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Brief Communication

Transcription factors NAC20 and NAC26 interact with RPBF to activate albumin accumulations in rice endosperm

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Storage proteins (SPs) and starch deposited in cereal endosperms are the most important sources of human food. SPs are classified into glutelins, prolamins, albumins and globulins according to their solubility in various solvents (Müntz, 1998). Most of these proteins are encoded by multiple genes and deposited specifically in endosperms through the regulation of transcription factors (TFs) (Liu *et al*., 2022). Among them, albumin is the most abundant water-soluble protein (Müntz, 1998), and the most important allergenic protein in the rice endosperm (Matsuda *et al*., 1991). Thus, understanding molecular machinery underlying albumin accumulations is critically important.

In maize, NAC TFs, NAC128/130, regulate starch and SP accumulations (Zhang *et al*., 2019). *NAC20* and *NAC26* (*NAC20/26* hereafter) are specifically expressed in developing rice endosperm, and mutations of both genes led to floury endosperm, together with compromised accumulations of starch and SP (Wang *et al*., 2020). In this study, a *NAC20/26* double knockout mutant, named *nac20/26-3*, was generated in a *Japonica* rice variety Zhonghua 11 (ZH11; Figure S1), which exhibited similar floury endosperm phenotype as reported (Wang *et al*., 2020; Figure S2). SDS-PAGE analyses of total proteins extracted from mature *nac20/26-3* endosperms showed that a 16-kDa band was disappeared, and a 26-kDa band was attenuated greatly when compared with ZH11 (Figure 1a). Previous studies suggest that the former is prolamin, and the latter is α-globulin (αGlb; Wang *et al*., 2020). Immunoblotting performed in this study showed that αGlb accumulation was significantly reduced, while prolamin was only slightly reduced in *nac20/26-3* (Figure S3), which is inconsistent with the SDS-PAGE result (Figure 1a). Subsequently, mass spectrometry analysis of the 16-kDa band excised from gel showed that abundances of four albumins encoded by *Os07g11360* (*Alb1*), *Os07g11380* (*Alb2*), *Os07g11330* (*Alb3*) and *Os07g11410* (*Alb4*) were decreased sharply in *nac20/26-3* (Table S1). Immunoblot analysis using anti-Albs antibody confirmed that albumin accumulations were almost disappeared in *nac20/26-3* (Figure 1a). As expected, when water-soluble proteins were extracted and analysed by SDS-PAGE, the albumin band was hardly detectable in *nac20/26-3* (Figure 1b). Systematic analyses of reported 16-kDa albumins and allergenic proteins, together with whole-genome sequencing data, showed that 16-kDa albumins in rice are encoded by five genes including *Alb1~4* and *Os07g11510* (*Alb5; Alb1~5* hereafter; Figure S4; Wakasa *et al*., 2011; Zhou *et al*., 2017). qRT-PCR analyses revealed that expressions of *Alb1~5* were decreased dramatically in *nac20/26-3* endosperms (Figure 1c), suggesting that NAC20/26 may regulate albumin deposits in rice endosperm through transcriptional activations.

To confirm if NAC20/26 activate directly the expressions of *Alb1~5*, a dual-luciferase (LUC) transactivation assay was performed in rice protoplasts by co-transformations pairwisely of effector constructs of *p35S:NAC20* and *p35S:NAC26*, and reporter constructs of either *pAlb1:LUC*, *pAlb2:LUC*, *pAlb3:LUC* or *pAlb4:LUC* or *pAlb4:LUC5*. Results showed that NAC20/26 activated *Alb1~5* expressions, in which NAC26 was more effective than NAC20 (Figure 1d; Figure S5). Domain swapping experiment, together with transactivation assay in rice protoplasts, showed that the intermediate region of NAC26, from 149th to 214th amino acid (AA), attributed to its higher efficiency (Figure 1e; Figure S6). CACG as the core binding motif of NAC TFs is present in promoter regions of 26-kDa *globulin*, 16-kDa *prolamin*, *GluA-1* and *GluB4/5* (Wang *et al*., 2020; Figure S7). CACG motifs (Figure S8), some with conserved flanking sequences (Figure S9), were identified in 5′ upstream sequences of *Alb1~5*. Bindings of NAC20/26 to *Alb1~2* 5′ upstream sequences, as representatives, were confirmed by electrophoretic mobility shift assay (EMSA; Figure 1f). Competition assay with probes carrying single-nucleotide substitutions covering CACG motif and its flanking nucleotides in *Alb1* promoter, as a representative, showed that ACG was critical for NAC20/26 bindings (Figure S10). These results together demonstrate that NAC20/26 regulate albumin expressions through binding to ACG motif.

We then used NAC20 to identify interacting proteins through yeast two-hybrid. As the full-length NAC20 exhibited auto-activation in yeast, a fragment from 86th to 320th AA was selected as the bait. Among 65 putative interaction clones identified (Table S2), two encoded Rice Prolamin Binding Factor (RPBF) that regulates starch and SP accumulations in rice (Kawakatsu *et al*., 2009). Firefly

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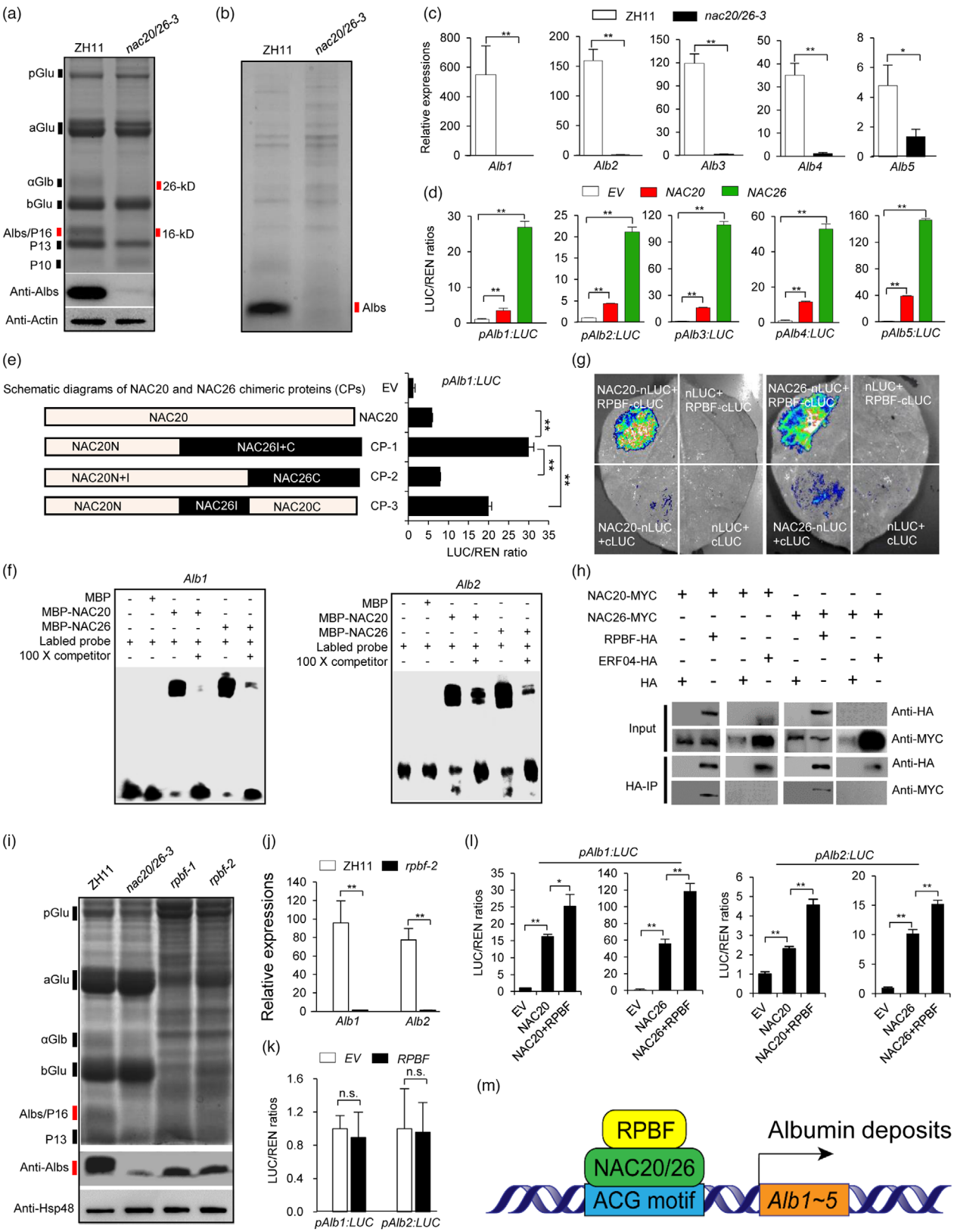

luciferase complementation imaging (LCI) and bimolecular fluorescence assays performed in tobacco leaves showed that NAC20/26 interacted with RPBF (Figure 1g; Figure S11). Co-immunoprecipitation (Co-IP) analysis showed that both NAC20 and NAC26 precipitated RPBF (Figure 1h).

Two independent mutant alleles, *rpbf-1* and *rpbf-2*, were generated by gene-editing, and their grains exhibited shrunken phenotype as reported (Kawakatsu *et al.*, 2009; Figure S12). SDS-PAGE analysis revealed that intensities of major SPs including albumin were reduced in their endosperms (Figure 1i, upper

Figure 1 NAC20/26, together with RPBF, regulate albumin accumulations in rice endosperm. (a) SDS-PAGE (upper) and immunoblot analysis in total proteins extracted from mature ZH11 and *nac20/26-3* endosperms. Anti-Actin antibody as a control. pGlu, Glutelin precursor; aGlu, acidic glutelins; bGlu, basic glutelins; 26-kDa, α-globulin (αGlb); 16-kDa, Albs/prolamin (P16); P13, 13-kDa prolamin; P10, 10-kDa prolamin. (b) SDS-PAGE analysis of water-soluble proteins in matured endosperms to show greatly reduced abundance of 16-kDa albumins in *nac20/26-3*. (c) qRT-PCR to analyse expressions of *Alb1~5* in ZH11 and *nac20/26-3* endosperms at 9 days after pollination (DAP). (d) Transactivation assay to show that NAC20/26 activated *Alb1~5* expressions in rice protoplasts. EV, empty vector. (e) Domain swapping analyses in rice protoplasts showed that the intermediate region in NAC26 attributed to its higher activation activity. (f) EMSA to show that NAC20/26 bind to *Alb1~Alb2* promoters. LCI (g) and Co-IP assays (h) performed in tobacco leaves to show that NAC20/26 interacted with RPBF. A non-relevant TF ERF04 used as a negative control (i) SDS-PAGE (upper) and western blot of total proteins from mature endosperms to show compromised SPs accumulations in *rpbf-1* and *rpbf-2*, compared with ZH11 and *nac20/26-3*. Anti-Hsp48 as a control. (j) qRT-PCR to show decreased *Alb1~2* expressions in *rpbf-2* endosperms at 9 DAP. (k, l) Transactivation assay performed in rice protoplasts to show that RPBF did not activate expressions of *Abl1~2* (k), but enhanced activities of NAC20/26 (l). n.s., not significance. (m) A proposed model of RPBF-NAC20/26 complex that regulates albumin accumulations in rice endosperm. Data were shown as means ± SD, $n = 3$; $*P < 0.05$; $**P < 0.01$; Student's *t*-test.

panel). Immunoblot analysis using anti-Albs confirmed that, compared with ZH11, albumin accumulation was significantly reduced in *rpbf-1* and *rpbf-2* (Figure 1i, middle panel). qRT-PCR analyses performed in *rpbf-2* endosperms confirmed that expressions of *Alb1~2* (as representatives) were dramatically decreased (Figure 1j), suggesting that RPBF regulates albumin accumulations through transcriptional activation. It has been showed that RPBF activates *Alb1* expression in rice callus protoplasts (Yamamoto *et al*., 2006). Although a confined RPBF-binding motif AAAG was frequently identified in promoter regions of *Alb1~5*, no intact P-box (TGTAAAG) has been found (Figure S8). Dual-luciferase transactivation assay performed in seedling protoplasts by co-transformations pairwisely of effector construct *p35S:RPBF* with reporter constructs of either *pAlb1:LUC* or *pAlb2:LUC* showed that RPBF activated expressions of *Alb1~2* (Figure 1k). However, when *p35S:RPBF* was included in transactivation assays of *p35S:NAC20* with *pAlb1:LUC* or *pAlb2:LUC*, and *p35S:NAC26* with *pAlb1:LUC* or *pAlb2:LUC*, increased transactivation activities were observed (Figure 1l), suggesting that RPBF may act as a cofactor for NAC20/26. It is plausible that the transcriptional activity observed in callus protoplasts may cause by faint expressions of *NAC20* or *NAC26*, or the shorter promoter sequence used (Yamamoto *et al*., 2006).

In summary, we demonstrate that NAC20/26 act redundantly, with different efficiencies, on ACG motif of *Alb* promoters to regulate their expressions, while RPBF functions as a cofactor to enhance NAC20/26 on transcriptions (Figure 1m). It has been reported that RPBF regulates the expressions of starch and SP genes in rice endosperms through interactions with RISBZ1, a bZIP-type TF (Kawakatsu *et al*., 2009). In maize, it has been showed that the RPBF orthologue of PBF functions additively with a bZIP TF O2, regulating expressions of multiple SPs (Zhang *et al*., 2015), and NAC128/130, maize orthologues of NAC20/26, regulate expression of SPs (Zhang *et al*., 2019). If PBF/RPBF function as a general cofactor, instead of specific TF, to coordinate activities of other TFs in storage product accumulations in cereal endosperms remain to be investigated. Further, since albumin is the most important allergenic protein in rice (Matsuda *et al*., 1991), the discovery of this study may provide a unique avenue to cope with the allergenic problem for rice consumers.

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Conflicts of interest

The authors declare no conflict of interest.

Author contributions

C.M.L. designed and supervised the project; M.W.W. performed most of the experiments; X.B. and W.Q.C. performed transactivation and Co-IP assays; M.W.W., Y.R. and W.Q.C. performed immunoblot analyses; other authors provided technical supports; M.W.W., J.L. and C.M.L. wrote the paper.

References

Kawakatsu, T., Yamamoto, M.P., Touno, S.M., Yasuda, H. and Takaiwa, F. (2009) Compensation and interaction between RISBZ1 and RPBF during grain filling in rice. *Plant J.* **59**, 908–920.

Liu, J., Wu, M.W. and Liu, C.M. (2022) Cereal endosperms: development and storage product accumulation. *Annu. Rev. Plant Biol.* **73**, 255–291.

Matsuda, T., Nomura, R., Sugiyama, M. and Nakamura, R. (1991) Immunochemical studies on rice allergenic proteins. *Agric. Biol. Chem.* **55**, 509–513.

Müntz, K. (1998) Deposition of storage proteins. *Plant Mol. Biol.* **38**, 77–99.

Wakasa, Y., Hirano, K., Urisu, A., Matsuda, T. and Takaiwa, F. (2011) Generation of transgenic rice lines with reduced contents of multiple potential allergens using a null mutant in combination with an RNA silencing method. *Plant Cell Physiol.* **52**, 2190–2199.

Wang, J., Chen, Z., Zhang, Q., Meng, S. and Wei, C. (2020) The NAC transcription factors OsNAC20 and OsNAC26 regulate starch and storage protein synthesis. *Plant Physiol.* **184**, 1775–1791.

Yamamoto, M.P., Onodera, Y., Touno, S.M. and Takaiwa, F. (2006) Synergism between RPBF Dof and RISBZ1 bZIP activators in the regulation of rice seed expression genes. *Plant Physiol.* **141**, 1694–1707.

Zhang, Z., Yang, J. and Wu, Y. (2015) Transcriptional regulation of zein gene expression in maize through the additive and synergistic action of opaque2, Prolamine-Box Binding Factor, and O_2 heterodimerizing proteins. *Plant Cell*, **27**, 1162–1172.

Zhang, Z., Dong, J., Ji, C., Wu, Y. and Messing, J. (2019) NAC-type transcription factors regulate accumulation of starch and protein in maize seeds. *Proc. Natl. Acad. Sci. USA*, **116**, 11223–11228.

Zhou, W., Wang, X., Zhou, D., Ouyang, Y. and Yao, J. (2017) Overexpression of the 16-kDa alpha-amylase/trypsin inhibitor RAG2 improves grain yield and quality of rice. *Plant Biotechnol. J.* **15**, 568–580.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1 Materials and methods.
Figure S1–S12 Supplementary Figures.
Table S1–S3 Supplementary Tables.