DAP) from transgenic line 2-3. (C) Spatial expression pattern of *Nud* in various organs from transgenic line 5-2. Hulls and caryopses were sampled at 7DAP. wt, caryopsis from wild-type (Nipponbare).

(DAP) in all five T_0 transgenic lines (Fig. 2A). The level of expression varied with the line: transgenic lines 2-1 and 5-4 showed a lower level of expression than the other three lines. A presumed unspliced transcript of *Nud* was also detected as a faint band that corresponds to the size of amplicon from the genomic DNA. In the developing caryopses, maximum expression of the *Nud* transgene was detected at 3DAP (Fig. 2B). The level of expression then decreased at 7DAP and 10DAP, while expression was hardly detectable at

21DAP (Fig. 2B). This pattern of expression was common to all five lines, and thus the expression profile of line 2-3 is representatively shown in Fig. 2B. This is in contrast to the temporal pattern of expression of the native *Nud* gene in barley caryopses: *Nud* is expressed only in the caryopsis with a peak at 2 weeks after anthesis; expression continues to 3 weeks after anthesis (Taketa *et al.* 2008). The tissue specificity of the *Nud* transgene was not as strict as that of the native *Nud* gene in barley: weak expression was also detected in hulls and roots (Fig. 2C).

Our previous paper reported the presence of two *Nud* homologs in rice, named *Os06ERF* (locus ID Os06g0604000) and *Os02ERF* (Os02g0202000), the former of which was assumed to be the rice ortholog of *Nud* based on a neighbor-joining tree constructed using the deduced amino acid sequences (Taketa *et al.* 2008). We confirmed that a hybridizing band in Fig. 1B (indicated by arrow) corresponds to *Os06ERF* based on a high stringency wash of the DNA blot probed with the gene (data not shown). We analyzed expression of the *Nud* homologs in wild-type rice by RT-PCR (Fig. 3). The analysis showed that the rice *Os06ERF* gene had a spatial and temporal pattern of expression similar to that of *Nud* in barley: the expression was specific to caryopses (Fig. 3, left) and the level of expression gradually increased during the development of the caryopsis (Fig. 3, right). However, it is important to note that the duration of expression in caryopses differs between *Nud* and *Os06ERF*. Transcripts of *Os06ERF* could not be detected in rice caryopses at 3 weeks after anthesis (21DAP), whereas expression of *Nud* in barley caryopses continues to this stage despite a small decrease in expression level (Taketa *et al.* 2008). The same pattern of temporal expression of *Os06ERF* was also confirmed in caryopses of transgenic rice (data not shown). On the other hand, another *Nud* homolog, *Os02ERF* (referred to as *OsSHN1* in the paper by Aharoni *et al.* 2004), showed a very low level of expression with little tissue specificity (Fig. 3). In developing caryopses, *Os02ERF* was expressed at a low level until one week after anthesis (7DAP) but could not be detected after 10DAP.

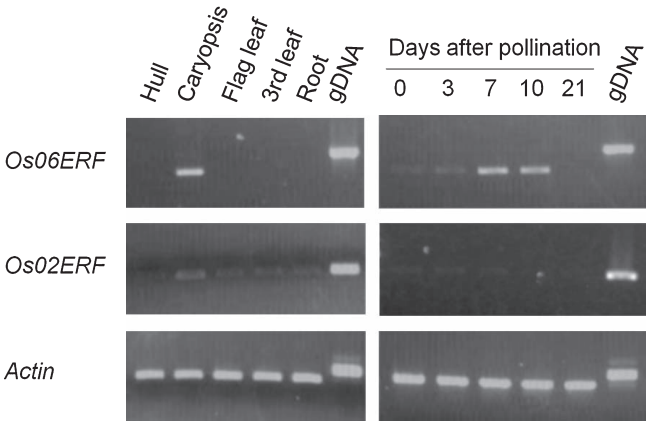

Fig. 3. Expression of *Nud* homologs in wild-type rice. RT-PCR patterns of *Os06ERF* and *Os02ERF* genes in different tissues (left) and developing caryopses at different stages (right) from Nipponbare. Hulls and caryopses in left panels were sampled from grains at 7DAP. All caryopses were dehulled before sampling. gDNA, genomic DNA.

Phenotype and histochemical analyses of caryopses

The phenotype of transgenic rice caryopses was examined at immature and mature stages (Fig. 4, upper and lower panels). No visible change was observed at any stage of caryopsis development between wild-type and transgenic lines. In covered barley, a sticky adhesive substance appears on the surface of immature caryopses before adhesion with the hull (Harlan 1920). However, we did not detect any stickiness on the surface of caryopses from any transgenic lines. At maturation, the caryopses of all transgenic lines were easily dehulled, displaying no particular difference compared to wild-type. Thus, the naked caryopsis phenotype remained unchanged in the transgenic rice. A noticeable phenotypic change was observed only in transgenic line 5-4: it showed a semi-dwarf plant type and low fertility (data not shown). This could be due to deleterious effects caused by the high copy number of transgenes in this line (Fig. 1B).

A distinctive feature of the caryopsis in covered barley is strong staining with the lipophilic dye Sudan black B on the dehulled surface at 2 to 3 weeks after anthesis (Taketa *et al.* 2008). However, none of the immature caryopses from any of the transgenic lines was stained with the dye at any stage (see examples at 14DAP in the upper panel of Fig. 4). We also tested staining of the caryopsis surface with Toluidine blue and Calcofluor white, which were used to detect changes in cuticular wax and surface permeability of *Arabidopsis* mutants (Bessire *et al.* 2007, Tanaka *et al.* 2004). We did not observe any difference in the pattern of staining with either dye between wild-type and transgenic lines (data not shown). In tissue sections of 3-week-old caryopses from covered barley, Sudan black B staining allowed detection of a clear lipid layer on the pericarp surface (Taketa *et al.* 2008; Fig. 5A). This lipid layer was not detected in caryopsis sections from either wild-type (Fig. 5B) or transgenic rice (Fig. 5C), which was similar to the case in naked barley (Taketa *et al.* 2008). In this study, we also stained the caryopsis sections with Calcofluor white (Fig. 5D–F). This staining showed tight adhesion between the hull and the pericarp in covered barley (Fig. 5D), which was apparently absent in the caryopses of both wild-type (Fig. 5E) and transgenic rice (Fig. 5F).

Scanning electron microscopy was used to investigate the surface of caryopses (Fig. 6). We did not find any differences between the surface of developing caryopses at 3 weeks after anthesis (21DAP) for wild-type (Fig. 6A) and transgenic rice (Fig. 6B): both surfaces were smooth and showed little wax deposition. We also observed the caryopsis surface of covered barley. At 3 weeks after anthesis, the hull had not yet adhered to the caryopsis but a sticky substance was detectable. At this stage, however, the surface of dehulled caryopses in covered barley was as smooth as that of rice caryopses and had no covering wax deposit (Fig. 6C). At

Fig. 4. Phenotype of transgenic rice caryopses. (Upper panel) Immature caryopses at 14DAP stained with Sudan black B. (Lower panel) Mature grains with hulls (left) and dehulled grains (right). wt, wild-type (Nipponbare).

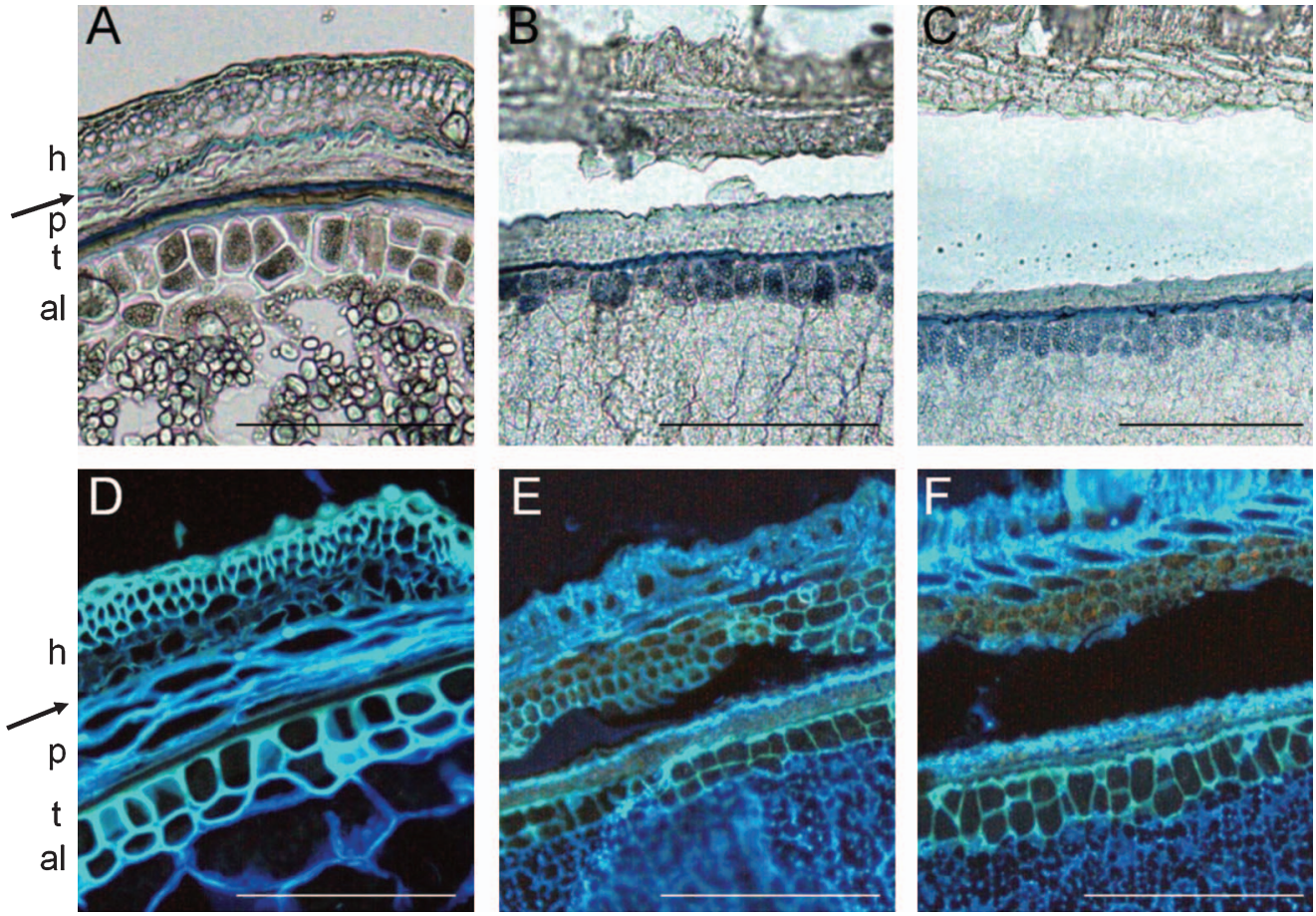

Fig. 5. Caryopsis sections stained with Sudan black B (A–C) and Calcofluor white (D–F). Caryopses at 3 weeks after anthesis (21DAP) from covered barley (Golden Promise) (A, D), and those at 2 weeks after anthesis (14DAP) from wild-type rice (Nipponbare) (B, E) and transgenic rice (line 2-3) (C, F). h, hull; p, pericarp; t, testa; al, aleurone layer. Arrows in (A) and (D) indicate the hull-caryopsis adhesion region observed in covered barley. Scale bars indicate 1 mm.

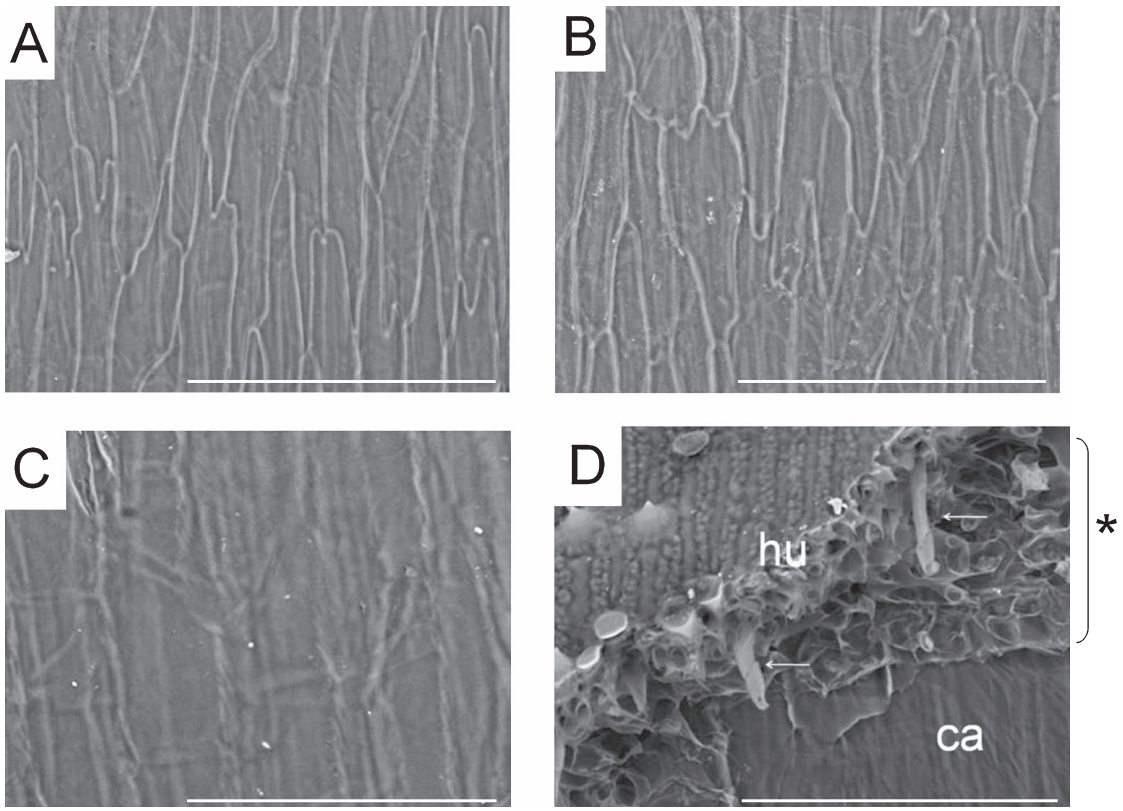

Fig. 6. The caryopsis surface observed by scanning electron microscopy. (A) Wild-type rice (Nipponbare). (B) Transgenic rice (line 2-3). (C, D) Covered barley (Golden Promise). Dehulled caryopses at 3 weeks after anthesis (21DAP) were used in (A–C). The hull (*hu*)-caryopsis (*ca*) adhesion region (marked by an asterisk) in covered barley at 4 weeks after anthesis (28DAP) is shown in (D), where the adhering hull was peeled away from the caryopsis. Arrows indicate hair-like structures inside the hull. Scale bars indicate 100 μm.

4 weeks after anthesis, the hull-caryopsis adhesion started to form and the caryopsis became difficult to dehull in covered barley. Fig. 6D shows the boundary zone between the hull and caryopsis at this stage, where a portion of the adhering hull was peeled away from the caryopsis. Hair-like structures (arrows) appeared inside the hull. However, it seemed that the dehulled part of the caryopsis surface (marked 'ca' in Fig. 6D) was still smooth and unchanged from that of the 3-week-old caryopsis (Fig. 6C).

Surface lipid and permeability analyses

Surface lipids were extracted from 2-week-old dehulled caryopses and separated by TLC (Fig. 7; details are in methods section). Using this protocol, we could detect a clear difference between covered (C) and naked (N) barley (Bowman isogenic lines): a covered barley-specific spot (indicated by arrow) appeared in the region where hydrocarbons (HC) migrated. Wild-type and transgenic rice lines had a couple of HC spots, one of which appeared at nearly the same position as the covered barley-specific spot. The intensity of this spot varied with the transgenic line: the spots of transgenic lines 2-3 and 5-2 were much more intense than that of wild-type, whereas that of line 1-3 was least intense. There was also a little difference in the intensity of another HC spot (a lower one) among transgenic lines. Several additional spots that were not clearly observed in barley (indicated by SE, W, TG, FA in Fig. 7) were detected from rice caryopses, but no remarkable difference was found in the patterns of these spots between wild-type and transgenic rice.

To examine whether the surface permeability of caryopses was altered in transgenic rice, we performed chlorophyll leaching and water-loss assays. Both assays have previously suggested that the pericarp epidermis in covered barley has significantly enhanced permeability compared to naked barley (Taketa *et al.* 2008). Here, such experiments using 2-week-old caryopses from wild-type and transgenic rice were repeated. However, neither of the assays provided a clear indication that the permeability of caryopses from transgenic lines was altered compared to wild-type (data not shown).

Discussion

In this study, we have shown that expression of the barley *Nud* gene in transgenic rice caryopses did not alter the naked caryopsis phenotype. This indicates that the barley Nud transcription factor is not sufficient to change the rice caryopsis to be hulled, although we could not completely rule out the possibility that a functional form of the Nud protein was not

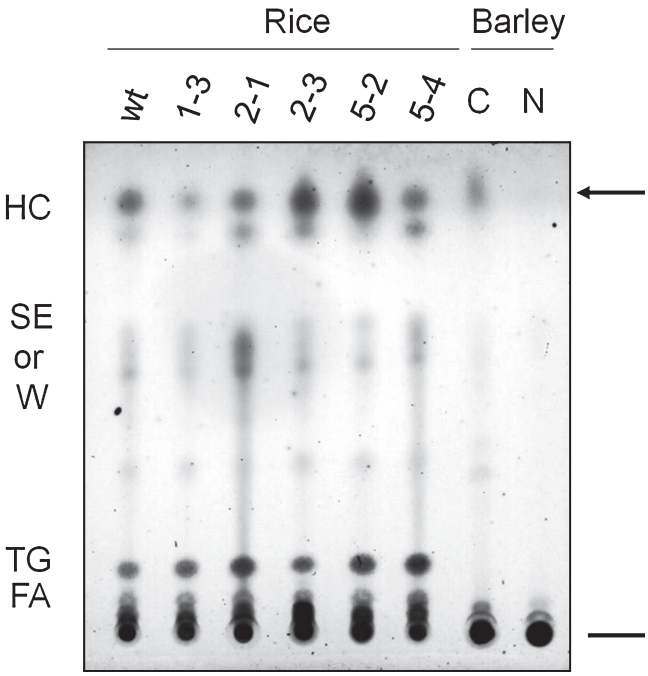

Fig. 7. Surface lipid analysis by TLC. Surface lipids were extracted with hexane from 2-week-old dehulled caryopses in wild-type (wt) and transgenic rice, and from covered (C) and naked (N) barley. After TLC separation, lipids were detected with sulfuric acid. Bar indicates the origin. Covered barley-specific spot is marked by an arrow. HC, Hydrocarbons; SE, Sterol esters; W, Waxes; TG, Triglycerides; FA, Fatty acid.

produced in transgenic rice plants. The Nud protein is homologous to WIN1/SHN1, a member of the *Arabidopsis* ERF (AP2/EREBP) family of transcription factor proteins (Aharoni *et al.* 2004, Broun *et al.* 2004). Of the two rice homologs (*Os02ERF* and *Os06ERF*) belonging to the same ERF gene family, *Os06ERF* was predicted to be the ortholog of *Nud* based on amino acid homology (Taketa *et al.* 2008). This conclusion was supported by the specific expression of *Os06ERF* in developing caryopses observed in this study, which was similar to the expression pattern of *Nud* in barley. The predicted Os06ERF protein shows a 74% amino acid identity to Nud and does not carry any apparent mutation that would cause a loss of function. Together with the results obtained from transgenic rice expressing the *Nud* transgene, the observation that rice caryopses expressing the endogenous *Nud* ortholog (*Os06ERF*) showed no hull-caryopsis adhesion suggests the hypothesis that the Nud family of transcription factors may not be sufficient to change the rice caryopsis to be hulled. Members of the WIN1/SHN1 family of transcription factors are known to activate wax biosynthesis in general, and are thought to have diverse functions by regulating metabolism of lipid and/or cell wall components (Aharoni *et al.* 2004). The barley *Nud* gene may have a highly specialized function in hull-caryopsis adhesion based on its very limited expression, in the testa of developing caryopses (Taketa *et al.* 2008). Our preliminary analysis showed that several species in the Triticeae (*Secale*, *Aegilops*, and *Triticum*, which are more closely related to barley than rice) carrying a naked caryopsis also have *Nud* homologs that seemingly carry no dysfunctional mutations (unpublished results). Although it remains to be investigated whether these homologs are expressed appropriately, it may be speculated that in naked grass species where the *Nud* ortholog is conserved, the *Nud*-mediated pathway does not function, possibly by the loss or mutation of other unknown components involved in the pathway.

Another possible cause for the unchanged naked phenotype in transgenic rice caryopses may be concerned with the timing of expression of *Nud* and its homologous genes (*Nud* member genes). In this study we found a slight but obvious difference in the temporal pattern of expression between barley *Nud* and its rice ortholog (*Os06ERF*). In the developing caryopses of covered barley, expression of *Nud* continues until at least 3 weeks after anthesis (Taketa *et al.* 2008). At this stage, hull-caryopsis adhesion begins, and later becomes firmly established, at 4 weeks after anthesis. In contrast, we found that expression of *Os06ERF* reached a maximum level much earlier (7DAP) than *Nud* (14DAP, Taketa *et al.* 2008) and ceased during an earlier stage (prior to 3 weeks after anthesis) of development in rice caryopses. This temporal offset of expression might cause *Os06ERF* to fail to activate the lipid pathway that is necessary and sufficient for the subsequent adhesion process, even though the Os06ERF protein could retain functions equivalent to Nud. Interestingly, despite the *Nud* transgene being driven by its native promoter, expression of the *Nud* transgene in transgenic rice caryopses disappeared earlier (21DAP) than that of the native *Nud* gene, which was similar to the expression of the endogenous *Os06ERF* gene. This suggests the possibility that the transcriptional control of *Nud* member genes might differ in rice and barley caryopses.

It has been reported that mutations in transcription factors play important roles in crop domestication (Doebley *et al.* 2006). Of these, a mutation in the rice domestication gene *qSH1*, which encodes a HOX transcription factor responsible for seed shattering (Konishi *et al.* 2006), represents the importance of a change in expression of a transcription factor gene. A single nucleotide polymorphism in the cis-regulatory element led to the loss of *qSH1* expression specific to the abscission layer; thereby, grains could remain attached to the spike at harvest in cultivated rice (Konishi *et al.* 2006). To address whether the period of expression of *Nud*/*Os06ERF* is critical for hull-caryopsis adhesion in rice, it will be useful to analyze the effects of overexpression of the gene throughout caryopsis development. To elucidate the mechanism of hull-caryopsis adhesion, it is essential to analyze the chemical properties of the unknown adhesive substance that appears on the caryopsis surface. Although it was not clear whether or not the level of synthesis of particular lipids increased in developing caryopses of our transgenic rice plants, overexpression lines could be used efficiently for such lipid analyses. Thus, together with efforts toward

biochemical characterization of covered barley-specific lipids, work is in progress to produce and analyze overexpression transgenic lines.

Acknowledgments

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