

Functions of the CCCH type zinc finger protein OsGZF1 in regulation of the seed storage protein *GluB-1* from rice

Yi Chen · Aijun Sun · Mei Wang · Zhen Zhu · Pieter B. F. Ouwkerk

Received: 25 July 2013 / Accepted: 20 November 2013 / Published online: 27 November 2013
© Springer Science+Business Media Dordrecht 2013

Abstract Glutelins are the most abundant storage proteins in rice grain and can make up to 80 % of total protein content. The promoter region of *GluB-1*, one of the glutelin genes in rice, has been intensively used as a model to understand regulation of seed-storage protein accumulation. In this study, we describe a zinc finger gene of the Cys3His1 (CCCH or C3H) class, named *OsGZF1*, which was identified in a yeast one-hybrid screening using the core promoter region of *GluB-1* as bait and cDNA expression libraries prepared from developing rice panicles and grains as prey. The *OsGZF1* protein binds specifically to the bait sequence in yeast and this interaction was confirmed in vitro. *OsGZF1* is predominantly expressed in a confined domain surrounding the scutellum of the developing embryo and is localised in the nucleus. Transient

expression experiments demonstrated that *OsGZF1* can down-regulate a *GluB-1*-GUS (β -glucuronidase) reporter and *OsGZF1* was also able to significantly reduce activation conferred by *RISBZ1* which is a known strong *GluB-1* activator. Furthermore, down-regulation of *OsGZF1* by an RNAi approach increased grain nitrogen concentration. We propose that *OsGZF1* has a function in regulating the *GluB-1* promoter and controls accumulation of glutelins during grain development.

Keywords *GluB-1* · Glutelin · CCCH · Zinc finger · Repressor · Rice

Introduction

Rice (*Oryza sativa*) grain is one of the most important staple food sources consumed everyday by billions of people in the world. Because rice grain contains between 5 and 12 % of seed storage protein (Villareal and Juliano 1978) it is of significant importance for daily protein intake in developing countries and improvement and any increase of protein content, thus of the nutritional value of rice, is therefore of great significance. Other than their important nutritional value for mankind, seed storage proteins also have crucial functions on the texture of the grain. Protein-starch interactions impede starch gelatinization and disruption of the structure of proteins increases the stickiness of cooked rice (Hamaker et al. 1991; Kang et al. 2006; Saleh and Meullenet 2007). Up to 80 % of the total seed storage protein (Yamagata et al. 1982) in rice is glutelin, therefore, this protein has a decisive role in determining grain quality and cooking traits. Encoded by about 15 genes per haploid genome, glutelin genes are divided into four subfamilies, named GluA, B, C and D (Takaiwa 1987, 1991b; Okita et al. 1989; Mitsukawa et al.

Electronic supplementary material The online version of this article (doi:10.1007/s11103-013-0158-5) contains supplementary material, which is available to authorized users.

Y. Chen · A. Sun · M. Wang · P. B. F. Ouwkerk (✉)
Sylvius Laboratory, Institute of Biology (IBL), Leiden University,
Sylviusweg 72, 2333 BE, PO Box 9505, 2300 RA Leiden,
The Netherlands
e-mail: p.b.f.ouwerkerk.2@gmail.com

Present Address:

Y. Chen
Rothamsted Research, West Common, Harpenden AL5 2JQ, UK

A. Sun · Z. Zhu
State Key Laboratory of Plant Genomics, Institute of Genetics
and Developmental Biology, Chinese Academy of Sciences, No.1
West Beichen Road, Chaoyang District, Beijing 100101, China

M. Wang
SU BioMedicine TNO, Utrechtseweg 48, 3704 HE Zeist,
PO Box 360, 3700 AJ Zeist, The Netherlands

1998; Kusaba et al. 2003; Kawakatsu et al. 2008). All rice glutelin genes are expressed exclusively during seed development and their expression is reaching maximum levels during filling stage (10–16 days after flowering) (Okita et al. 1989; Takaiwa and Oono 1990, 1991; Mitsukawa et al. 1998; Duan and Sun 2005; Kawakatsu et al. 2008).

The expression pattern of glutelin is essentially the same as for prolamin and albumin, which are other seed storage proteins, suggesting that they are coordinately regulated and controlled by the same transcriptional regulatory machinery (Nakase et al. 1996; Duan and Sun 2005). Comparison of their promoters showed the presence of conserved *cis*-regulatory elements, not only in rice but also in the orthologous genes from other cereals. For instance, cereal prolamin genes have a conserved region at ~300 base pair (bp) upstream of the transcriptional start. This so-called endosperm box is essentially composed of two closely located motifs: A prolamine box (PROL) class endosperm motif (Vicente-Carbajosa et al. 1997) and a GCN4-like motif (Kreis et al. 1985). These sequences are so well conserved that they can also be recognised by transcription factors from other cereals. For instance, the GCN4 motif in the rice *GluB-1* promoter is recognised by the bZip protein Opaque-2 (O2) from maize (Wu CY et al. 1998; Hwang et al. 2004). O2 can also activate high levels of transcription from the wheat GCN4-like motif in plant protoplasts and yeast cells (Holdsworth et al. 1995). The prolamin-box binding factor (PBF) of maize is able to bind to the PROL motif and interacts with O2 protein in vivo (Vicente-Carbajosa et al. 1997). Therefore, it is likely that investigation of one particular *cis*-acting element or transcription factor in rice will also provide valuable information on regulation of seed storage proteins in general.

Other well-known *cis*-acting elements identified in promoters of seed storage protein genes include AACA, ACGT, G-box, GCAA, and Skn-1. In vivo transient assays and transgenic rice with promoter-GUS (β -glucuronidase) constructs confirmed that these sequences are essential for binding of transcription factors and are affecting expression of downstream reporter genes (Kim and Wu 1990; Takaiwa et al. 1991a, 1996; Zheng et al. 1993; Yoshihara and Takaiwa 1996; Yoshihara et al. 1996; Suzuki et al. 1998; Washida et al. 1999; Wu et al. 2000; Kawakatsu et al. 2008). The regulation of one particular member of the glutelins, *GluB-1*, has been studied into more detail and as a result a 245 bp core promoter was identified (Takaiwa et al. 1996). Within this region, the so-called GCN4, AACA, ACGT and PROL-boxes motifs are the critical elements driving seed-specific expression (Washida et al. 1999; Wu C et al. 2000).

Despite many studies on promoter regions of different storage proteins, only few corresponding transcription factors have been identified so far. O2 (Hartings et al. 1989; Schmidt et al. 1990) is one of the first intensively

investigated seed storage protein activators in cereals. Target binding sites of maize O2 include sequences with or without an ACGT core (Lohmer et al. 1991; Schmidt et al. 1992; Ueda et al. 1992; Cord Neto et al. 1995; Muth et al. 1996). The maize protein ZmTaxilin was reported to negatively regulate activation by O2 by direct interaction with this protein thereby changing the sub-cellular distribution (Zhang et al. 2012). In rice, a bZIP protein RITA-1 is capable of binding to an ACGT element thereby activating reporter gene expression in transient assays. RITA-1 is highly expressed in the aleurone of developing endosperm (Izawa et al. 1994). In addition, the AACA sequence in glutelin gene promoters is the target site for OsMYB5 (Suzuki et al. 1998). Five different bZIP proteins, RISBZ1 to RISBZ5 are able to interact with the GCN4 motif and RISBZ1 is capable of activating the expression of a reporter gene preceded by a minimal promoter fused to a pentamer of the GCN4 motif (Onodera et al. 2001; Kawakatsu et al. 2008). The Dof (DNA binding with one finger) prolamin box-binding factor (RPBF) is able to recognise AAAG/CTTT motifs in the *GluB-1* promoter. RISBZ1 and RPBF both can *trans*-activate GUS activity driven by promoters of different storage protein genes in transient assays, including *GluA-1*, *GluA-2*, *GluA-3*, *GluB-1*, *GluD-1*, 10 kD Prolamin, 13 kD Prolamin, 16 kD Prolamin, and α Globulin. Synergistic interactions between RISBZ1 and RPBF were also studied in transient assays, as well as by transgenic rice in which RISBZ1 and RPBF were silenced (Yamamoto et al. 2006; Kawakatsu et al. 2008, 2009).

Although several activators of seed storage proteins have been identified in cereals so far, only the maize protein ZmTaxilin can function as repressor. Here we report on a novel zinc finger protein of the CCCH type, named OsGZF1, which acts negatively on rice glutelin gene *GluB-1* expression. OsGZF1 can interact specifically with the promoter region of *GluB-1* and is highly expressed in seeds during the filling stage which overlaps with *GluB-1* expression. Transient assays in rice protoplasts showed the function of *OsGZF1* in down-regulation of the *GluB-1* promoter. Furthermore, it was found that OsGZF1 could significantly decrease the *GluB-1* activator RISBZ1-mediated activation of *GluB-1* promoter expression in a transient expression system. A loss-of-function approach using *OsGZF1*RNAi transgenic plants showed increased nitrogen concentration in the grain. Based on these results we conclude that OsGZF1 acts as a repressor of *GluB-1*.

Materials and methods

Plant materials

Two japonica cultivars Nipponbare and Zhonghua 11 were used in these studies. Zhonghua 11 was used in

follow-up experiments because we found it transformed more efficient than Nipponbare. Rice plants were grown in 10 × 10 cm pots in the green house at 28 °C and 80 % humidity with a light regime of 12 h day/12 h night. Plant material for DNA and total RNA extractions were frozen in liquid nitrogen immediately after harvesting and stored at –80 °C until use.

RNA isolation and cDNA library construction

Total RNA for cDNA library construction was isolated according to Gao et al. (2001), except that the extraction buffer was composed of 100 mM Tris–HCl, 100 mM LiCl, 10 mM EDTA, 1 % SDS (set pH to 9.0), 2 % PVP40, 2 % BSA and 10 mM DTT. mRNA was isolated using the PolyATtract mRNA Isolation System III (Promega, www.promega.com). cDNA libraries of panicle and seed of filling stage were constructed from 5 µg poly(A)⁺ RNA with the HybriZAP-2.1 XR cDNA Synthesis and Library Construction Kits (Stratagene, www.agilent.com). *E. coli*-yeast shuttle vector λACTII (Memelink 1997) was used instead of HybriZAP-2.1 from the kit. The average titration of five packagings was ~2 × 10⁵ and in total over 10⁶ plaques were generated for each primary library. Next, the primary libraries were amplified to 10⁹ pfu/ml culture and the phage libraries were then circularised into pACTII-based plasmid libraries via Cre-recombinase-mediated mass excision (Ouwkerk and Meijer 2001).

Yeast one-hybrid screening

A set of four GluB-1 bait constructs ProGluB-1-S1 (-227, -173), ProGluB-1-S2 (-182, -123), ProGluB-1-S3 (-132, -83) and ProGluB-1-S4 (-87, -33) was based on overlapping oligonucleotides with attached *NotI* and *XbaI* sites (Table S2). The annealed oligonucleotides were directly cloned into integrative vector pINT1-HIS3NB (GenBank Accession AY061966; Ouwkerk and Meijer 2011). Next, linearised fragments containing the *HIS3* reporter and the *APT1* marker were transformed into yeast strain Y187 (*MATα*, *ura3-52*, *his-Δ200*, *ade2-101*, *trp1-901*, *leu2-3*, *112 met⁻*, *gal4 gal80 URA3::GAL1_{UAS}-GAL1_{TATA}-lacZ*, *MEL1*) (Harper et al. 1993) and integrated at the non-essential *PDC6* locus via double cross-over. Positive colonies were selected on YAPD-G418 plates. Next, positive strains were grown on CM plates lacking histidine to test for leaky *HIS3* expression. Yeast one-hybrid screenings were performed according to Ouwkerk and Meijer (2001, 2011). One µg of pACTII-cDNA library was transformed and 10⁶ transformants were screened. Selection was performed on CM plates lacking leucine and histidine and containing 10 mM 3-aminotriazole (3-AT, a competitive inhibitor of His3p). Positive colonies were re-streaked on

the same selective plates and colonies still growing after this step were selected for plasmid isolation or colony PCR with primers COPCR-for and COPCR-rev (Table S1). The amplification was done with 98 °C 5 min, 98 °C 30 s, 52 °C 30 s, 72 °C 2 min, 35 cycles, 72 °C 10 min, 4 °C pause.

Electrophoretic mobility shift assays (EMSAs)

OsGZF1 cDNA was subcloned in frame with the GST-encoding sequence in expression vector pGEX-KG (Guan and Dixon 1991) as *NcoI/XhoI* fragment from pACTII/OsGZF1. Overnight *E. coli* XL-1 cultures (10 ml) containing the expression construct were diluted into 500 ml LB medium and grown to OD₆₀₀ = 0.5 at 37 °C. The culture was then induced by adding IPTG to 1 mM. After 3.5 h (OD₆₀₀ = 0.8) of growth at 29 °C, cells were pelleted, resuspended in 20 ml of 1 × PBS buffer, and lysed by mild sonication. Following centrifugation, the supernatants were filtered using a syringe through a 0.45 µm membrane filter. Samples were run over a column with 0.5 ml Glutathione-Sepharose (GE Healthcare, www.gelifesciences.com) and washed with 10 ml of 1 × PBS buffer. Next, the bound GST fusion proteins were eluted with eight times volume of 0.25 ml Glutathione Elution Buffer (10 mM glutathione, 50 mM Tris–HCl pH8.0) and frozen in liquid nitrogen after addition of 10 % glycerol and stored at –80 °C.

EMSAs were performed in a 10 µl system consisting of 0.1–0.2 µg of purified fusion protein, 0.1 ng P³²-end labeled probe (60 bp double strand oligonucleotides), a variable amount of poly (dIdC) (GE Healthcare) depending on the protein/probe combination, 2 µl five times nuclear extraction buffer (Green et al. 1989), and water up to 10 µl. The reaction mixtures were incubated at room temperature for 30 min and loaded on 5 % acrylamide/bisacrylamide (37.5:1) gels in 0.5 × TBE buffer under tension. After electrophoresis, gels were vacuum dried on DE81 Whatman paper at 65 °C for 1 h and autoradiographed. The DNA fragments were the same four sequences as used in the yeast one-hybrid assays, as well as 12 other mutant oligonucleotides derived from these with different mutations at the key motifs as described in the text and figure legend (sequences listed in Table S3).

Northern blotting

Total RNA of vegetative tissues were isolated with TRIzol Reagent (Gibco BRL, www.invitrogen.com). Total RNA of seeds was isolated according to Singh et al. (2003). Twenty µg of total RNA of different tissues of Nipponbare rice were separated by electrophoresis and transferred onto a cellulose membrane. *OsGZF1* and *GluB-1* specific probe was then hybridised on the same membrane after stripping

off the first probe. Blotting and hybridisation were as described (Memelink et al. 1994). The *OsGZF1* probe was excised from vector pHANNIBAL/*OsGZF1* (constructed for the RNAi experiment) with *KpnI-XhoI*. The *GluB-1* probe was amplified from Nipponbare genomic DNA with primers GBNOR-for and GBNOR-rev (Table S1).

Subcellular localisation of *OsGZF1*

A Green Fluorescent Protein (GFP)/*OsGZF1* fusion construct for localisation studies was made as following: A full length cDNA clone of *OsGZF1* was excised from pACTII/*OsGZF1* with *EcoRI/XhoI* and ligated into *BglIII/XhoI* digested pTH2-BN, (Kuijt et al. 2004) which is a derivative of pTH2 lacking the stop codon (Chiu et al. 1996), together with a *BglIII/EcoRI* adapter. The adapter was made by annealing of two oligonucleotides 5'-GATCTGTCGACG-3' and 5'-GAATTCGTCGACA-3'. The vector was then transformed into Nipponbare seedling protoplasts through a PEG-mediated method and incubated overnight in darkness at 25 °C. GFP was visualised with an LSM 5 Exciter on an AXIO Observer confocal laser scanning microscope (Zeiss, microscopy.zeiss.com).

Binary vectors construction, plant transformation and detection of GUS expression

The binary vector to make the *OsGZF1* RNAi plants was constructed as follows. A 771 bp *OsGZF1* specific region (coordinate 1–771 of the cDNA sequence of *OsGZF1* in Fig. S1) was amplified on construct pACTII-*OsGZF1* with primer pairs: sense fragment ZF1SN-for and ZF1SN-rev; anti-sense fragment ZF1ASN-for and ZF1ASN-rev (Table S1). The fragments were cloned into pCR-Blunt II TOPO (Invitrogen). Next, the sense and anti-sense segments were subcloned into vector pHANNIBAL (GenBank Accession AJ311872, Wesley et al. 2001) separately as *EcoRI/XhoI* and *Clal/BamHI* excised products resulting in construct pHANNIBAL/*OsGZF1*. Next, the fragment containing the CaMV 35S promoter, sense, anti-sense part and OCS (octopine synthase) terminator was excised with *SacI/SpeI* and ligated into vector pC1300intA (GenBank Accession AF294976) between corresponding sites.

Plasmids for expression analysis were constructed as follows. The 5' regulatory region of *OsGZF1* (–2061 to –1) was amplified from genomic DNA of Nipponbare using primers PROZF1-for and PROZF1-rev (Table S1). The PCR products were subcloned into pCAMBIA-1391Z (GenBank Accession AF234312, Hajdukiewicz et al. 1994) vector as *HindIII/NcoI* fragments.

Transformation, histochemical detection of GUS activity, cytological techniques and microscopy were performed

as previously described (Scarpella et al. 2000) except that *A. tumefaciens* strain LBA4404 was used instead of LBA1119. Pictures were recorded using a Leica MZ12 stereo microscope equipped with a Sony 3CCD Digital Photo Camera DKC-5000.

Construction of reporter and effector plasmids and transient assays

Construction of effector vectors was as follows, cDNAs of *OsGZF1* was amplified from pACTII/*OsGZF1* with primers ZF1PR-for and ZF1PR-rev (Table S1). *RISBZ1* cDNA was amplified from cDNA of 15 DAF rice panicles (Agalou et al. 2008) with RISBZ1PR-for and RISBZ1PR-rev (Table S1). The fragments were ligated into pCR-Blunt II TOPO (INVITROGEN). The cDNA of *OsGZF1* and *RISBZ1* were then subcloned into vector pRT101 (Töpfer et al. 1987) between *EcoRI* and *KpnI* to form the two effectors. Reporters were constructed as follows. The ProGluB-1-L promoter was digested out from ProGluB-1-L/pINT1-HIS3NB vector by *NotI/SpeI* and cloned into *EagI/SpeI*-digested GUSSH-47 (Pasquali et al. 1994) vector. ProGluB-1-S2 was cloned into *EagI/XbaI*-digested GUSSH-47 by annealing the oligonucleotides used in the yeast one-hybrid experiments.

Transient assays were performed as previously described using protoplasts isolated from 2 weeks old Nipponbare seedlings (Chen et al. 2006). GUS activity was measured with a Cytofluor 2350 fluorimeter (Millipore, www.Millipore.com) according to (Jefferson 1987). To equilibrate any transformation efficiency bias, a 35S- Ω -Luc vector (firefly luciferase gene driven by the *CaMV* 35S promoter and Ω leader) was transformed at the same time with the effector and reporter as internal control. Luciferase activity measurement was done according to the manufacturer's instructions (Promega) and the activity was read with a Perkin Elmer Victor 3. The luciferase standardised GUS activity of different effector and reporter combinations were linearised against the time interval and compared.

Activation assays in yeast

The open reading frame (ORF) of *OsGZF1* was cut from pACTII-*OsGZF1* with *NcoI/XhoI* and translationally fused with the GAL4 DNA-binding domain (BD) in vector pAS2-1 (Clontech, www.clontech.com). Next, *OsGZF1*/pAS2-1 was transformed into yeast strain PJ69-4A (*MAT α* , *trp1-901*, *leu2-3*, *ura3-52*, *his3- Δ 200*, *gal4 Δ* *gal80 Δ* , *LYS2::GAL1_{UAS}-GAL1_{TATA}-HIS3*, *GAL2_{UAS}-GAL21_{TATA}-ADE2*, *met2::GAL7-LacZ* (Clontech)). Positive colonies from CM-Trp plates were restreaked on CM-His, CM-His with 2 mM 3-AT and CM-Ade plates and grow at 30 °C for 1 week.

Measurement of nitrogen concentration in rice grains

Three random seeds from each plant were grinded into fine powder. Per sample, 4 mg of powder was weighed in a 5 × 8 mm tin capsule and the nitrogen content was measured against the dry weight by dry combustion on a Flash EA 2000 elemental analyser (Thermo Electron Corporation). The assays were performed with three replicates and nitrogen concentrations were analysed statistically by ANOVA using GeneStat software.

Protein isolation from rice grain and SDS-PAGE

Five random grains from different part of the mature panicle were weighed and grinded in mortar in isolation buffer (Kawakatsu et al. 2008) added according to the weight of the grains. The mixtures were incubated overnight at 28 °C under shaking (200 rpm) and centrifuged at 14,000 rpm for 10 min to get rid of debris. Finally, proteins were separated by 12 % SDS-PAGE and the gel was stained with Comma-ssie Brilliant Blue R-250.

Accession numbers

The cDNA sequence of OsGZF1 from this article can be found in the EMBL/GenBank databases with accession code KF289071.

Results

Identification of a CCCH zinc finger interacting with the *GluB-1* core-promoter

In order to further understand the regulatory mechanism controlling transcriptional expression of the rice storage protein *GluB-1*, yeast one-hybrid screenings were employed to search for novel transcription factors. For this, two cDNA expression libraries, derived from seeds and panicle harvested during the filling stage of rice, were constructed and used as preys. Based on the upstream region of *GluB-1* (Fig. 1a) in total seven bait constructs with different lengths were cloned in front of a *HIS3* reporter. The constructs were integrated into the genome of yeast strain Y187 through homologous recombination using the pINT1 yeast-one hybrid vector system (Meijer et al. 1998; Ouwerkerk and Meijer 2011). After checking on a CM medium lacking histidine, only the yeast strain with a 59 bp bait (ProGluB-1-S2) containing the GCN4 and PROL motifs was able to grow. Yeast strains harbouring the other six constructs did not grow which likely means that endogenous yeast proteins with repressing functions bound to the baits thereby preventing *HIS3* expression and

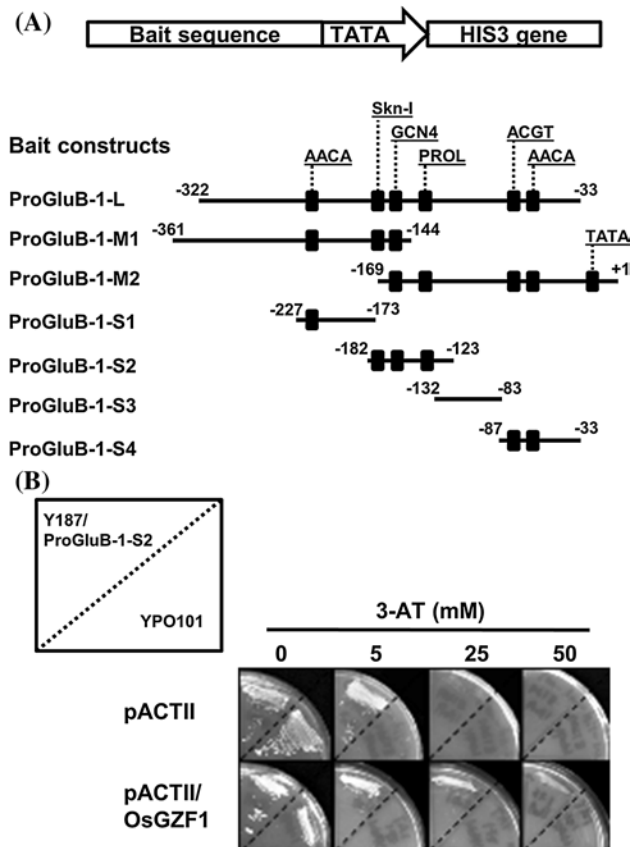


Fig. 1 Yeast one-hybrid screening using different fragments of *GluB-1* promoter as baits. **a** Schematic overview of the baits used in the yeast screening. **b** OsGZF1 exhibits strong interaction with bait ProGluB-1-S2. pACTII/OsGZF1 was transformed into yeast strain Y187/ProGluB-1-S2 and YPO101 (contains the *HIS3* reporter preceded only with a minimal promoter) and streaked on CM-Leu-His with increasing concentrations of 3-AT. Empty pACTII vector with the GAL4 AD was used as control. Growth was monitored after 7 days at 30 °C

thus growth. Therefore, these constructs were not suitable for further yeast one-hybrid screenings. Next, we continued with the analysis of construct ProGluB-1-S2 by titrating with 3-AT which is a competitive inhibitor of *HIS3* protein, for the minimum concentration which just suppresses leaky *HIS3* expression. As a result, 10 mM of 3-AT was found sufficient to suppress leaky expression of strain Y187/ProGluB-1-S2. Finally, using this condition, rice panicle and seed cDNA libraries were screened. Screening of 10^6 transformants of each library resulted in 131 positive colonies after re-streaking the primary positive colonies on the same selective medium. Positive colonies that could grow again after this initial selection step were further analysed either by colony PCR or plasmid rescue followed by sequence analysis. Finally, 42 PCR products and 27 plasmids were obtained, sequenced and BLAST-searched against GenBank (<http://www.ncbi.nlm.nih.gov>).

Most clones were ribosomal proteins that show up in yeast one-hybrid screens as false positives but five clones were identified that encoded the same type of zinc finger protein which is in frame with the Gal4p activation domain (Gal4 AD) sequence in the library vector pACTII. All clones have the same intact ORF as in GenBank Accession AK108315 from the KOME collection (Kikuchi et al. 2003). However, two clones have an extra 89 bp sequence at the beginning of the 5'-UTR. Two other clones have a 5'-UTR which is 13 bp shorter than AK108315 and the fifth clone has the shortest 5'-UTR which is 22 bp shorter than AK108315. We used our longest cDNA clone with a length of 1,341 bp (Fig. S1; GenBank Accession KF289071) for further studies. Sequence analysis and annotation (<http://plntfdb.bio.uni-potsdam.de/v3.0/>) showed that the gene encoded by these clones belong to the so-called CCCH zinc finger family with 67 members in rice (Wang et al. 2008a, b) and we named this gene *OsGZF1* after *Oryza sativa* *GluB-1*-binding Zinc Finger. Further sequence analysis and annotation of *OsGZF1* using the TIGR database (<http://rice.plantbiology.msu.edu/blast.shtml>) showed that *OsGZF1* is encoded by locus LOC_Os07g47240 on chromosome 7 and has two exons and only one intron near the C-terminus. LOC_Os07g47240 translates into a protein of 281 amino acids. *OsGZF1* has one CHCH motif and two CCCH motifs which are closely located together with 19 amino acids (AA) between the CHCH motif and the first CCCH motif and 16 AA between the two CCCH motifs (Fig. S1).

In order to further check the binding specificity of *OsGZF1*, the corresponding pACTII clone was retransformed into strain Y187/ProGluB-1-S2 and the control strain YPO101 (Ouwkerk and Meijer 2001, 2011) which contains the *HIS3* reporter preceded by a minimal promoter including a TATA box and transcriptional start site. A titration assay consisting of a series of 3-AT concentrations was used to verify the interaction of *OsGZF1* with the bait. As shown in Fig. 1b, Y187/ProGluB-1-S2 transformed with pACTII/*OsGZF1* was able to grow up to 50 mM of 3-AT whereas growth of YPO101 with pACTII/*OsGZF1* was totally suppressed at 5 mM of 3-AT (Fig. 1b). The results show that *OsGZF1* is able to interact specifically with the bait sequence ProGluB-1-S2.

OsGZF1 can interact with the *GluB-1* promoter in vitro

In order to verify the binding properties of *OsGZF1* to the core promoter of *GluB-1*, the four short baits from the yeast one-hybrid screens ProGluB-1-S1, 2, 3, 4 (S1a, S2a, S3a, S4a, Fig. 2a) were used in Electrophoretic Mobility Shift Assays (EMSA) in combination with *OsGZF1*-GST-tagged recombinant protein that was purified from *E. coli*. As shown in Fig. 2b, DNA–protein complexes were observed for all fragments tested with *OsGZF1*-GST protein, thereby

confirming the results of the yeast one-hybrid screens in vitro with sequence ProGluB-1-S2. Other authors (Takaiwa et al. 1996; Yoshihara et al. 1996; Wu CY et al. 1998, Wu C et al. 2000; Washida et al. 1999) showed the importance of the so-called AACA, GCN4, PROL and ACGT motifs in regulation of *GluB-1* expression. In order to validate if these motifs are also recognised by *OsGZF1*, single or double mutations were made in oligonucleotides ProGluB-1-S1, S2 and S4 (Fig. 2a). DNA–protein complexes were observed with all tested oligonucleotides, indicating the mutated motifs were not essential for binding to *OsGZF1* (Fig. 2c).

Subcellular localisation and expression pattern of *OsGZF1*

In order to validate the putative function of *OsGZF1* as a transcription factor, subcellular localisation of the protein was analysed using a transient expression system. We made a construct by fusing the ORF of *OsGZF1* in frame to the C-terminus of the GFP reporter. After transformation of rice seedling protoplasts, an *OsGZF1*/GFP signal was observed predominantly in the nucleus (Fig. 3a) which is consistent with a function in regulating *GluB-1* expression.

Northern blot analysis was employed to study the expression pattern of *OsGZF1* in more detail. *OsGZF1* was found to be highly expressed in developing seeds. As shown in Fig. 3b, mRNA expression of *OsGZF1* started to accumulate at DAF 5 (days after flowering) and peaked at 10 DAF. Afterwards, the expression decreased again between 15 DAF and 20 DAF. Besides in developing grains, *OsGZF1* was also found to be expressed weakly in seminal shoots of 3 days after germination but not at later stages of development.

In order to study the temporal and spatial expression pattern in more detail, a 2 kb upstream promoter region of *OsGZF1* was amplified from Nipponbare rice and cloned in front of the *GUS* reporter gene resulting in construct of Pro*OsGZF1*::*GUS* which was introduced into rice cultivars Nipponbare and Zhonghua 11. Five independent transgenic lines of each cultivar were histochemically analysed for *GUS* activity which was shown to be predominant in grains thereby confirming the data obtained from the northern blot study (Fig. 3b). *GUS* activity was detected mainly in a narrow endosperm layer around the scutellum region of the embryo during grain filling stages (5–10 DAF; Fig. 3c). A *GUS* signal was also observed on the surface of mature seeds (Fig. 3d), germinating embryos, seminal shoots and roots (Fig. 3e, f), indicating its diverse function in these tissues.

OsGZF1 down-regulates *GluB-1* promoter driven reporter gene expression

In order to elucidate the potential regulatory function of *OsGZF1* in gene transcription, further studies were

(A)

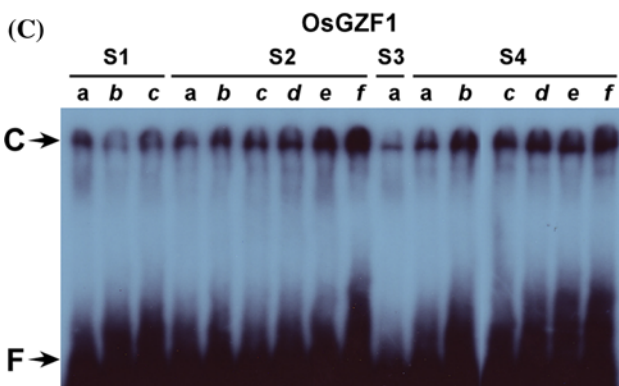
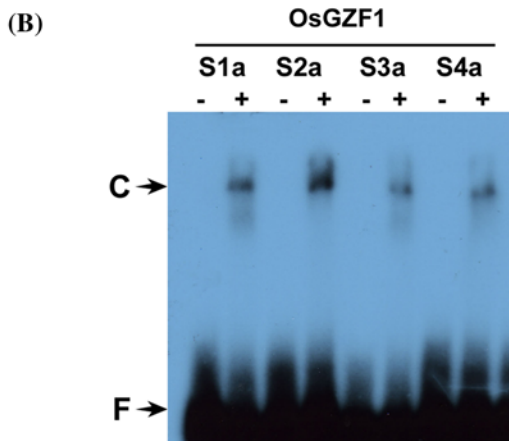
```

                AACA
S1a:  -----AACAAAC-----
S1b:  -----ATGTTTC-----
S1c:  -----ATCTATC-----

                GCN4          PROL
S2a:  -----TGAGTCA-----TGCAAAG-----
S2b:  -----TCTCAGA-----TGCAAAG-----
S2c:  -----TCACTGA-----TGCAAAG-----
S2d:  -----TGAGTCA-----TCGTTTG-----
S2e:  -----TGAGTCA-----TCCATATG-----
S2f:  -----TCACTGA-----TCCATATG-----

S3a:  -----

                ACGT    AACA
S4a:  ---GTACGTGCAACAAAC-----
S4b:  ---GTACGTGCATGTTTC-----
S4c:  ---GTACGTGCTAGATAG-----
S4d:  ---GATGCACCAACAAAC-----
S4e:  ---GAAGGAGGAACAAAC-----
S4f:  ---GAAGGAGGTAGATAG-----
    
```



performed in a yeast activation assay and a rice protoplast system. For the yeast activation assays, *OsGZF1* was cloned in frame with the GAL4 binding domain in vector pAS2-1 and transformed into yeast strain PJ69-4A containing the *HIS3* and *ADE2* reporters each preceded by a Gal4p binding sequence. If *OsGZF1* protein would have any activation properties then the fusion with the Gal4p BD would result in binding to activation of the *HIS3* and

◀ **Fig. 2** Interaction of *OsGZF1* with *GluB-1* promoter in vitro. **a** Oligonucleotide sequences used in EMSA. S1a, S2a, S3a and S4a represents the four baits ProGluB-1-S1, 2, 3, 4 used in the yeast one-hybrid assays. The other fragments were generated from the four short baits but with single or double mutations in the so-called *cis*-elements displayed in Fig. 1a. Single mutations were designed such that they converted the motifs into their complementary sequences (S1b, S2b, S2d, S4b, S4d) or every other nucleotide (S1c, S2c, S2e, S4c, S4e). Double mutations were created by converting both motifs into their complementary sequences every other nucleotide (S2f and S4f). **b** EMSAs were performed with recombinant GST-tagged *OsGZF1* protein and radio-labeled wild-type oligonucleotides. – or + represent without or with protein. **c** Interaction of *OsGZF1* to oligonucleotides without and with mutations. The arrow heads mark the positions of protein-DNA complexes (C) and free probes (F)

ADE2 reporters and thus in growth. The pAS2-1/*OsGZF1* transformants were cultured on selection medium (CM-His with 2 mM 3-AT) but did not show any growth (Fig. S2a). On plates without histidine and no 3-AT, there was some growth apparent but this was the same as with empty vector pAS2-1. The same results were obtained when selected for *ADE2* activation which is even a more stringent condition than selection for histidine auxotrophy (Fig. S2b). In agreement with the results obtained from the histidine selection assay, no growth was found indicating that in yeast, *OsGZF1* shows no strong activation properties.

Next, transient assays in rice protoplasts were employed to further determine the possible roles of *OsGZF1* in regulating the *GluB-1* promoter. For this, *OsGZF1* was cloned into expression vector pRT101 under control of *CaMV 35S* promoter to form the effector plasmid pRT101/*OsGZF1* (Fig. 4a). The *GluB-1* promoter fragments of ProGluB-1-L and ProGluB-1-S2 used in the yeast one-hybrid screens were cloned in front of the *GUS* reporter to generate the reporters ProGluB-1-L::GUS and ProGluB-1-S2::GUS (Fig. 4a). Next, combinations of effectors and reporters were transformed together into protoplasts as indicated in Fig. 4b. As shown, effector pRT101/*OsGZF1* down-regulated expression of the two *GluB-1* promoter reporter constructs each with 20 % when compared to the control vector. Thus, it seems *OsGZF1* can function as repressor.

OsGZF1 reduces activation by RISBZ1 on *GluB-1* promoter

From the protoplast experiments it became clear that *OsGZF1* has functions in repression of gene expression. To further study this in more detail, co-transformations were carried out with a *RISBZ1* overexpression construct. *RISBZ1* is a transcription factor of the bZip type and is a well known activator of *GluB-1* (Onodera et al. 2001; Yamamoto et al. 2006; Kawakatsu et al. 2009). We transformed pRT101/*RISBZ1* and pRT101/*OsGZF1* effector or empty pRT101, and also one of the reporters

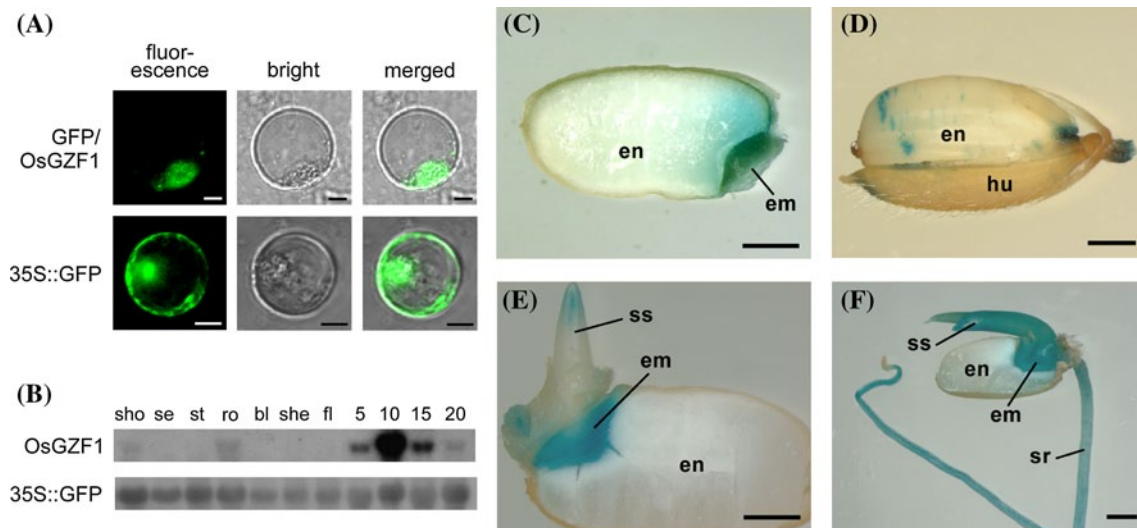


Fig. 3 Nuclear localisation and expression pattern of *OsGZF1*. **a** Observed under fluorescence. *Middle* with bright field. *Right* merger of fluorescence and bright field microscopy. **b** Total RNAs were isolated from seminal shoot (*sho*), seedling (*se*), stem (*st*), root (*ro*), leaf blade (*bl*), sheath (*she*), flower (*fl*), grains of 5, 10, 15 and 20 DAF respectively. *OsGZF1* and *GluB-1* probes were hybridised on the same membrane one after the other. **c** to **f** Histochemical stain-

ing of Pro*OsGZF1*::GUS plants with X-Gluc. Seeds were cut longitudinally prior to staining with X-Gluc solution. **c**, developing seeds of 10 DAF. **d** Mature seeds. **e** Seeds at 1 day after germination (DAG). **f** Seeds at 3 DAG. *al* Aleurone layer, *em* embryo, *en* endosperm, *hu* husk, *pe* pericarp, *sr* seminal root, *ss* seminal shoot. Bar (**a**) = 10 μ m bar (**c** to **f**) = 1,000 μ m

into protoplasts respectively (Fig. 4c). pRT101/RISBZ1 could increase GUS activity 52 and 82 times when co-transformed with either Pro*GluB-1-L*::GUS or Pro*GluB-1-S2*::GUS respectively. However, when co-transformed with *OsGZF1*, this factor strongly down-regulated activation by RISBZ1 of construct Pro*GluB-1-L*::GUS by 50 % and only 25 times of activation remained. However, no difference was found on the effect of RISBZ1 on Pro*GluB-1-S2*::GUS (Fig. 4c). The results confirm that *OsGZF1* can repress gene expression but also that the interactions with other transcription factors can be specific for the *cis*-regulatory element.

Loss-of-function of *OsGZF1* increases seed nitrogen concentration in rice

To further investigate the biological roles of *OsGZF1* in planta, an RNAi strategy was used to create transgenic loss-of-function rice since we were not able to identify a suitable T-DNA or transposon mutant in the available collections. To enable the RNAi approach, a 770 bp fragment specific for *OsGZF1* was selected and expressed using the pHANNIBAL system (Wesley et al. 2001) in Nipponbare rice. Five transgenic plants with single copy insertion lines were identified using Southern blot analysis (data not shown). Compared to Nipponbare plants, we did not observe any obvious visual phenotypical abnormalities of the grains or the vegetative organs from the RNAi plants (Fig. 5a). As

shown in the northern blot presented in Fig. 5b, expression of *OsGZF1* in the 10 DAF seeds of lines 54, 61 and 79 was reduced compared to the untransformed control. In two other transgenic lines (55, 60) *OsGZF1* expression was not reduced. Furthermore, the weight of 20 grains from the five transgenic *OsGZF1* RNAi lines and two Nipponbare wild type plants was compared and no significant difference was found (Fig. 5c). In order to study the potential regulatory function of *OsGZF1* in rice developing grains, we first checked the expression level of *GluB-1* in grains of *OsGZF1* RNAi plants (10 DAF). A northern blot analysis was done on whole grains since at this developmental stage it is very difficult to separate the region where *OsGZF1* is highly expressed from the rest of the milky grain where expression is lower (Fig. 3b, c). No obvious changes in both *OsGZF1* reduced and non-silenced lines were apparent (Fig. S3). We speculate that this may be due to the highly specific and limited expression domain of *OsGZF1* in the developing grain which is in the embryo and in the endosperm flanking the embryo (Fig. 3c), which may make the northern blot data of the whole grain not fully reflect the effect of *OsGZF1* on *GluB-1* in its expressing domain.

Because of the high similarity of the promoter regions and expression pattern of grain glutelin genes, most likely *OsGZF1* is also involved in regulation of other glutelin genes besides *GluB-1*. To further study the potential function of *OsGZF1* in the grain protein accumulation, we measured the nitrogen concentration of *OsGZF1*-RNAi

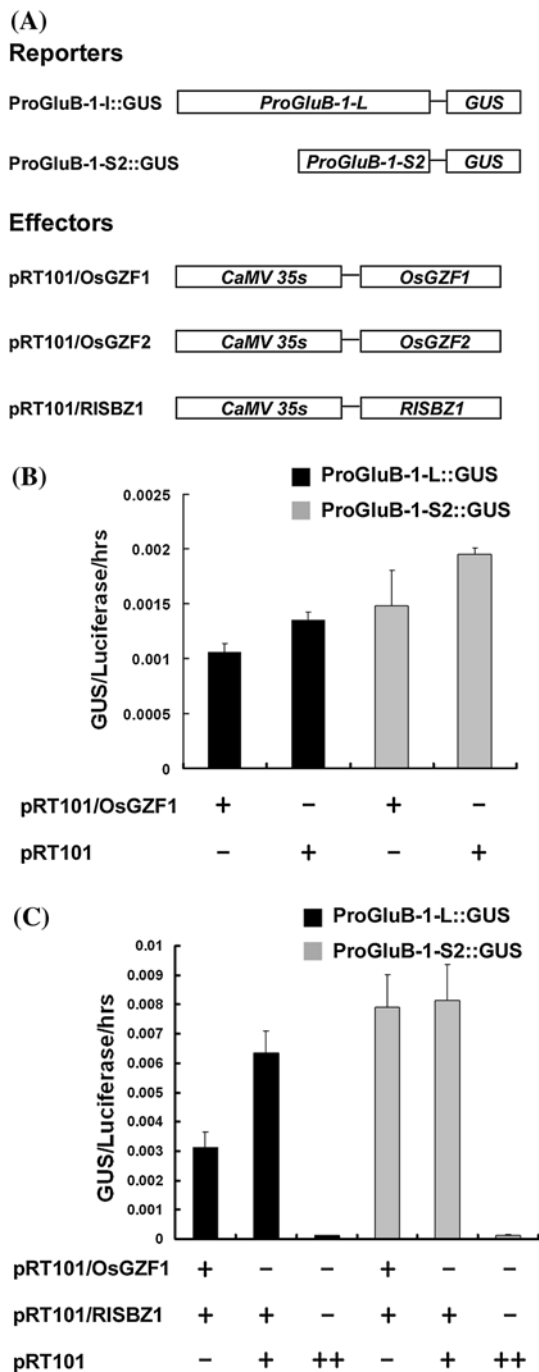


Fig. 4 OsGZF1 down-regulates *GluB-1* promoter in transient assays. Rice protoplasts were co-transformed with different combinations of reporter and effector constructs as indicated. **a** Schematic overview of reporter and effector constructs. **b** OsGZF1 represses GUS activity driven by the *GluB-1* promoter. **c** Effects of OsGZF1 on the activation of ProGluB-1-L::GUS or ProGluB-1-S2::GUS by RISBZ1

transgenic seeds. As shown in Fig. 5d, the nitrogen concentrations of lines 54 and 61 are significantly higher than the transgenic control plants 55 and 60. Nonetheless line 79, in which *OsGZF1* is lower expressed did not show a

significant change compared to the transgenic controls. Compared to two different grain lots from wild type Nipponbare (named NB-1 and NB-2), line 54 also exhibited significantly increased nitrogen concentration. But the nitrogen content of line 61 is just significantly higher than NB-1 but not NB-2. We hypothesise that the reason why not every *OsGZF1* down-regulated line showed increased grain nitrogen level may be related to the limited expression domain and time where and when *OsGZF1* is expressed in the endosperm and embryo and thus can be down-regulated by the RNAi approach. We also applied SDS-PAGE to monitor protein content in *OsGZF1* RNAi grains in more detail. As shown in Fig. 5e, no obvious changes were found in the number and location of the storage protein bands in *OsGZF1* down-regulated seeds. But increased intensities of glutelin precursor and the two basic and acidic subunit bands were found in grains from line 54 compared to both Nipponbare and transgenic control plants, which is consistent with the nitrogen concentration results in Fig. 5d. However it is difficult to judge from the SDS-PAGE if there are any significant changes in lines 61 and 79.

Discussion

Functions of OsGZF1 in regulating *GluB-1* expression

Here we report on a novel zinc finger protein named OsGZF1 which we identified from a yeast one-hybrid screen using part of the *GluB-1* promoter as a bait. Using transient expression experiments with rice protoplasts we showed that OsGZF1 can down-regulate expression of the *GluB-1* promoter and moreover that it significantly repressed activation properties of RISBZ1, which is known to be a strong activator of *GluB-1*. All transcription factors known so far to be involved in regulation of storage protein genes, for instance *O2*, *RISBZ1* and *PBF1*, are activators except for the recently reported repressor ZmTaxilin of *O2*, in maize (Zhang et al. 2012). Several in vitro and in vivo experiments provided evidence for a function of OsGZF1 in regulation of the *GluB-1* promoter. First, both the yeast one-hybrid assays as well as the EMSAs demonstrated that OsGZF1 can interact with the *GluB-1* promoter. Sequence analysis showed that OsGZF1 has three zinc finger motifs which is consistent with a role in DNA binding and gene regulation. Second, using a GFP-tagged construct, OsGZF1 showed nuclear localisation which further supports a function in transcriptional gene regulation.

Further evidence for a role for OsGZF1 in regulation of *GluB-1* is found in the overlapping expression patterns of *GluB-1*, *RISBZ1* and *OsGZF1*. According to the northern blot analysis, the expression of *GluB-1* peaks from 10 to 15 DAF which confirms data of others (Onodera et al. 2001;

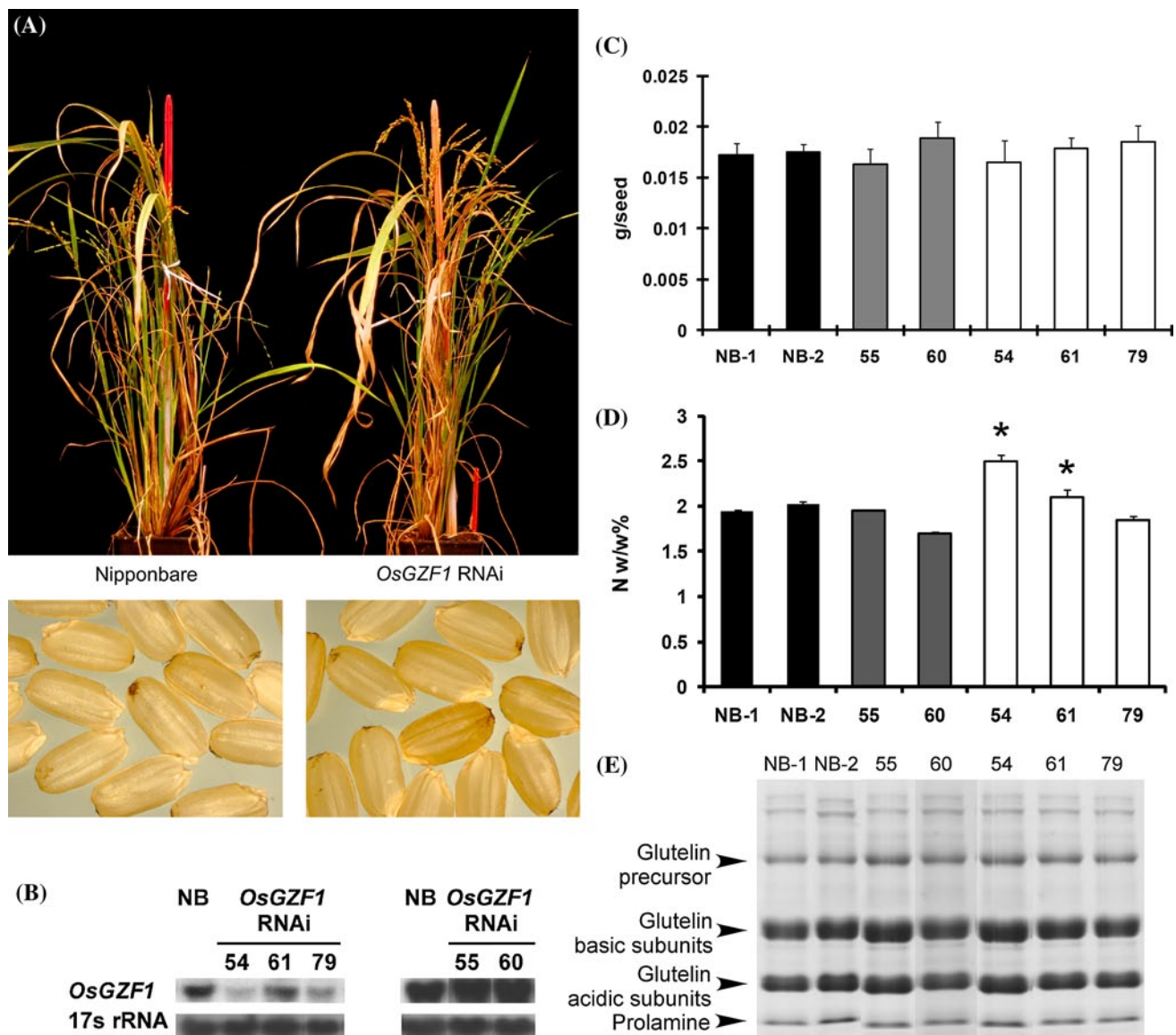


Fig. 5 *OsGZF1* RNAi plants showed increased grain protein content. **a** Upper panel phenotypes of Nipponbare and *OsGZF1* RNAi plants; below, seeds from the same plants. **b** Northern blot analysis of *OsGZF1* RNAi plants. Total RNA of 10 DAF grains was isolated and hybridised with an *OsGZF1* specific probe. **c** Twenty grains of each plant line were peeled and weighted after drying at 50 °C for 1 week. Two control Nipponbare lines were used (NB-1 and 2). Lines 55 and 60 do not show down-regulation and serve as transformation

control. Lines 54, 61 and 79 show down-regulation on the RNA level. Mean \pm SE of the weight of *OsGZF1* RNAi grains is indicated in the bars. Grain weights of the plant lines tested were not significantly different. **d** Mean \pm SE of the nitrogen concentrations of the *OsGZF1* RNAi grains. Lines 54 and 61 are significantly different than the others and are indicated with an asterisk ($p < 0.05$). **e** SDS-PAGE of proteins from *OsGZF1* RNAi grains

Duan and Sun 2005; Yamamoto et al. 2006). *RISBZ1* starts to accumulate from 5 DAF and stays at the same level till 15 DAF before declining (Onodera et al. 2001; Yamamoto et al. 2006) which is inconsistent with *GluB-1* expression. However *OsGZF1* is highly expressed during from 5 to 10 DAF and peaks at 10 DAF, which is earlier than the peak of *GluB-1*. Promoter-GUS results also revealed that the expression region of *RISBZ1* (Onodera et al. 2001) partially overlaps with *OsGZF1*. These data suggest that

the activity of *RISBZ1* before 10 DAF may be controlled by *OsGZF1* thus negatively regulates the accumulation of *GluB-1* mRNA in the confined area of the endosperm where *OsGZF1* is expressed.

Furthermore, the gain-of-function approach using the transient expression system clearly showed that *OsGZF1* alone could repress expression of the *GluB-1* promoter. Considering that *GluB-1* is exclusively expressed during grain development, experiments in seedling protoplasts

may not fully reflect its full transcriptional properties as in seed tissue but we had to choose for this system since it seemed to be difficult in making good protoplasts from embryogenic calli or cell suspensions in which seed-storage proteins are also expressed. Therefore, to demonstrate the validity and usefulness of our protoplast system, we reconstituted a *GluB-1* regulatory system by adding the well known transcriptional activator RISBZ1 of *GluB-1* into the assay. RISBZ1 too is predominantly active in developing seeds and is functional through the GCN4 box in the core promoter of *GluB-1* (Onodera et al. 2001). Addition of the OsGZF1 overexpression construct in the protoplast assays reduced activation of RISBZ1 on the *GluB-1* promoter-GUS constructs. OsGZF1 has three zinc fingers and can interact with multiple sites as discussed earlier. One possibility is that the binding of OsGZF1 to the *GluB-1* promoter blocks the GCN4 box from interacting with RISBZ1. Such competition in DNA binding is one of the main mechanisms in transcriptional regulation in eukaryotes to limit the rate of transcription initiation from nearby promoters (Johnson 1995). The main repressing effects of OsGZF1 may act through affecting the function of other activators like RISBZ1. A similar mode of action has been demonstrated before for regulation of the expression of storage protein genes in bean where it was found that a bZIP protein named ROM1 functions as a DNA-binding repressor of genes *DLEC2* and *PHS β* (coding for phytohemagglutinin L-subunit and β -phaseolin) by reducing the activation of a *trans*-activator PvALF of *DLEC2* and *PHS β* (Chern et al. 1996).

Important *in planta* evidence supporting a role of OsGZF1 as repressor of *GluB-1* was from the analysis of grain nitrogen concentration in *OsGZF1* loss-of-function plants made with RNAi technology. The northern blot showed no obvious change in expression of *GluB-1* on the whole grain level which could be explained by the observation that *OsGZF1* is only expressed in a limited domain in rice grains. Therefore, regulation and expression of *GluB-1* by OsGZF1 in this domain needs to be further studied by a more precise method in the future such as with laser capture microdissection. However, an increased nitrogen concentration was found in grains from two out of three RNAi lines in which *OsGZF1* was down-regulated. Such increase was further confirmed in the RNAi lines by SDS-PAGE as increased intensities of glutelin bands were observed. The changes in nitrogen concentration and glutelin bands also indicate that OsGZF1 may be involved in the regulation of other glutelin genes which in rice are the most abundant seed-storage proteins. Since RNAi plants were generated through tissue culture, somaclonal variation may have been generated. To minimise the effect of such variation we used *OsGZF1* RNAi seeds in which expression was not affected, as controls. Meanwhile we also used Nipponbare wild type plants grown in the same condition at the same time

for comparison. The two types of controls showed variable nitrogen concentrations. In addition, the comparison was based on individual plants grown in single pots. We cannot entirely exclude the effects of environment factors such as light and fertilizer which may affect composition and content of seed storage proteins. Future field replicated trials with *OsGZF1* RNAi plants is needed to further validate the results.

Interaction of OsGZF1 with the *GluB-1* promoter

CCCH type zinc finger proteins belong to an ancient conserved group that is present both in pro- and eukaryotes. Most animal CCCH proteins investigated were shown to play important roles in post-transcriptional regulation of mRNAs by binding to the AU-rich element in the 3'-UTR (e.g. Guo et al. 2004; Kelly et al. 2007; Prouteau et al. 2008; Stumpo et al. 2009). In plants much less information is known about their biological function, but several CCCH proteins were also identified as RNA-binding proteins (Li et al. 2001; Cheng et al. 2003; Addepalli and Hunt 2007, 2008; Pomeranz et al. 2009, 2010; Jan et al. 2013). Compared to the well described protein-RNA interactions of CCCH proteins, knowledge on protein-DNA and protein-protein interactions of the CCCH zinc finger proteins is yet very poorly understood. PEI1 is a CCCH gene from Arabidopsis and is able to bind to specific DNA sequences (Li and Thomas 1998). AtTZF1 can bind to both DNA and RNA *in vitro* (Pomeranz et al. 2009) and seems to have effects on plant growth, development and abiotic stress responses which may be related to GA and ABA metabolism (Lin et al. 2011; Pomeranz et al. 2011). From GhZFP1 it is known that it can also interact with other proteins (Guo et al. 2009). In rice, OsLIC (Wang et al. 2008a, b) and C3H12 (Deng et al. 2012) displayed binding activity to both double-stranded and single-stranded DNA and for Ehd4 from rice a role in flowering time was identified (Gao et al. 2013).

Classic C2H2 type zinc finger proteins contain three or more repeating finger units and bind as monomers. Each finger binds a specific triplet base-pair sequence and tandemly linked fingers bind sequential triplets and interact with the same DNA strand. In this way, a strong and specific DNA-protein interaction is created. However, it is not well understood if the CCCH zinc finger can bind specifically to DNA sequences in the same way C2H2 fingers do or that additional DNA sequences are needed. However, through a random binding site selection method it was demonstrated that recombinant PEI1 protein from Arabidopsis can specifically interact with DNA sequences (Li and Thomas 1998). Moreover, many CCCH zinc finger proteins have tandemly arranged fingers too which is indicative of a high specificity of interaction.

According to our EMSA results, OsGZF1 can interact not only with the bait used in the yeast one-hybrid screens, but also binds to three other segments of the *GluB-1* core promoter region. Taken into account that OsGZF1 has three zinc finger motifs, and that all of them are located with certain length of gaps in between, we speculate that these zinc fingers could interact with different parts of the *GluB-1* promoter region at the same time. Nonetheless, mutations of any of the oligonucleotides, which have been demonstrated to be important for downstream reporter gene expression (Yoshihara et al. 1996; Washida et al. 1999; Wu C et al. 2000), did not affect the interactions with OsGZF1 *in vitro*. This indicates that either these motifs are not the binding targets for OsGZF1, or mutations at one or two sites are not sufficient to prevent interactions since OsGZF1 has three zinc finger motifs for binding.

Potential roles of OsGZF1 in grain development

So far there is little known on biological functions of plant CCCH family members. AtTZF1 in Arabidopsis affects ABA- and GA-mediated growth, stress and gene expression responses (Lin et al. 2011) whereas AtTZF2 and AtTZF3 are involved in ABA and JA responses (Lee et al. 2012). GhZFP1 from cotton is involved in salt stress tolerance and fungal disease resistance (Guo et al. 2009). A rice protein, C3H12 is involved in the resistance to *Xanthomonas oryzae pv oryzae* (Deng et al. 2012) and another protein OsTZF1 confers delayed senescence and stress tolerance in rice (Jan et al. 2013). An Arabidopsis homologue of OsGZF1, PEI1, is highly conserved to OsGZF1 in the zinc finger domain (Fig. S4). PEI1 is an embryo-specific zinc finger protein gene required for heart-stage embryo formation in Arabidopsis (Li and Thomas 1998; Wang et al. 2008a), however, we did not observe any expression within embryos in the ProOsGZF1::GUS plants thus apparently OsGZF1 has a different function. The cell differentiation of the embryo is highly active during the early stage after fertilisation. At around 10 DAF, the size of the embryo increases rapidly and morphological differentiation is finished. Any transport of nutrients to the embryo during its development and during germination is through the endosperm tissue. Endosperm cells close to the embryo do not only supply nutrients to the embryo but are at some stage also digested and absorbed by the embryo in order to further provide in nutrient supply (Matsuo and Hoshikawa 1993). Our data suggests that OsGZF1 can down-regulates the promoter of *GluB-1* and affect the function of the *GluB-1* activator RISBZ1 in a protoplast system. We therefore hypothesise that regulation of *GluB-1* and possibly also of *RISBZ1* is also occurring *in vivo* in the confined region in the endosperm close to the embryo where *OsGZF1* is expressed and that the function of OsGZF1

is in controlling levels of GluB-1 and perhaps also other glutelins and seed-storage proteins. This hypothesis is supported by the finding that down-regulation of OsGZF1 which counteracts repression is resulting in an increase of grain N level. The expression pattern of OsGZF1 close to the embryo may also suggest a function in nutrient supply but needs to be further studied. It is very well possible that several other storage protein genes are also regulated by OsGZF1 considering the high similarity in core promoter sequences.

Acknowledgments This research was supported by the Programme for Strategic Scientific Alliances (04-PSA-BD-04) between China and the Netherlands of the Royal Netherlands Academy of Arts and Sciences (KNAW) for MW, PBF0, ZZ and the KNAW China Exchange Programmes (04CDP022, 06CDO033, 07CDP005, 08CDP042) for PBF0, MW, ZZ and YC.

References

- Addepalli B, Hunt AG (2007) A novel endonuclease activity associated with the Arabidopsis ortholog of the 30-kDa subunit of cleavage and polyadenylation specificity factor. *Nucleic Acids Res* 35:4453–4463
- Addepalli B, Hunt AG (2008) Ribonuclease activity is a common property of Arabidopsis CCCH-containing zinc-finger proteins. *FEBS Lett* 582:2577–2582
- Agalou A, Purwantomo S, Övernas E, Johannesson H, Zhu X, Estiati A, de Kam RJ, Engstrom P, Slamet-Loedin IH, Zhu Z, Wang M, Xiong L, Meijer AH, Ouwerkerk PBF (2008) A genome-wide survey of HD-Zip genes in rice and analysis of drought-responsive family members. *Plant Mol Biol* 66:87–103
- Chen S, Tao L, Zeng L, Vega-Sanchez M, Wang GL, Umemura K (2006) A highly efficient transient protoplast system for analyzing defence gene expression and protein–protein interactions in rice. *Mol Plant Pathol* 7:417–427
- Cheng Y, Kato N, Wang W, Li J, Chen X (2003) Two RNA binding proteins, HEN4 and HUA1, act in the processing of AGAMOUS pre-mRNA in Arabidopsis thaliana. *Dev Cell* 4:53–66
- Chern MS, Eiben HG, Bustos MM (1996) The developmentally regulated bZIP factor ROM1 modulates transcription from lectin and storage protein genes in bean embryos. *Plant J* 10:135–148
- Chiu W, Niwa Y, Zeng W, Hirano T, Kobayashi H, Sheen J (1996) Engineered GFP as a vital reporter in plants. *Curr Biol* 6:325–330
- Cord Neto G, Yunes JA, da Silva MJ, Vettore AL, Arruda P, Leite A (1995) The involvement of Opaque 2 on beta-prolamin gene regulation in maize and Coix suggests a more general role for this transcriptional activator. *Plant Mol Biol* 27:1015–1029
- Deng H, Liu H, Li X, Xiao J, Wang S (2012) A CCCH-type zinc finger nucleic acid-binding protein quantitatively confers resistance against rice bacterial blight disease. *Plant Physiol* 158:876–889
- Duan M, Sun SS (2005) Profiling the expression of genes controlling rice grain quality. *Plant Mol Biol* 59:165–178
- Gao J, Liu J, Li B, Li Z (2001) Isolation and purification of functional total RNA from blue-grained wheat endosperm tissues containing high levels of starches and flavonoids. *Plant Mol Biol Rep* 19:185–186
- Gao H, Zheng X-M, Fei G, Chen J, Jin M, Ren Y, Wu W, Zhou K, Sheng P, Zhou F et al (2013) Ehd4 encodes a novel and Oryza-genus-specific regulator of photoperiodic flowering in rice. *PLoS Genet* 9:1–12

- Green P, Kay S, Lam E, Chua NH (1989) In vitro DNA footprinting. In: Gelvin SB, Schilperoort RA (eds) Plant molecular biology manual. Kluwer, Dordrecht, pp B11/11–B11/22
- Guan KL, Dixon JE (1991) Eukaryotic proteins expressed in *Escherichia coli*: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. *Anal Biochem* 192:262–267
- Guo X, Carroll JW, Macdonald MR, Goff SP, Gao G (2004) The zinc finger antiviral protein directly binds to specific viral mRNAs through the CCCH zinc finger motifs. *J Virol* 78:12781–12787
- Guo YH, Yu YP, Wang D, Wu CA, Yang GD, Huang JG, Zheng CC (2009) GhZFP1, a novel CCCH-type zinc finger protein from cotton, enhances salt stress tolerance and fungal disease resistance in transgenic tobacco by interacting with GZIRD21A and GZIPR5. *New Phytol* 183:62–75
- Hajdukiewicz P, Svab Z, Maliga P (1994) The small, versatile pPZP family of *Agrobacterium* binary vectors for plant transformation. *Plant Mol Biol* 25:989–994
- Hamaker BR, Griffin VK, Moldenhauer KAK (1991) Potential influence of a starch granule-associated protein on cooked rice stickiness. *J Food Sci* 56:1327–1329
- Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge SJ (1993) The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* 75:805–816
- Hartings H, Maddaloni M, Lazzaroni N, Di Fonzo N, Motto M, Salamini F, Thompson R (1989) The O2 gene which regulates zein deposition in maize endosperm encodes a protein with structural homologies to transcriptional activators. *EMBO J* 8:2795–2801
- Holdsworth MJ, Munoz-Blanco J, Hammond-Kosack M, Colot V, Schuch W, Bevan MW (1995) The maize transcription factor Opaque-2 activates a wheat glutenin promoter in plant and yeast cells. *Plant Mol Biol* 29:711–720
- Hwang YS, Ciceri P, Parsons RL, Moose SP, Schmidt RJ, Huang N (2004) The maize O2 and PBF proteins act additively to promote transcription from storage protein gene promoters in rice endosperm cells. *Plant Cell Physiol* 45:1509–1518
- Izawa T, Foster R, Nakajima M, Shimamoto K, Chua NH (1994) The Rice bZIP transcriptional activator RITA-1 is highly expressed during seed development. *Plant Cell* 6:1277–1287
- Jan A, Maruyama K, Todaka D, Kidokoro S, Abo M, Yoshimura E, Shinozaki K, Nakashima K, Yamaguchi-Shinozaki K (2013) OsTZF1, a CCCH-Tandem zinc finger protein, confers delayed senescence and stress tolerance in rice by regulating stress-related genes. *Plant Physiol* 16:1202–1216
- Jefferson R (1987) Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Mol Biol Rep* 5:387–405
- Johnson AD (1995) The price of repression. *Cell* 81:655–658
- Kang HJ, Hwang IK, Kim KS, Choi HC (2006) Comparison of the physicochemical properties and ultrastructure of japonica and indica rice grains. *J Agric Food Chem* 54:4833–4838
- Kawakatsu T, Yamamoto MP, Hirose S, Yano M, Takaiwa F (2008) Characterization of a new rice glutelin gene GluD-I expressed in the starchy endosperm. *J Exp Bot* 59:4233–4245
- Kawakatsu T, Yamamoto MP, Touno SM, Yasuda H, Takaiwa F (2009) Compensation and interaction between RISBZ1 and RPBFB during grain filling in rice. *Plant J* 59:908–920
- Kelly SM, Pabitt SA, Kitchen CM, Guo P, Marfatia KA, Murphy TJ, Corbett AH, Berland KM (2007) Recognition of polyadenosine RNA by zinc finger proteins. *Proc Natl Acad Sci USA* 104:12306–12311
- Kikuchi S, Satoh K, Nagata T, Kawagashira N, Doi K, Kishimoto N, Yazaki J, Ishikawa M, Yamada H, Ooka H et al (2003) Collection, mapping, and annotation of over 28,000 cDNA clones from japonica rice. *Science* 301:376–379
- Kim SY, Wu R (1990) Multiple protein factors bind to a rice glutelin promoter region. *Nucleic Acids Res* 18:6845–6852
- Kreis M, Shewry PR, Forde BG, Forde J, Mifflin BJ (1985) Structure and evolution of seed storage proteins and their genes with particular reference to those of wheat, barley and rye. In: Mifflin B (ed) Oxford surveys of plant molecular and cell biology. Oxford University Press, Oxford, pp 253–317
- Kuijt SJH, Lamers GEM, Rueb S, Scarpella E, Ouwerkerk PBF, Spaik HP, Meijer AH (2004) Different subcellular localization and trafficking properties of KNOX class I homeodomain proteins from rice. *Plant Mol Biol* 55:781–796
- Kusaba M, Miyahara K, Iida S, Fukuoka H, Takano T, Sassa H, Nishimura M, Nishio T (2003) Low glutelin content 1: a dominant mutation that suppresses the glutelin multigene family via RNA silencing in rice. *Plant Cell* 15:1455–1467
- Lee SJ, Jung HJ, Kang H, Kim SY (2012) Arabidopsis zinc finger proteins AtC3H49/AtTZF3 and AtC3H20/AtTZF2 are involved in ABA and JA responses. *Plant Cell Physiol* 53:673–686
- Li Z, Thomas TL (1998) PEI1, an embryo-specific zinc finger protein gene required for heart-stage embryo formation in Arabidopsis. *Plant Cell* 10:383–398
- Li J, Jia D, Chen X (2001) HUA1, a regulator of stamen and carpel identities in Arabidopsis, codes for a nuclear RNA binding protein. *Plant Cell* 13:2269–2281
- Lin PC, Pomeranz MC, Jikumaru Y, Kang SG, Hah C, Fujioka S, Kamiya Y, Jang JC (2011) The Arabidopsis tandem zinc finger protein AtTZF1 affects ABA- and GA-mediated growth, stress and gene expression responses. *Plant J* 65:253–268
- Lohmer S, Maddaloni M, Motto M, Di Fonzo N, Hartings H, Salamini F, Thompson RD (1991) The maize regulatory locus Opaque-2 encodes a DNA-binding protein which activates the transcription of the b-32 gene. *EMBO J* 10:617–624
- Matsuo T, Hoshikawa K (1993) Science of the rice plant, vol 1: morphology. Food and Agriculture Policy Research Center, Tokyo
- Meijer AH, Ouwerkerk PBF, Hoge JHC (1998) Vectors for transcription factor cloning and target site identification by means of genetic selection in yeast. *Yeast* 14:1407–1415
- Memelink J (1997) Two Yeast/*Escherichia coli* lambda/plasmid vectors designed for yeast one- and two-hybrid screens that allow directional cDNA cloning. Technical Tips Online TT01111 (<http://research.bmn.com/>). *Trends Genet* 13:376
- Memelink J, Swords KMM, Staehelin LA, Hoge JHC (1994) Southern, northern and western blot analysis. In: Gelvin SB, Schilperoort RA (eds) Plant molecular biology manual. Kluwer, Dordrecht, pp F1–F23
- Mitsukawa N, Hayashi H, Yaiyamamoto K, Kidzu K, Konishi R, Masutvlura T, Tanaka K (1998) Molecular cloning of a novel glutelin cDNA from rice seeds. *Plant Biotechnol* 15:205–211
- Muth JR, Muller M, Lohmer S, Salamini F, Thompson RD (1996) The role of multiple binding sites in the activation of zein gene expression by Opaque-2. *Mol Gen Genet* 252:723–732
- Nakase M, Yamada T, Kira T, Yamaguchi J, Aoki N, Nakamura R, Matsuda T, Adachi T (1996) The same nuclear proteins bind to the 5'-flanking regions of genes for the rice seed storage protein: 16 kDa albumin, 13 kDa prolamin and type II glutelin. *Plant Mol Biol* 32:621–630
- Okita TW, Hwang YS, Hnilo J, Kim WT, Aryan AP, Larson R, Krishnan HB (1989) Structure and expression of the rice glutelin multigene family. *J Biol Chem* 264:12573–12581
- Onodera Y, Suzuki A, Wu CY, Washida H, Takaiwa F (2001) A rice functional transcriptional activator, RISBZ1, responsible for endosperm-specific expression of storage protein genes through GCN4 motif. *J Biol Chem* 276:14139–14152
- Ouwerkerk PBF, Meijer AH (2001) Yeast one-hybrid screening for DNA-protein interactions. In: Ausubel FM et al (eds) Current protocols in molecular biology 12.12.1–12.12.22

- Ouwerkerk PBF, Meijer AH (2011) Yeast one-hybrid screens for detection of transcription factor DNA interactions. *Methods Mol Biol* 678:211–227
- Pasquali G, Ouwerkerk PBF, Memelink J (1994) Versatile transformation vectors to assay the promoter activity of DNA elements in plants. *Gene* 149:373–374
- Pomeranz MC, Hah C, Lin PC, Kang SG, Finer JJ, Blackshear PJ, Jang JC (2009) The Arabidopsis tandem zinc finger protein AtTZF1 traffics between the nucleus and cytoplasmic foci and binds both DNA and RNA. *Plant Physiol* 152:151–165
- Pomeranz MC, Zhang L, Finer J, Jang J-C (2011) Can AtTZF1 act as a transcriptional activator or repressor in plants? *Plant Signal Behav* 6:719–722
- Prouteau M, Dageron MC, Seraphin B (2008) Regulation of ARE transcript 3' end processing by the yeast Cth2 mRNA decay factor. *EMBO J* 27:2966–2976
- Saleh MI, Meullenet J-F (2007) Effect of protein disruption using proteolytic treatment on cooked rice texture properties. *J Texture Stud* 38:423–437
- Scarpella E, Rueb S, Boot KJ, Hoge JHC, Meijer AH (2000) A role for the rice homeobox gene *Oshox1* in provascular cell fate commitment. *Development* 127:3655–3669
- Schmidt RJ, Burr FA, Aukerman MJ, Burr B (1990) Maize regulatory gene *opaque-2* encodes a protein with a “leucine-zipper” motif that binds to zein DNA. *Proc Natl Acad Sci USA* 87:46–50
- Schmidt RJ, Ketudat M, Aukerman MJ, Hoschek G (1992) *Opaque-2* is a transcriptional activator that recognizes a specific target site in 22-kD zein genes. *Plant Cell* 4:689–700
- Singh G, Kumar S, Singh P (2003) A quick method to isolate RNA from wheat and other carbohydrate-rich seeds. *Plant Mol Biol Rep* 21:93
- Stumpo DJ, Broxmeyer HE, Ward T, Cooper S, Hangoc G, Chung YJ, Shelley WC, Richfield EK, Ray MK, Yoder MC, Aplan PD, Blackshear PJ (2009) Targeted disruption of *Zfp3612*, encoding a CCCH tandem zinc finger RNA-binding protein, results in defective hematopoiesis. *Blood* 114:2401–2410
- Suzuki A, Wu CY, Washida H, Takaiwa F (1998) Rice MYB protein OSMYB5 specifically binds to the AACA motif conserved among promoters of genes for storage protein glutelin. *Plant Cell Physiol* 39:555–559
- Takaiwa F (1987) Gene for a rice storage protein glutelin. *Tanpakushitsu Kakusan Koso*, pp 247–260
- Takaiwa F, Oono K (1990) Interaction of an immature seed-specific trans-acting factor with the 5' upstream region of a rice glutelin gene. *Mol Gen Genet* 224:289–293
- Takaiwa F, Oono K (1991) Genomic DNA sequences of two new genes for new storage protein glutelin in rice. *Jpn J Genet* 66:161–171
- Takaiwa F, Oono K, Kato A (1991a) Analysis of the 5' flanking region responsible for the endosperm-specific expression of a rice glutelin chimeric gene in transgenic tobacco. *Plant Mol Biol* 16:49–58
- Takaiwa F, Oono K, Wing D, Kato A (1991b) Sequence of three members and expression of a new major subfamily of glutelin genes from rice. *Plant Mol Biol* 17:875–885
- Takaiwa F, Yamanouchi U, Yoshihara T, Washida H, Tanabe F, Kato A, Yamada K (1996) Characterization of common cis-regulatory elements responsible for the endosperm-specific expression of members of the rice glutelin multigene family. *Plant Mol Biol* 30:1207–1221
- Töpfer R, Matzeit V, Gronenborn B, Schell J, Steinbiss HH (1987) A set of plant expression vectors for transcriptional and translational fusions. *Nucleic Acids Res* 15:5890
- Ueda T, Wawerczak W, Ward K, Sher N, Ketudat M, Schmidt RJ, Messing J (1992) Mutations of the 22- and 27-kD zein promoters affect transactivation by the *Opaque-2* protein. *Plant Cell* 4:701–709
- Vicente-Carbajosa J, Moose SP, Parsons RL, Schmidt RJ (1997) A maize zinc-finger protein binds the prolamin box in zein gene promoters and interacts with the basic leucine zipper transcriptional activator *Opaque2*. *Proc Natl Acad Sci USA* 94:7685–7690
- Villareal RM, Juliano BO (1978) Properties of glutelin from mature and developing rice grain. *Phytochemistry* 17:177–182
- Wang D, Guo Y, Wu C, Yang G, Li Y, Zheng C (2008a) Genome-wide analysis of CCCH zinc finger family in Arabidopsis and rice. *BMC Genomics* 9:44
- Wang L, Xu Y, Zhang C, Ma Q, Joo SH, Kim SK, Xu Z, Chong K (2008b) OsLIC, a novel CCCH-type zinc finger protein with transcription activation, mediates rice architecture via brassinosteroids signaling. *PLoS ONE* 3:e3521
- Washida H, Wu CY, Suzuki A, Yamanouchi U, Akihama T, Harada K, Takaiwa F (1999) Identification of cis-regulatory elements required for endosperm expression of the rice storage protein glutelin gene *GluB-1*. *Plant Mol Biol* 40:1–12
- Wesley SV, Helliwell CA, Smith NA, Wang M, Rouse DT, Liu Q, Gooding PS, Singh SP, Abbott D, Stoutjesdijk PA, Robinson SP, Gleave AP, Green AG, Waterhouse PM (2001) Construct design for efficient, effective and high-throughput gene silencing in plants. *Plant J* 27:581–590
- Wu CY, Suzuki A, Washida H, Takaiwa F (1998) The GCN4 motif in a rice glutelin gene is essential for endosperm-specific gene expression and is activated by *Opaque-2* in transgenic rice plants. *Plant J* 14:673–683
- Wu C, Washida H, Onodera Y, Harada K, Takaiwa F (2000) Quantitative nature of the Prolamin-box, ACGT and AACA motifs in a rice glutelin gene promoter: minimal cis-element requirements for endosperm-specific gene expression. *Plant J* 23:415–421
- Yamagata H, Sugimoto T, Tanaka K, Kasai Z (1982) Biosynthesis of storage proteins in developing rice seeds. *Plant Physiol* 70:1094–1100
- Yamamoto MP, Onodera Y, Touno SM, Takaiwa F (2006) Synergism between RPBF Dof and RISBZ1 bZIP activators in the regulation of rice seed expression genes. *Plant Physiol* 141:1694–1707
- Yoshihara T, Takaiwa F (1996) Cis-regulatory elements responsible for quantitative regulation of the rice seed storage protein glutelin *GluA-3* gene. *Plant Cell Physiol* 37:107–111
- Yoshihara T, Washida H, Takaiwa F (1996) A 45-bp proximal region containing AACA and GCN4 motif is sufficient to confer endosperm-specific expression of the rice storage protein glutelin gene, *GluA-3*. *FEBS Lett* 383:213–218
- Zhang N, Qiao Z, Liang Z, Mei B, Xu Z, Song R (2012) Zea mays *Taxilin* protein negatively regulates *opaque-2* transcriptional activity by causing a change in its sub-cellular distribution. *PLoS ONE* 7:e43822
- Zheng Z, Kawagoe Y, Xiao S, Li Z, Okita T, Hau TL, Lin A, Murai N (1993) 5' distal and proximal cis-acting regulator elements are required for developmental control of a rice seed storage protein glutelin gene. *Plant J* 4:357–366