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Sucrose signaling regulates storage protein synthesis in maize endosperm

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Abstract

Sucrose plays a crucial role as a primary carbon source during seed development. In addition to this metabolic function, sucrose and its breakdown products (hexoses) serve as important signaling molecules that regulate cell fate and seed development in maize. However, the precise molecular mechanisms through which sucrose influences storage protein synthesis during seed development remain poorly understood. In this study, we identified a novel allelic mutant of *Incw2*, named *vitreous endosperm 2* (*ven2*). The loss-of-function mutation in *Incw2* led to a significant reduction in grain weight, accompanied by a marked increase in sucrose levels in the basal endosperm. This was also associated with substantial increases in the accumulation of the 19 kDa and 22 kDa α-zein proteins. In contrast, overexpression of *Incw2* resulted in lower sucrose and zein contents. Furthermore, we discovered that the transcription factor Sugar Response Factor 1 (SRF1), which is responsive to sucrose, regulates the expression of the *19 kDa* and *22 kDa α-zein* genes by directly binding to their promoters. These findings suggest that disruption of sucrose metabolism due to the *Incw2* mutation modulates *α-zein* gene expression and affects protein synthesis during seed development.

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Introduction

In maize (*Zea mays* L.) seeds, starch, and storage proteins represent the primary components of the endosperm, which is a crucial tissue for nutrient storage and seed development. Starch constitutes the largest proportion, accounting for approximately 70%–75% of the seed dry weight, and serves as the main energy reserve for the germinating seedling. Storage proteins, primarily zeins, make up around 8%–10% of the dry weight and play an essential role in providing amino acids required for early growth. Together, starch and storage proteins support the metabolic needs of the seed during germination and early seedling development. The balance and accumulation of these components are tightly regulated through complex molecular pathways involving enzymes and transcription factors, which ensure that maize seeds have the necessary nutrients to sustain growth and development upon germination. Understanding the genetic and biochemical mechanisms behind starch and protein biosynthesis in the endosperm is critical for improving the yield and quality of maize seeds[1]. Seed texture and quality are determined by the ratio of vitreous to starchy endosperm. The vitreous endosperm, located in the outer layer of seeds, enhances resistance to pests and diseases and protects the grain from mechanical damage during harvest and transport[2]. The abundance of zein gene expression plays a crucial role in forming vitreous endosperm during seed maturation[3–5]. Zeins, which comprise over 60% of maize proteins, are classified into four major groups: α-zeins (19 and 22 kDa), β-zein (15 kDa), γ-zeins (16, 27, and 50 kDa), and δ-zeins (10 and 18 kDa) based on their amino acid sequences[6]. While α-zeins are encoded by a multigene family, other zeins are encoded by single-copy or low-copy genes[7,8]. The expression patterns and regulation of these genes are critical for zein accumulation in maize seeds.

Recent studies have demonstrated that specific transcription factors are central to regulating the biosynthesis of starch and storage proteins in the seed endosperm[9]. These transcription factors function as master regulators, orchestrating the expression of genes involved in the synthesis and accumulation of both starch and storage proteins, such as zeins. The coordinated activity of these factors ensures the proper balance between starch production, which provides the energy reserves for germination, and storage protein synthesis, which supplies essential amino acids. These transcription factors often respond to environmental cues, metabolic signals, and developmental signals, allowing the plant to adjust its seed composition to optimize seed viability and growth potential. For example, factors like WRI1, LEC1, and FUS3 have been implicated in the regulation of genes responsible for storage protein and starch synthesis[10]. By modulating the expression of key enzymes, these transcription factors help control the timing and extent of starch deposition and protein accumulation, which are critical for seed development and subsequent seedling growth. The intricate regulation of these processes by transcription factors is essential for improving crop yield and quality, offering potential targets for genetic engineering to enhance seed nutrient content[9]. The first zein regulator identified in the classic opaque endosperm mutants was the bZIP protein OPAQUE2 (O2), a central regulator of nearly all zein gene families[11–13]. O2 regulates zein gene expression through additive and synergistic interactions with multiple transcription factors (TFs), including O2-heterodimerizing proteins (OHPs), Prolamin-box binding factor 1 (PBF1), bZIP transcription factor 22 (bZIP22), and MADS-box transcription factor 47 (MADS47)[14–17]. ZmNAC128 and ZmNAC130, in coordination with O2, activate the transcription

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of *γ-zein* genes (50, 27, and 16 kDa) and starch metabolism genes, including *Brittle2*, *pullulanase-type starch debranching enzyme*, *granule-bound starch synthase I*, *starch synthase I*, *starch synthase II*, and *sucrose synthase I*, thereby regulating the accumulation of storage proteins and starch in maize seeds[18,19]. Additionally, Opaque11 (O11), a key TF in endosperm development and nutrient metabolism, directly regulates other key TFs involved in protein deposition, such as O2 and PBF1[20]. ZmABI19 also directly regulates several essential grain-filling TFs, including O2, O11, NAC130, ZmbZIP22, and PBF1, in the endosperm nutrient reservoir[21]. However, the signaling pathways that regulate these TFs and zein genes remain unclear.

Sucrose plays a dual role in plant growth and development, serving as both a vital source of energy and carbon and as a key signaling molecule that regulates various developmental and metabolic processes[22,23]. It has been shown to synergistically regulate starch synthesis, storage protein accumulation, and grain filling[24–27]. In maize, sucrose and ABA jointly regulate the starch biosynthesis-related gene *ZmSSIIIa* via the transcription factor ZmEREB156, driving starch accumulation in the endosperm[28]. Sucrose also promotes the expression of *O2*, a hub gene that coordinates the accumulation of storage reserves in the endosperm[29]. Similarly, in potato, sucrose induces the expression of the *patatin* gene, which encodes a storage protein constituting 30%–40% of the total soluble proteins[30]. However, whether sucrose signaling influences zein protein accumulation in maize endosperm remains unclear. In this study, we identified the gene underlying the *ven2* mutant and confirmed it as a mutant allele of *Mn1/Incw2*, which encodes a cell wall invertase expressed in the basal endosperm[31]. Beyond its role in seed size regulation, we demonstrate that *Mn1/Incw2* also affects seed texture and quality through sucrose metabolism.

Materials and methods

Plant materials

The mutants *ven2* and *ven2-1* were identified in the ethyl methane sulfonate (EMS) mutagenesis populations of the inbred lines B73 and HuangZao4, respectively. The fine mapping population was developed using the F_2 progenies of C733 × *ven2*. *OE-Incw2* is a gift from Prof. Guangtang Pan (Sichuan Agricultural University, Sichuan, China), which is *Incw2* gene driven by *27 kDa γ-zein* promoter specifically over-expressed in the endosperm cells[32]. The *ven2-2* mutant was in W22 inbred line background obtained from the Maize Genetic Cooperation Stock Center.

Measurement of starch and protein contents

Twelve mature seeds of wild type (WT) and mutants were selected for further research, respectively. The pericarp and embryo of seeds were removed and the endosperm were ground into fine flour. Then, 50 mg of each sample was used for starch and proteins measurements according to previous described[33]. SDS-PAGE gel with Coomassie Brilliant Blue (CBB)-staining was performed to analyze the accumulation patterns of zein proteins in WT, *ven2*, *ven2-1*, and *OE-Incw2*.

Scanning electron microscopy (SEM)

For scanning electron microscopy, mature WT and *ven2* seeds were rifted longitudinally with a razor. Samples were critical-point dried, spray-coated with gold and then observed under a scanning electron microscopy (JSM-6610LV) at Shandong Agricultural University (Shandong, China).

Map-based cloning

To clone the causative gene for *ven2* phenotype, the homozygous mutants were out-crossed with maize inbred line C733, and then self-pollinated the offspring to generate the F_2 segregating population. A total of 1,547 homozygous *ven2* mutant seeds from F_2 segregating population with vitreous endosperm phenotype were used for gene mapping. Polymorphic molecular markers listed in Supplementary Table S1 were used to localize the *Ven2* locus to a 280-kb region between the molecular markers 28z-27 and c-3799-2 containing four annotated genes. The corresponding DNA fragments of candidate genes were amplified from WT and *ven2* seeds using KOD DNA polymerase (Toyobo, Japan) and sequenced. Finally, allelism tests were carried out among *ven2*, *ven2-1*, and *ven2-2*.

RNA extraction and reverse-transcription quantitative PCR (qRT-PCR)

Total RNA was extracted from plant tissues using the Ultrapure RNA Kit (cwbiotech, CW0581M). After DNase treatment to remove genomic DNA, 2-μg of total RNA per sample was subjected to synthesize first-strand cDNA using FastQuant RT Kit (TIANGEN) according to the instructions. Next, qRT-PCR was performed using SYBR Green qRT-PCR kit (TIANGEN) on a Light Cycler 96 (Roche Diagnostics) detection system. The gene expression was normalized to the maize *Actin* gene (Zm00001d010159) with three biological replicates. The primers used in qRT-PCR are listed in Supplementary Table S1.

Measurement of sucrose, glucose, and fructose

The developing seeds of WT, *ven2*, CK, and *OE-Incw2* at 6, 12, 18, and 24 DAP were cut longitudinally transversely along the base of the embryo into two parts (upper and basal seeds section). Each sample was ground into a fine flour with liquid nitrogen. First, the total soluble sugar in the sample was extracted three times by 80% ethanol at 80 °C. And then, the resulting soluble sugar solution was dried into flour at 60–80 °C. The fructose, glucose, and sucrose were differentiated by HPLC systems (LC-20AT, Ultimate 3000) using XBridge® Amide Column (4.6 mm × 250 mm, 3.5 μm, Waters) at Shandong Analysis Test Center. The operating temperature was 25 °C, and mobile phases A and B were acetonitrile and H_2O, respectively. And the flow rate was 0.8 mL/min.

Transient expression assays, exogenous sugar induction, and LUC activity assay in maize endosperm

Approximately 1,500 bp regions region of *19 kDa α-zein* and *22 kDa α-zein* were amplified with the primers listed in Supplementary Table S2. Then these two sequences were introduced into the pGreenII0800-LUC vector, respectively, yielding the construct *pro19 kDa-LUC* and *pro22 kDa-LUC*. Endosperms at 16 DAP were excised and sterilized with 75% (v/v) ethanol and cultivated on MS medium at 28 °C, with a 4-h dark photoperiod. Then the prepared endosperm was subject to bombardment using a helium biolistic gun transformation system (PDS-1000, Bio-Rad) according to the manufacturer's instructions and the methods previously described[34]. The bombarded endosperm is cultivated on MS medium supplied with 200 nM mannitol (as the control), 200 nM sucrose, 200 nM glucose, and 200 nM fructose, respectively, at 28 °C, for 24-h dark photoperiod to analyze LUC activity. For the LUC activity assay, both LUC and REN activities were measured using the Dual-Luciferase Reporter Assay System (Promega, USA) according to the manufacturer's instructions on the Enzyme label instrument (PerkinElmer EnSpire). Relative LUC activity was calculated by normalizing the LUC activity to the REN activity.

Transient expression assay in leaves of *N. benthamiana*

Approximately 1,500 bp regions of the *19 kDa and 22 kDa α-zein* gene promoter were cloned into the vector pGreenII0800-LUC to generate a reporter. The full-length coding sequences of *SRF1* (Zm00001d021947) were inserted into vector pGreenII62-SK under

the control of the *35S* promoter to generate the effectors. pGreenII62-SK under the control of the *35S* promoter was used as the negative control effector. The reporter and the effector were introduced into *Agrobacteriumtumefaciens* GV3101 strain (pSoup-p19, Catalog No. AC1003, Shanghai Weidi Biotechnology Co., Ltd., Shanghai, China) and then were co-infiltrated into the leaves of *N. benthamiana* as described previously[35]. The LUC/REN activity ratio was measured using a Dual-luciferase Reporter Assay System (Promega).

Recombinant protein expression and purification

The full coding sequences of *SRF1* were fused with GST in the expression vectors pGEX-4T-1, and expressed in *E. coli* BL21 (DE3) strain. The recombinant proteins were induced with 0.2 mM isopropyl-β-D-thiogalactoside (IPTG) for 16 h induction at 16 °C and were purified with glutathione beads from GE Healthcare. Primers are listed in Supplementary Table S2.

Electrophoretic mobility shift assay (EMSA)

Oligonucleotide probes of the *19 kDa* and *22 kDa α-zein* gene promoter was synthesized and labeled with biotin at the 5' end by Sangon Biotech. Biotin-labeled DNA was detected according to the instructions of the LightShift Chemiluminescent EMSA Kit (Thermo Fisher Scientific, Catalog No. 20158) according to previous studies[36,37].

Results

The *ven2* mutant produced small seeds characterized by vitreous endosperm

A mutant was derived from an EMS-mutagenized B73 population. Homozygous mutants were crossed with B73 and C733 inbred lines, respectively. Phenotypic segregation in the F_2 seeds followed a 3:1 ratio of wild-type (WT, +/+ and *ven2*/+) to mutant (*ven2*) phenotypes, confirming that it is a recessive, nuclear monogenic mutation (Supplementary Table S2). The mutant seeds were smaller, shrunken, and vitreous compared to WT seeds (Fig. 1a; Supplementary Fig. S1a, b), exhibiting a harder endosperm phenotype. So, the mutant was named *vitreous endosperm 2* (*ven2*). Longitudinal dissection showed that *ven2* seeds had an increased ratio of hard to soft endosperm (Fig. 1b). The hundred-seed weight of *ven2* was only 23% of WT (Supplementary Fig. S1c). Starch content in *ven2* seeds was 32% lower than that in WT seeds (Supplementary Fig. S1d), while zein protein levels were significantly higher in *ven2* seeds, with non-zein proteins remaining unchanged (Fig. 1c–e). Correspondingly, transcript levels of most zein genes, particularly those encoding 19-kDa and 22-kDa α-zeins, were markedly elevated in the *ven2* endosperm at 15 DAP (Supplementary Fig. S2). The results of SEM showed that the central region of *ven2* endosperm was densely packed with smaller, smooth starch granules (SGs) embedded in a protein matrix. In contrast, WT endosperm was loosely packed with

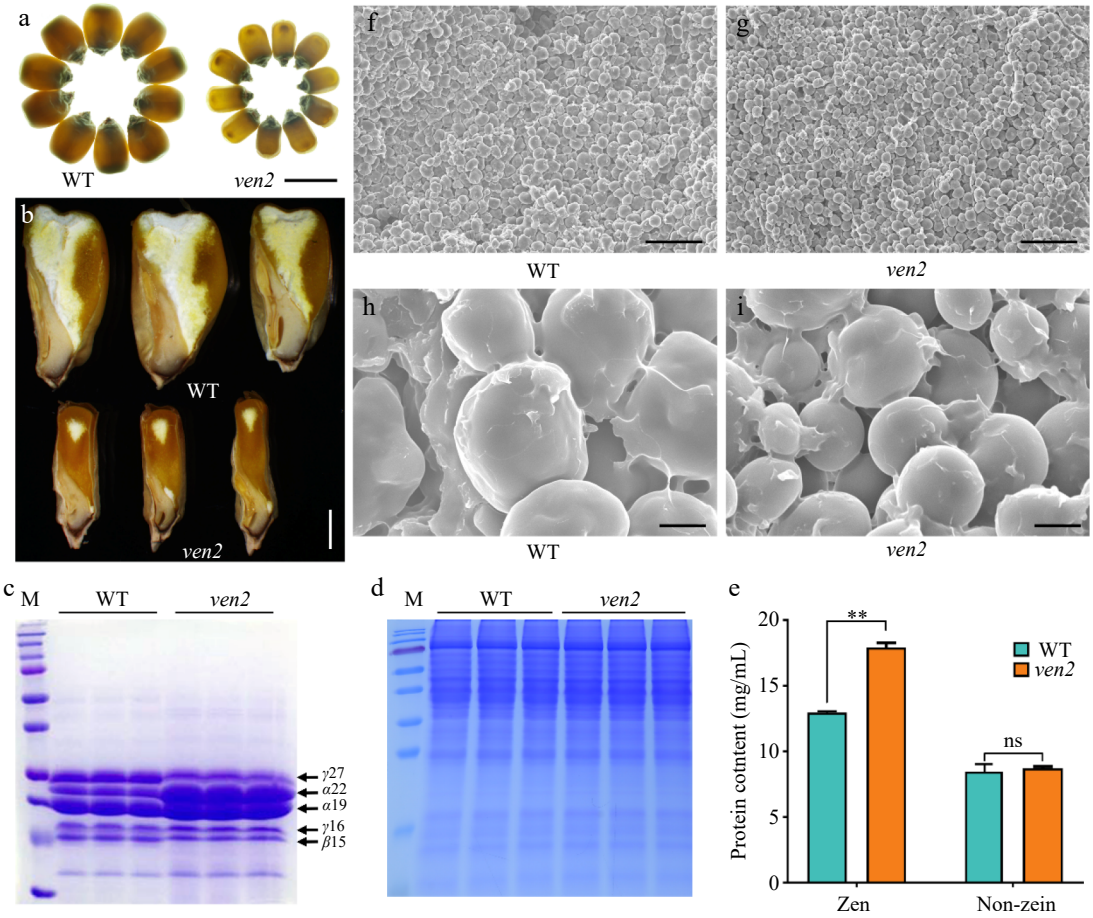

Fig. 1 Seed phenotypes of the WT and *ven2* mutant. (a) WT and *ven2* mature seeds viewed on a light box. Scale bars, 2 mm. (b) Sagittal sections of WT and *ven2* mature seeds. Scale bars, 2 mm. SDS-PAGE analysis of (c) endosperm zein and (d) non-zein protein accumulation in WT and *ven2* mutant. (e) Quantification of zein, non-zein protein contents in WT and *ven2* mature endosperm. (f), (g) SEM of the WT and the *ven2* endosperm cells at maturity. Scale bars, 50 μm. (h), (i) The magnified sections from the (f) and (g) panels. Scale bars, 5 μm.

spherical SGs and lacked the protein matrix seen in *ven2* (Fig. 1f–i, Supplementary Fig. S3).

Map-based cloning of *Ven2* gene

To identify the gene responsible for the phenotype of *ven2* mutant, we conducted genetic mapping using an F_2 population derived from a cross between C733 and *ven2*. After analyzing 1,547 F_2 plants displaying the *ven2* phenotype with molecular markers (Supplementary Table S2), we narrowed the locus to a 0.28 Mb genomic interval on Chromosome 2, located between the markers 28z-27 and c-3799-2. According to the maize version 4 database, four open reading frames (ORFs) were identified within this mapped region (Supplementary Fig. S4). Sequencing revealed an EMS-induced G/A transition in the second exon of the Zm00001d003776 gene, leading to a conversion from aspartate (Asp) to asparagine (Asn) (Fig. 2a). Thus, the Zm00001d003776 gene is the candidate gene and designed as *Ven2*, which was the same gene as *cell wall invertase II* (*INCW2*) in maize.

To confirm that the identified mutation in *Ven2* is the causal gene for the *ven2* phenotype, we obtained two additional mutant alleles: a spontaneous small kernel mutation in maize, *ven2-1* (from the HuangZao4 background) and *ven2-2* (from the W22 background) from the Maize Genetic Cooperation Stock Center, which are new allelic mutants. In the *ven2-1* mutant, a deletion of 37 bp from positions 952 to 988 in *Ven2* gene leads to early termination of protein translation, resulting in significantly increased zein protein levels (Fig. 2a; Supplementary Fig. S5). The mutant *ven2-1* exhibited a higher ratio of vitreous endosperm, smaller starch granules (SGs), and more protein bodies compared to WT (HuangZao4) (Supplementary Fig. S6). Thus, *ven2-1* shares a similar phenotype with *ven2*. In the *ven2-2* mutant, a single nucleotide change (A to G) occurs in the *Ven2* gene, resulting in a codon change from CAG (Gln/N) to CGG (Arg/R) (Fig. 2a). Allelism tests were conducted by crossing *ven2-1* and *ven2-2* with the *ven2* mutant. All F_1 seeds exhibited the *ven2* mutant phenotype (Fig. 2b–d). These results confirm that the loss of function in *Ven2* is responsible for the *ven2* mutant phenotype.

Ven2/*INCW2* is primarily functions to hydrolyze sucrose into hexoses[31,38,39]. To evaluate sugar content in WT and *ven2* seeds, we employed a sensitive HPLC-based detection method. Our results showed that fructose (Fru) and glucose (Glc) levels decreased in the basal sections of *ven2* seeds at 6, 12, 18, and 24 DAP. Conversely, sucrose (Suc) levels increased in the basal *ven2* seeds (Supplementary Fig. S7). Interestingly, Glc and Fru levels increased in the upper sections of *ven2* seeds at 6, 12, and 18 DAP. However, Suc levels remained unchanged at 6 and 12 DAP. Overall, there was no significant change in Glc and Fru levels in the entire seeds, but Suc levels increased in *ven2* seeds (Supplementary Fig. S7). These findings confirmed that the loss of function of Ven2/Incw2 results in sucrose accumulation during early seed development.

19 kDa α-zein and *22 kDa α-zein* are regulated by a sucrose-inducible promoter

Sugars play a dual role in plants, serving as both energy sources and signaling molecules that regulate gene expression and development[40]. To investigate whether the accumulation of zein in the *ven2* mutant is linked to the changes in sugar content, we analyzed the promoters of the *19 kDa α-zein* and *22 kDa α-zein* genes. Notably, the promoters of both genes contain several W-box *cis*-elements that respond to sugar signals (Fig. 3a).

To determine if sugar induces the expression of these genes, we cultured 16 DAP endosperm from WT in Murashige and Skoog (MS) medium supplemented with mannitol, sucrose, glucose, and fructose, respectively. The expression levels of the *19 kDa* and *22 kDa α-zein* genes were subsequently measured. The results indicated that transcripts for both genes accumulated in the endosperm treated with sucrose (Fig. 3b). To further explore the role of the W-box element in regulating zein gene expression, we conducted transient assays using chimeric promoters of the *19 kDa* and *22 kDa α-zein* genes, fused to the luciferase (LUC) reporter gene in transformed maize endosperm. The total length of about 1.5 kb promoters was linked to the LUC reporter, and the transformed endosperm was cultured on various sugar-treated media. Results revealed a significant increase in relative LUC activity in endosperm treated with

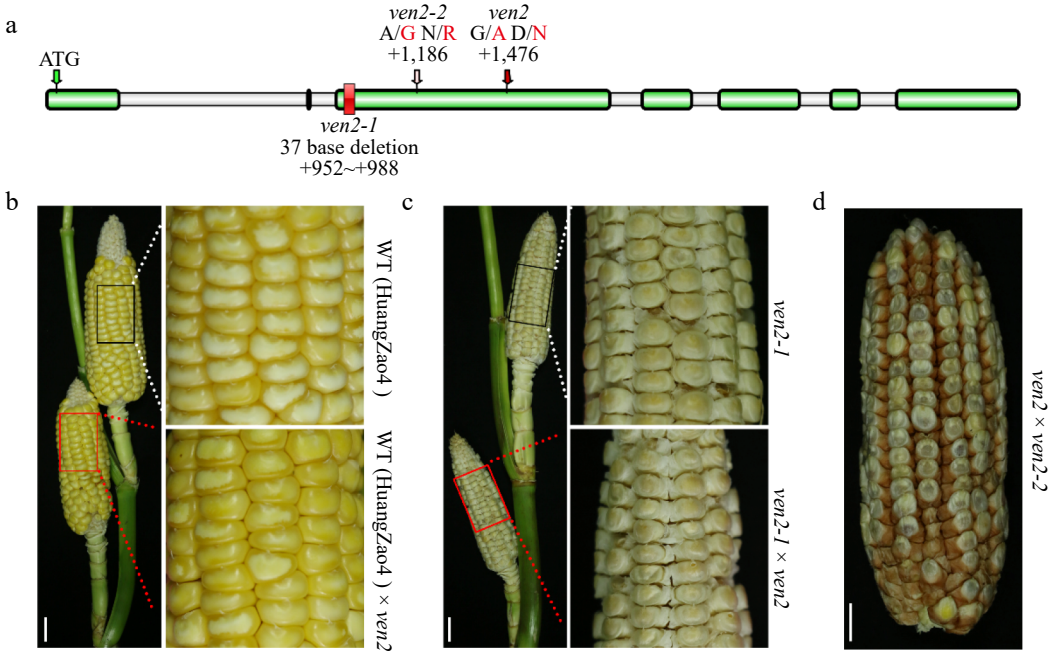

Fig. 2 Positional cloning and identification of *Ven2*. (a) Structure and mutation site of the Zm00001d003776 gene. Gray and green boxes represent introns and exons, respectively. (b) Allelism test using homozygous WT, *ven2*, *ven2-1*, and *ven2-2*. Scale bars, 3 cm.

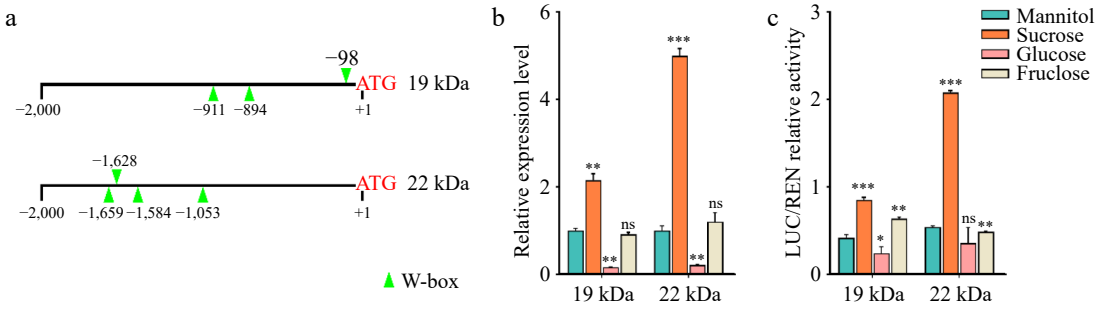

Fig. 3 Sucrose induces *19 kDa α-zein* and *22 kDa α-zein* gene expression. (a) Promoter analysis of *19 kDa α-zein* and *22 kDa α-zein* gene. W-box is a *cis*-acting element that responds to sugar signals. (b) Expression analysis of *19 kDa α-zein* and *22kDa α-zein* in different sugar treatment by qRT-PCR. Normalization was performed against the maize *Actin* gene (Zm00001d010159). Values represent the mean and SD of three biological replicates. **, $p < 0.01$; ***, $p < 0.001$; ns, no significant difference; student's *t*-test. (c) The LUC/REN relative activity of the promoter fragments including W-box of *19 kDa α-zein* and *22 kDa α-zein* gene. The expression level of REN was used as an internal control. Values represent the mean and SD of three biological replicates. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, no significant difference; student's *t*-test.

sucrose (Fig. 3c). Collectively, these findings suggest that sucrose acts as a signaling molecule regulating the expression of the *19 kDa* and *22 kDa α-zein* genes, consistent with the observed over accumulation of zein in the *ven2* mutant.

Overexpression of *Ven2/Incw2* gene decreased the contents of sucrose and zein

To further explore the role of sucrose in regulating zein, we examined seed storage proteins in the *Ven2/Incw2* overexpression transgenic line controlled by the *27 kDa γ-zein* promoter (*OE-Incw2*). Longitudinal analysis of dry seeds from both the control (CK) and *OE-Incw2* lines revealed a decreased ratio of hard to soft endosperm in the *OE-Incw2* transgenic seeds (Fig. 4a, b). Transverse sections of mature seeds displayed a significantly thinner layer of vitreous endosperm in *OE-Incw2* compared to CK (Fig. 4c, d). CBB-staining of protein gels indicated a marked reduction in the levels of 19 kDa α-zein and 22 kDa α-zein proteins in *OE-Incw2*, a finding confirmed by quantitative measurements of protein content (Fig. 4e, f). In line with these results, qRT-PCR analysis revealed that the transcript levels of both *19 kDa α-zein* and *22 kDa α-zein* were significantly down-regulated in the endosperm of *OE-Incw2* compared to CK (Fig. 4g).

Moreover, we analyzed the sucrose content of seeds at various developmental stages. The results showed a significant decrease in sucrose levels in *OE-Incw2* seeds during early development (Fig. 4h–j). This reduction in sucrose may explain the decreased zein content and lower transcript levels of *19 kDa α-zein* and *22 kDa α-zein* observed in *OE-Incw2* seeds.

SRF1 responds to sugar signals

To investigate the developmental defects in the *ven2* mutant and the regulatory relationship between sugar metabolism and storage proteins, we conducted RNA sequencing (RNA-seq) on endosperm samples from both WT and *ven2* at 13 DAP. We identified a total of 2,501 significant differentially expressed genes (DEGs) between WT and the *ven2* mutant, with 1,481 genes upregulated and 1,020 genes downregulated in *ven2* (Supplementary Fig. S8a). Gene ontology (GO) enrichment analysis showed that DEGs were primarily associated with metabolic, catalytic, and cellular processes (Supplementary Fig. S8b). Additionally, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis revealed that DEGs were mainly clustered in the biological processes of starch and sucrose metabolism and amino acid biosynthesis (Supplementary Fig. S8c). Notably, the *ven2* mutant endosperm exhibited upregulation of genes related to the synthesis and metabolism of storage proteins, while genes involved in starch biosynthesis were downregulated. This pattern aligns with the observed phenotype of reduced starch and increased protein content in the mutant (Supplementary Fig. S9). These findings suggest that the altered starch and protein contents in the *ven2* mutant may be partially due to the differential regulation of genes associated with starch and protein biosynthesis.

To investigate the mechanism by which sugar signals regulate zein expression, we analyzed the expression levels of transcription factors from the transcriptome data (Supplementary Fig. S9). We utilized the PLACE database (https://sogo.dna.affrc.go.jp/cgi-bin/sogo.cgi) to examine the promoters of these transcription factors and discovered that the promoter of the WRKY transcription factor SRF1 contains several *cis*-acting elements responsive to sugar signals (Supplementary Fig. S10a). To confirm that the expression of *SRF1* is regulated by sugar signaling, we treated wild-type endosperm with sucrose, using mannitol as a control. The results indicated that *SRF1* expression was significantly elevated in endosperm treated with sucrose (Supplementary Fig. S10b). Additionally, the *ven2* mutant seeds, which exhibited increased sucrose content compared to WT, also showed a significant increase in *SRF1* expression (Supplementary Fig. S10c). Conversely, the decrease of sucrose content in endosperm was accompanied by the decrease of *SRF1* gene expression in *OE-Incw2* endosperm (Supplementary Fig. S10d). These findings suggest that sugar signals positively regulate the expression of *SRF1* in maize.

SRF1 transcriptionally regulates *19 kDa α-zein* and *22 kDa α-zein* gene expression

To determine whether the transcription factor SRF1 directly activates the expression of the *α-zein* genes, we performed a dual-luciferase transcriptional activity assay. This assay utilized a firefly luciferase (LUC) reporter system, specifically designed with the promoters of the *19 kDa* and *22 kDa α-zein* genes. By co-expressing SRF1 along with the LUC reporter in tobacco leaves, we aimed to assess any resultant changes in luciferase activity. The experimental results revealed a significant increase in LUC activity in the presence of SRF1, indicating that this transcription factor indeed enhances the transcriptional activity of the *α-zein* promoters (Fig. 5a).

To further elucidate the binding interaction between SRF1 and the W-box element within the promoters, we employed eight biotin-labeled probes that were derived from the specific promoter regions of the *19 kDa* and *22 kDa α-zein* genes in EMSA. It clearly demonstrated that SRF1 can bind directly to the W-box in the promoters of both *α-zein* genes *in vitro* (Fig. 5b, c). This finding supports the hypothesis that SRF1 plays a critical role in regulating the transcription of *α-zein* genes through direct interaction with their promoter regions.

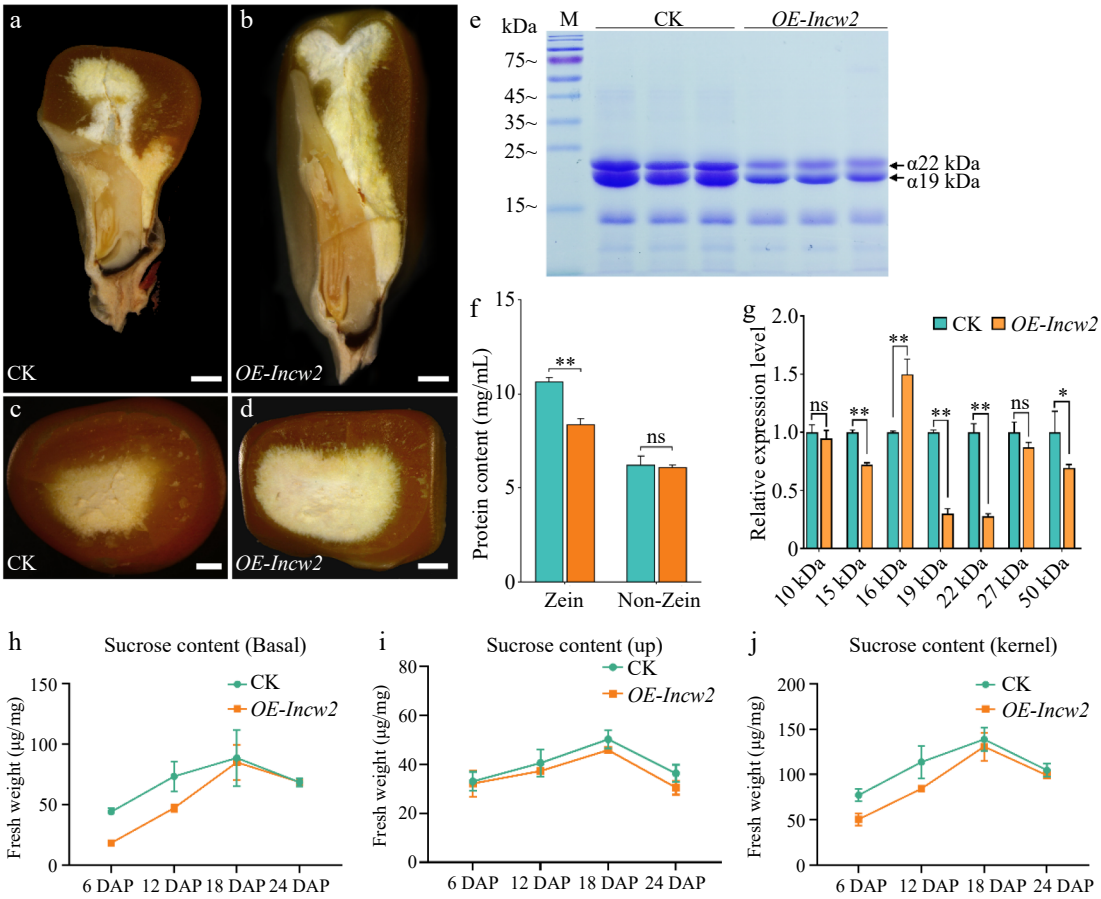

Fig. 4 Seed phenotypes of the CK and *OE-Incw2*. (a), (b) Longitudinal dissection of CK and *OE-Incw2* mature seeds. CK, non-transgenic control. Scale bars, 2 mm. (c), (d) Transection of CK and *OE-Incw2* mature seeds. Scale bars, 2 mm. (e) SDS-PAGE analysis of zein in CK and *OE-Incw2*. (f) Quantification of zein and non-zein contents in CK and *OE-Incw2* mature endosperm. Values represent the mean and SD of three biological replicates. **, $p < 0.01$; ns, no significant difference; student's *t*-test. (g) qRT-PCR analysis of the expression of zein genes. *, $p < 0.05$; **, $p < 0.01$; ns, no significant difference; student's *t*-test. (h)–(j) Sucrose contents of CK and *OE-Incw2* developing seeds. Values represent the mean and SD of three biological replicates.

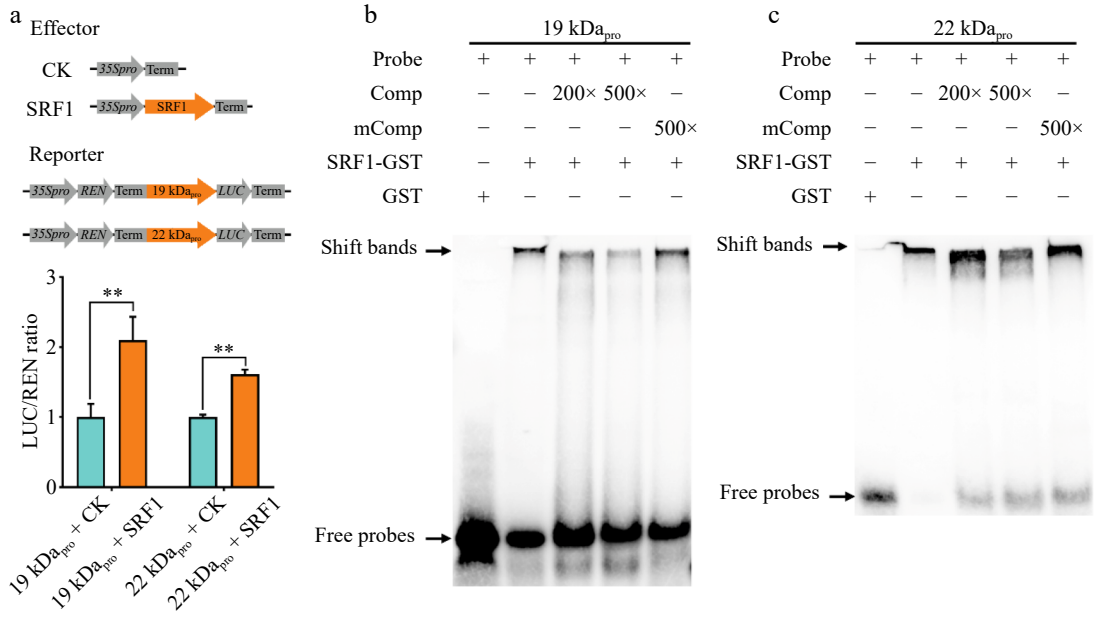

Fig. 5 Identification of *cis*-elements and the associated SRF1 for *19 kDa* and *22 kDa* promoter transactivation. (a) DLR assay of transactivation of the *19 kDa* and *22 kDa zein* genes promoter by SRF1 in tobacco leaves. (b), (c) EMSA of the specific binding of SRF1-GST to the W-box motifs in the *19 kDa* and *22 kDa zein* genes promoter. The normal and mutant probes were labeled with biotin. Unlabeled intact probes were used for competition. Comp, competing probes unlabeled with biotin.

Discussion

Impaired INCW2 function leads to abnormal sugar metabolism and storage protein synthesis

Impaired INCW2 function disrupts normal sugar metabolism and storage protein synthesis, leading to significant alterations in seed development. INCW2, which encodes a cell wall invertase, plays a crucial role in the breakdown of sucrose into its component hexoses, which are essential for energy metabolism and proper biosynthesis of starch and proteins. When INCW2 is nonfunctional, sucrose accumulation in the basal endosperm is elevated, while the conversion of sucrose to hexoses is impaired[39]. This disruption not only affects energy availability for storage but also leads to an imbalance in the metabolic pathways responsible for synthesizing storage proteins, such as zeins, which are critical for seed structure and nutrient storage. As a result, the altered sugar and protein metabolism negatively impacts seed size, quality, and overall development, highlighting the essential role of INCW2 in maintaining metabolic homeostasis during seed maturation.

Dysfunction of INCW2 leads to reduced invertase activity, resulting in impaired metabolic flux of sugar utilization in the developing endosperm[39,41]. The altered sugar profile in *ven2* is attributed not only to the loss of catalytic activity of the cell wall invertase but also to the regulation of sugar transport-related genes. During the filling stage, sucrose can be directly transferred to the endosperm through the BETL via specific sucrose transporters, such as SUGCAR1, ZmSUT1, and ZmSWEET11/13a[42–44]. In the *ven2* endosperm, several DEGs associated with starch biosynthesis, including *Brittle2* (*Bt2*), *Amylose extender1* (*Ae1*), and *Shrunken2* (*Sh2*)[45], were significantly downregulated (Supplementary Fig. S9). This downregulation inhibits the conversion of sugar to starch, resulting in elevated sugar levels in the endosperm of *ven2*. Additionally, the increase in sugar content in *ven2* may also be partially due to the upregulation of sugar transport-related genes, such as *Sucrose transporter1* (*SUT1*) (Supplementary Fig. S9). In summary, INCW2 plays a crucial role in regulating seed weight and starch content through its influence on sugar metabolism.

Previous research indicated that mutations in *INCW2*, localized in the basal endosperm transfer cell layer, resulted in a loss of over 70% of seed weight at maturity due to reductions in both cell number and size during endosperm development[31,46–48]. Further investigations revealed that the decreased cell number and size in the *mn1* mutant were attributed to deficiencies in various phytohormones[47,49]. In our study, we observed that the zein protein content in the *ven2* and *ven2-1* mutants was significantly increased, particularly for the 19 kDa and 22 kDa zein proteins, compared to the WT (Fig. 1c–e; Supplementary Fig. S5). In contrast, zein protein content decreased when *INCW2* was specifically overexpressed in maize BETLs (Fig. 4e, f). Unlike previous studies, our findings demonstrate that INCW2 influences not only seed weight but also storage protein levels. Collectively, these results indicate that INCW2 has a pleiotropic effect on seed development.

Transcriptional regulation of zein genes by SRF1

Transcriptional regulation of zein genes by SRF1 involves a complex mechanism by which this transcription factor modulates the expression of genes responsible for synthesizing storage proteins in maize seeds. SRF1 is a key regulator that responds to changes in sugar levels, particularly sucrose, which acts as both an energy source and a signaling molecule during seed development. When sucrose levels increase, SRF1 is activated and binds directly to the promoter regions of the *19 kDa and 22 kDa α-zein* genes, which encode major storage proteins in the endosperm. Through this binding, SRF1 enhances the transcription of these genes, facilitating the production of α-zeins, which are critical for seed protein storage. This regulatory pathway ensures that adequate storage proteins are synthesized to support seed growth and development. In addition to its role in regulating zein gene expression, SRF1 also integrates signals from other metabolic pathways, providing a coordinated response to nutrient availability and developmental cues, which is essential for optimizing seed quality and nutrient content.

Several transcription factors, including O2, PBF1, MADS47, and others have been reported to directly activate *19 kDa* and *22 kDa α-zein* proteins genes and have been shown to play crucial roles in regulating the biosynthesis of maize endosperm storage proteins[9,12,15,16]. However, the external signals that influence the storage protein biosynthesis remain largely unknown. In this study, we identified a new allelic mutant of *INCW2*, characterized by increased levels of the 19 kDa and 22 kDa α-zein proteins, along with elevated sugar content. During seed development, approximately 90% of carbon is derived from sucrose, whose levels exhibit diurnal variations that lead to the diurnal transcription of O2-regulated zein genes[25]. Collectively, these findings suggest that sucrose serves not only as a primary carbon source but also as a critical signal in regulating starch and storage protein synthesis in

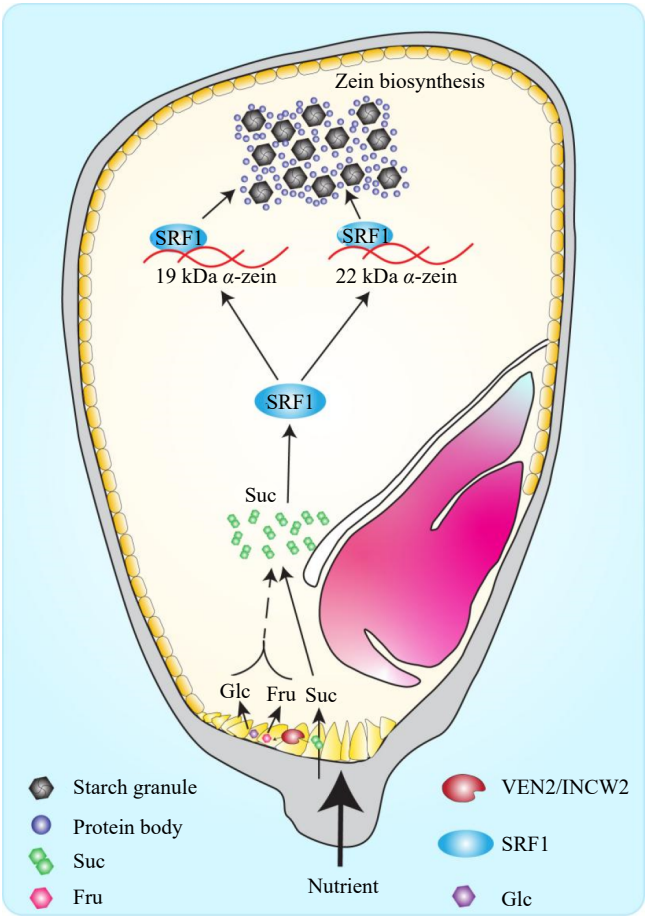

Fig. 6 A proposed model of sucrose content affects zein biosynthesis mediating by transcription factor SRF1. In the cell wall ingrowths, sucrose is hydrolyzed into hexoses (glucose and fructose) by VEN2/INCW2 and the apoplasmic sucrose and hexoses are translocated into endosperm cell. The transcription factor SRF1 responds to sucrose and regulates the expression of *19 kDa* and *22 kDa α-zein* by binding directly to their promoters, thereby mediating zein biosynthesis. Suc, sucrose; Fru, fructose; Glc, glucose.

plants. In *ven2* mutant, transcription factors, such as, O2, PBF1, and MADS47 show no significant changes in transcriptome data. This suggests that, in addition to the known transcription factors such as O2, PBF1, and MADS47, there may be other potential transcription factors that are regulated by the sucrose signal and are involved in the regulation of zein protein synthesis.

Over the past few decades, various transcription factors, including MYB domain proteins, basic leucine zippers (bZIPs), and WRKY proteins, have been identified as key regulators of sugar responses[40,50–52]. In the *ven2* mutant, elevated sucrose levels lead to increased expression of *SRF1*, which subsequently upregulates the transcription of genes involved in zein biosynthesis. Sequence analysis indicates that SUSIBA2 in barley and SRF1 in maize are closely related members of the WRKY superfamily of plant transcription factors. SUSIBA2 plays a role in sugar signaling in barley by binding to sugar-responsive (SURE) and W-box elements within the *iso1* promoter[52,53]. Our promoter sequence analysis revealed multiple W-box *cis*-elements in the α-*zein* gene, which are bound by the transcription factor SRF1 (Fig. 3a). Additionally, the results from dual-luciferase transcriptional activity assays and electrophoretic mobility shift assays confirmed that SRF1 is essential for activating the expression of the *19 kDa* and *22 kDa* α-*zein* genes (Fig. 5). In conclusion, we proposed a model for sucrose content affects zein biosynthesis mediating by transcription factor SRF1 (Fig. 6). These findings establish SRF1 as a potential candidate regulatory transcription factor in the synthesis of storage proteins, underscore the role of a WRKY protein in the sucrose response. Therefore, further exploration of SRF1 and its regulatory mechanisms will help to deepen our understanding of the regulatory network of maize zein protein synthesis and pave the way for its future application in maize improvement.

Conclusions

In this work, we characterize a WRKY transcription factor SRF1, which responds to sucrose signal and regulates *19 kDa* and *22 kDa* α-*zein* gene expression, thereby affecting zein protein synthesis during seed development.

Author contributions

The authors confirm contribution to the paper as follows: study conception and design: Zhao X; data collection: Wei Y, Zang J, Shao D, Wang B, Zheng G, Yan R, Wu J; analysis and interpretation of results: Wei Y, Zang J, Zhao Y, Zhang C, Zhou C; draft manuscript preparation: Wei Y, Zhao X, Zhang X. All authors reviewed the results and approved the final version of the manuscript.

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information files. Further information is available from the corresponding author on reasonable request.

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Conflict of interest

The authors declare that they have no conflict of interest.

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