GmUFO1 Regulates Floral Organ Number and Shape in Soybean

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Posted Date: 11 April 2023

doi: 10.20944/preprints202304.0187.v1

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Article

GmUFO1 Regulates Floral Organ Number and Shape in Soybean

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Abstract: The *UNUSUAL FLORAL ORGANS* (*UFO*) gene is an essential regulatory factor of class B genes and plays a vital role in the process of inflorescence primordial and flower primordial development. We investigated the role of *UFO* genes in soybean to better understand the development of floral organs through gene cloning, expression analysis, and gene knockout. In situ hybridization demonstrated predominant expression of the *GmUFO1* gene in the flower primordium. Phenotypic observation of *GmUFO1* knockout mutant lines (*Gmufo1*) showed obvious alteration in floral organ number and shape, and mosaic organ formation. In contrast, *GmUFO2* knockout mutant lines (*Gmufo2*) showed no obvious difference in floral organs. However, the *GmUFO1* and *GmUFO2* double knockout lines (*Gmufo1ufo2*) showed a more severe mutant phenotype than the *Gmufo1* lines. Gene expression analysis also showed differences in the expression of major ABC function genes in the knockout lines. Based on the phenotypic and expression analysis, our results suggest the major role of *GmUFO1* in the regulation of flower organ formation in soybean and that *GmUFO2* does not have any direct effect, but it might have an interaction role with *GmUFO1* in the regulation of flower development. In conclusion, the present study identified *UFO* genes in soybean and improved our understanding of floral development that could be useful for flower design in hybrid soybean breeding.

Keywords: *GmUFOs*; knockout; floral organ number; floral organ shape; soybean

1. Introduction

Cell division control is necessary for the proper arrangement of flower primordia and is regulated by the plant hormones auxin and cytokinin. The vast majority of angiosperm flowers have four-wheeled floral organs, which consist of sepals, petals, stamens, and the carpel, in that order, moving from the outside to the interior of the flower. The well-known "ABC model" is the functional model of floral organ attribute genes in *Arabidopsis thaliana* [1, 2]. This model correctly describes the molecular process of flower organ primordium attributes, and it has been the subject of a great deal of research. The A functional genes *AP1* (*APETALA1*) and *AP2*, B functional genes *AP3* and *PI* (*PISTILLATA*), C functional gene *AG* (*AGAMOUS*), and E functional gene *SEP* (*SEPALLATA*) are the most typical genes studied in model plants [3]. This well-known "ABC model" is a scientific model of the process through which a pattern of gene expression is produced by flowering plants in their meristems, which in turn leads to the appearance of an organ oriented toward sexual reproduction [4]. This model correctly describes the molecular process of flower organ primordium attributes, and it has been the subject of a great deal of research.

 doi:10.20944/preprints202304.0187.v1

The *UFO* gene is an F-box gene that encodes E3 ubiquitin ligase and was first discovered in *Arabidopsis thaliana*, where mutant flowers lack typical petals and stamens [1]. In *Arabidopsis thaliana*, the *UFO* gene co-activates the expression of the downstream *AP3* gene with the *AP1* gene as a co-activator of the *LFY* gene, thus preserving petal characteristics and petal development. *LFY* controls the temporal specificity of *AP3* expression, while *UFO* and *AP1* control the spatial pattern [5, 6]. UFO has been reported to interact with the SCF E3 ubiquitin ligase subunits, ASK1 and CUL1, as well as subunits of the COP9 signalosome, which suggest the possible role of UFO in the ubiquitination of proteins that are involved in flower development [7, 8]. The interaction of the UFO and ASK1 proteins may enhance the degradation of the downstream B functional gene *AP3* and the *PI* inhibitor by the *UFO* gene, resulting in the activation and production of the B functional gene [5, 9, 10]. Strong loss-of-function mutations in the *UFO* gene cause flower abnormalities in all whorls. In sepals and petals, homeotic changes, organ number, and phyllotaxis are the most severe [8, 11]. Sepals replace petals, and carpels replace stamens; however, mosaic organs are also prevalent in both whorls of the *ufo* mutant. *UFO*, similar to its *Antirrhinum* homolog *FIM*, is expressed in a complicated temporal and geographic pattern throughout floral development [12, 13]. In floral meristems, transcription is inhibited in stage 1 and triggered in stage 2 in the central area. By stage 3, the core meristem loses expression but extends laterally in cone form. By stage 5, expression is confined to the petal primordia and remains there throughout floral organ development. *AP3* and *PI* patterns are formed during stage 3 [14, 15] before petal/stamen organ activation during stage 5, supporting UFO's significance in B class function [16].

Soybean (Glycine max) is a globally important crop that is a rich source of edible protein and oil [17]. The reproductive period of soybean (starting from flower initiation till maturity) is essential for higher yield and quality [18]. Identifying and understanding the molecular mechanism of the genes involved in the regulation of the reproductive period and flower development in soybean are key targets for yield and quality improvement in soybean. In the present study, we reported that *GmUFOs* play a crucial role in regulating flower development, especially the quantity and characteristics of petals in connection with the development of the stamen. The *GmUFOs* mutation caused floral organ morphological defects and altered the expression of ABC flower development genes.

2. Results

2.1. Identification of UFO Genes

In the present study, we used the *AtUFO* gene (*AT1G30950.1*) of *Arabidopsis thaliana* as a query to search its orthologs in soybean. We identified two orthologues, *Glyma.05G134000* and *Glyma.08G088700*, and named them *GmUFO1* and *GmUFO2*, respectively. For phylogenetic analysis, we used *GmUFO1* and *GmUFO2*, as well as another nine *UFO* genes from eight species, to construct the phylogenetic tree. The phylogenetic alignment showed that *GmUFO1* and *GmUFO2* were more closely related to the *UFOs* of leguminous plants (Figure 1A). Amino acid sequence alignment and protein 3D model analysis showed that the UFO protein domain was relatively conserved in these species (except cotton), and both GmUFO proteins in soybean had a conserved F-box (Figure 1B). Protein physical property analysis showed that the amino acid length of UFOs in these species was between 357 and 444, and the polypeptide isoelectric points were almost constant (Table S2).

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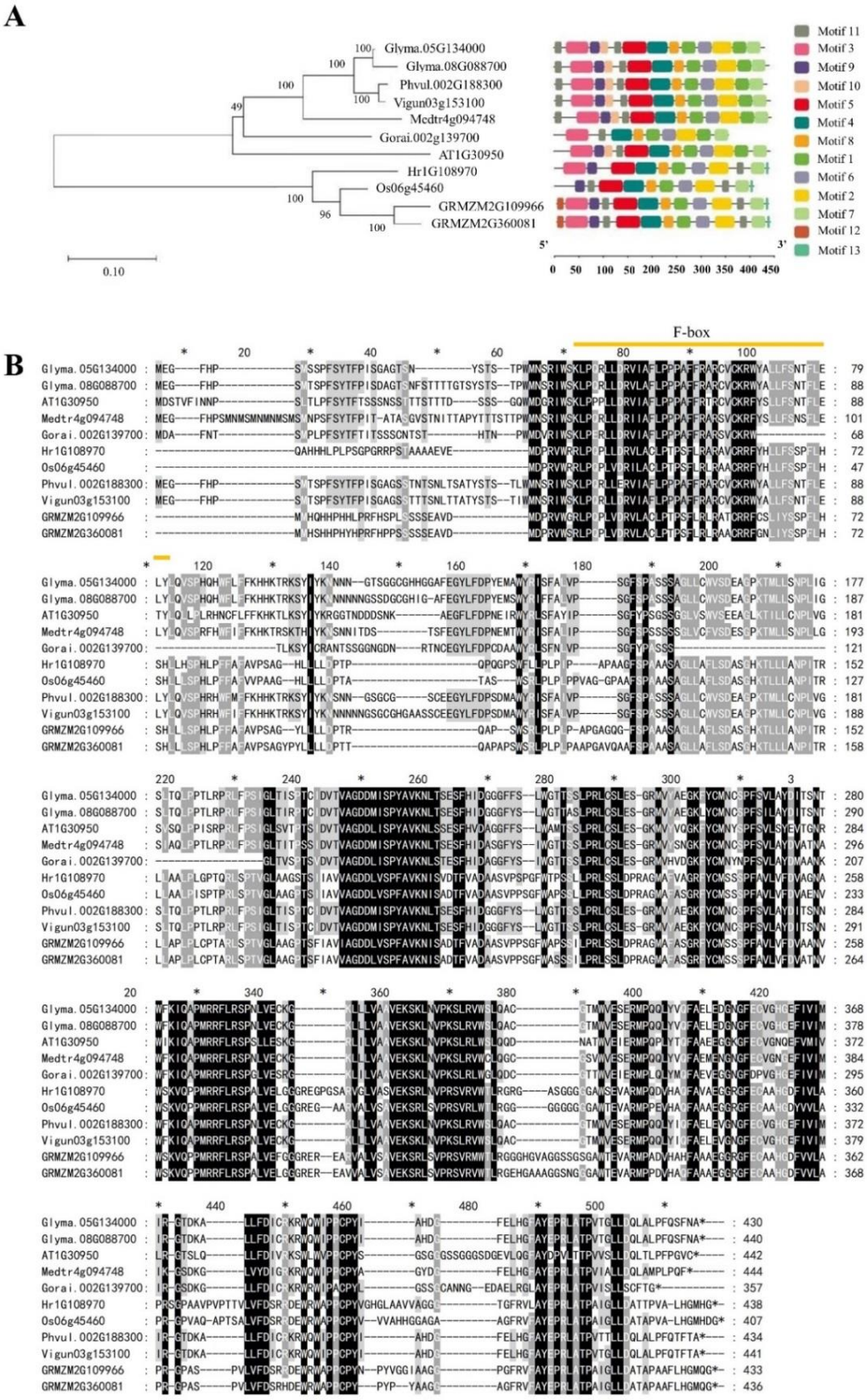

Figure 1. (**a**) Phylogenetic analysis of the selected 11 UFO proteins from nine species, namely Arabidopsis thaliana (AT1G30950), Glycine max (Glyma.05G134000, Glyma.08G088700), Medicago truncatula (Medtr4g094748), Vigna unguiculate (Vigun03g153100), Phaseolus vulgaris (Phvul.002G188300), Gossypium raimondii (Gorai.002G139700), Oryza sativa (Os06g45460), Zea mays (GRMZM2G109966, GRMZM2G360081), and Hordeum vulgare (Hr1G108970). The neighbor-

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joining tree was constructed using MEGA11, and the bootstrap value was set at 1000 replicates. (**b**) Amino acid sequence alignment of UFOs in soybean and other species. The orange line represents the F-box domain.

2.2. Spatial and Temporal Expression Profiles of the GmUFO1 Gene in Soybean Flower Development

RNA in situ hybridization was used to detect *GmUFO1* expression in the soybean flower primordium at different development stages (Figure 2A, B). In stage 1, the flower meristem formation stage, *GmUFO1* was expressed in the central area of the whole flower meristem. In stage 2, which represents the sepal primordium formation stage, *GmUFO1* was expressed between the sepal primordium and the carpel primordium, and the signal was also detected near the central dome of the sepal primordium. In stage 3, the petal primordium formation stage, *UFO1* was mainly expressed in the petal primordium, but a few signals were detected in the sepal primordium. Stage 4 represents the stamen and carpal primordium formation stage, and in this stage, *UFO1* was expressed in the petal primordia and almost absent in elongating sepal primordia. In stage 5, when the formation of all flower organs occurs, *UFO1* was expressed at the boundary region of the petal between sepal and stamen.

We also studied the subcellular localization of GmUFO1, and the GFP fluorescence of GFP-GmUFO1 fusion protein was located in the nucleus of Arabidopsis protoplasts, which was co-localized with AtAHL22, which was confirmed as a nuclear marker gene [19] (Figure 2C).

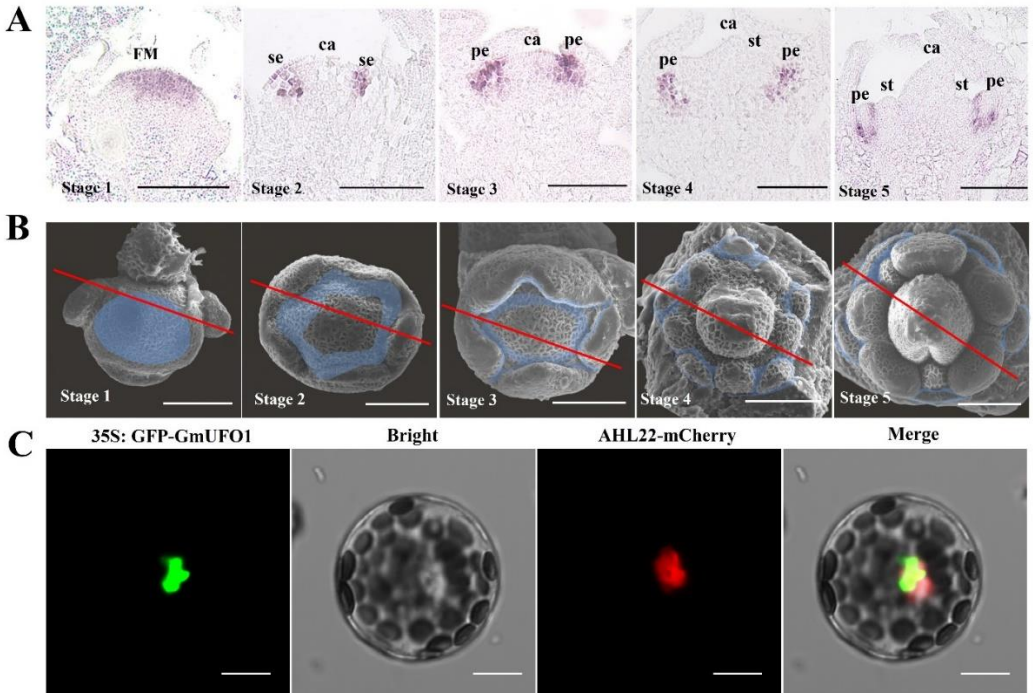

Figure 2. (**a**) In situ hybridization of the *UFO1* gene in the soybean flower primordium. The periods from left to right are stage 1: flower primordium initiation, stage 2: sepal primordium formation stage, stage 3: petal primordium formation stage, stage 4: stamen and carpal primordium formation, and stage 5: formation of all flower organs. Bar = 100 μm. (**b**) Scanning electron microscopy (SEM) observations of in situ hybridization and stereoscopic models of *GmUFO1* gene expression at five developmental stages. Bar = 100 μm. (**c**) Expression location of GmUFO1-GFP in *Arabidopsis thaliana*. Bar = 10 μm.

2.3. CRISPR/Cas9 System Generated Target Mutations for GmUFO Genes

To obtain the *GmUFO* mutants, CRISPR/Cas9-mediated genome editing of the *GmUFO* genes was performed, and the target site for both *GmUFO1* and *GmUFO2* was located ahead of the F-box domain (Figure 3A, B). We obtained four T0 generation transgenic lines and 40 T1 generation

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transgenic lines. In the T1 generation, the *GmUFOs* were sequenced, and based on the *GmUFO* target site sequencing data, we identified 10 *Gmufo1* single mutants, six *Gmufo2* single mutants, and three *Gmufo1ufo2* double mutants (Figure 3C). These mutants were used for further investigation in our study.

Figure 3. Summary of CRISPR/Cas9-mediated genome editing of *GmUFO* genes. (**a**) Structural diagram of the CRISPR/Cas9 vector. (**b**) Target sites designed for *GmUFO1* and *GmUFO2* in CRISPR/Cas9. (**c**) Summary of mutation frequency in the T1 generation. (**d-f**) Seedling phenotype of *Gmufo1* single mutant lines (**d**), *Gmufo2* single mutant lines (**e**), and *Gmufo1ufo2* double mutant lines (**f**) compared with the wild type. Scale bar = 10 cm.

2.4. Knockout of the GmUFO1 Gene Caused Abnormal Flower Organs

All 10 mutant plants obtained in *Gmufo1* of the T1 knockout lines showed heterozygosity at the *GmUFO1* target site, whereas no mutation was found at the *GmUFO2* gene, and three of the mutants were selected for further analysis (Figure 4). The *Gmufo1* seedlings showed no obvious differences compared with the wild type (Figure 3D), whereas the floral organ was different from the wild type (Figure 4A). Compared with the wild type, *Gmufo1* showed floral organ number and shape alterations

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in sepals, petals, and stamens, and also mosaic flower organ formation with green sepal-like petals (Figure 4A, B).

Furthermore, five ABC function genes, namely *GmAP1a*, *GmAP2*, *GmAP3*, *GmPI04G*, and *GmAG*, were selected, and their expression was detected using qPCR in the three *Gmufo1* lines and the wild type. The expression levels of *GmAP1a*, *GmAP3*, *GmPI04G*, and *GmAG* decreased in all three lines, while the expression levels of *GmAP2* increased (Figure 4C). We also examined the expression of *GmUFO1* and *GmUFO2* gene transcripts in *Gmufo1* and found that they did not differ from the wild type (Figure 4D).

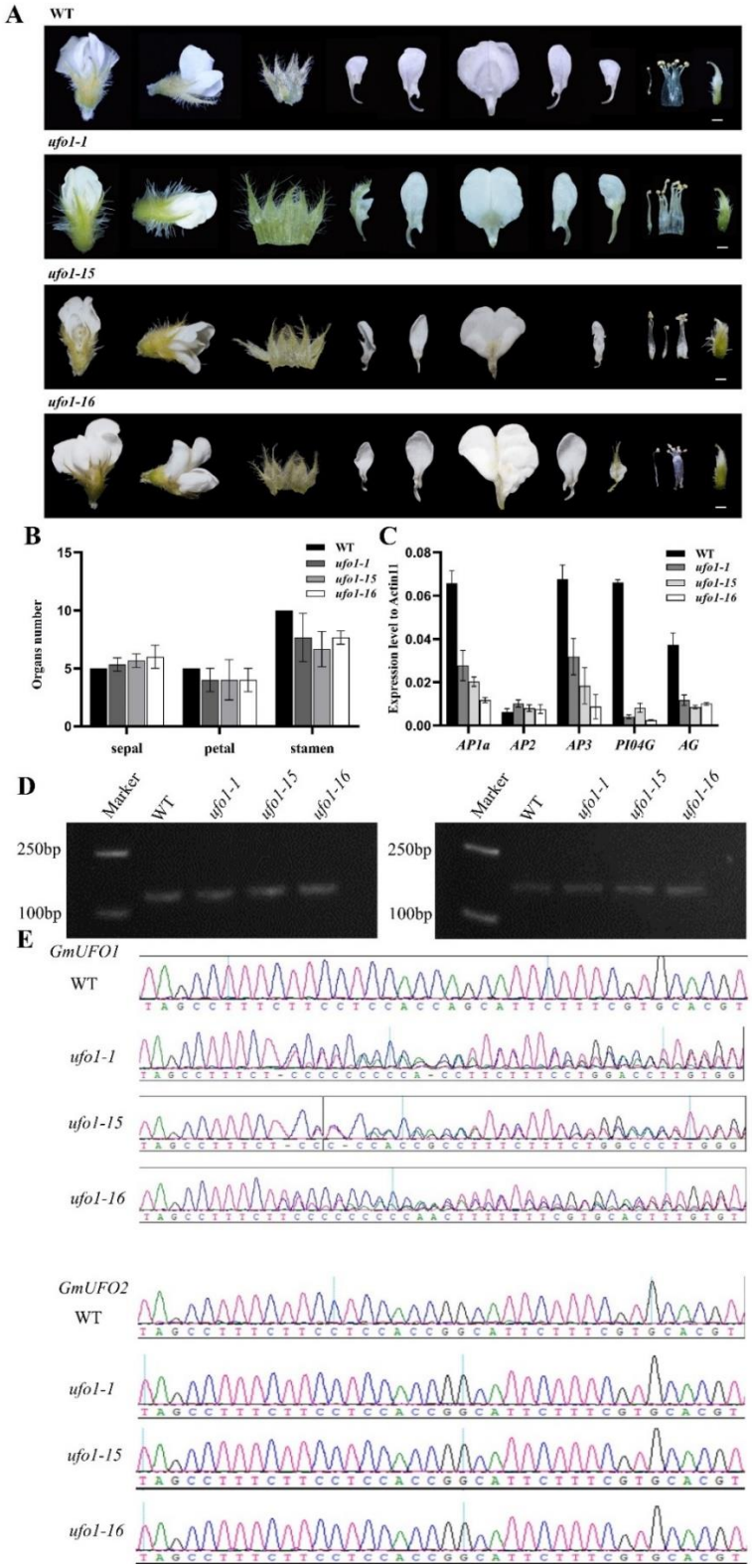

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Figure 4. (**a**) Floral organ phenotype of the three *Gmufo1* mutant lines compared with the wild type. Scale bar = 1 mm. (**b**) Statistics of the number of sepals, stamens, and petals in *Gmufo1* mutant lines. (**c**) ABC function gene expression was analyzed in the flowers of 'Williams 82', *Gmufo1-1*, *Gmufo1-15*, and *Gmufo1-16*. All data presented are mean ± SD for three biological replicates. (**d**) *GmUFO1* and *GmUFO2* expression level in three mutant lines and the wild type. (**e**) CRISPR/Cas9 target site sequencing of *GmUFO1* and *GmUFO2* in the three selected lines.

2.5. Knockout of the GmUFO2 Gene Had no Effect on Flower Organs

Six positive plants obtained in *Gmufo2* of the T1 knockout lines showed heterozygosity at the *GmUFO2* target site, whereas no mutants possessed at the *GmUFO1* gene, and three of them were selected for further analysis (Figure 5). *Gmufo2* seedlings had no obvious differences compared with the wild type (Figure 3E) and did not show differences in floral organ shape or number (Figure 5A). Further statistics also showed that the number of *Gmufo2* organs to the wild type had not changed (Figure 5A, B). The five ABC functional genes (i.e., *GmAP1a*, *GmAP2*, *GmAP3*, *GmPI04G*, and *GmAG*) in the three *Gmufo2* lines were detected using qPCR. The *GmPI04G* expression decreased, while *GmAP2* expression increased in all three *Gmufo2* lines (Fig. 5C). We examined the expression of *GmUFO1* and *GmUFO2* gene transcripts in *Gmufo2* and found that they did not differ from the wild type (Figure 5D).

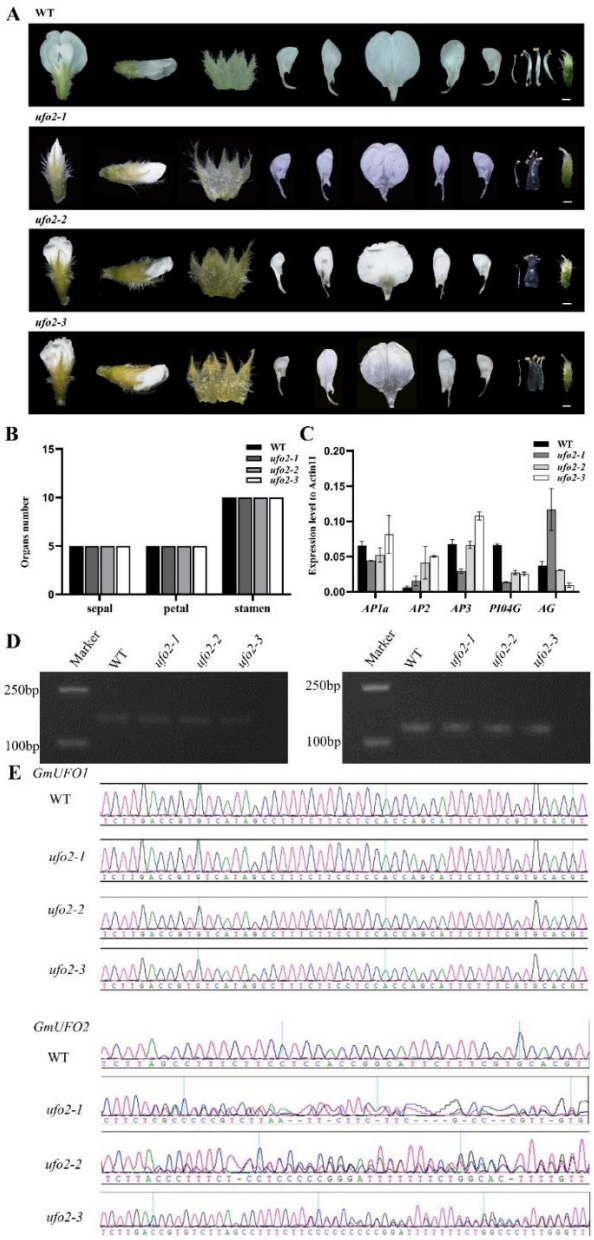

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Figure 5. (**a**) Floral organ phenotype of the three *Gmufo2* mutant lines compared with the wild type. Scale bar =1 mm. (**b**) Statistics of the number of sepals, stamens, and petals in *Gmufo2* mutant lines. (**c**) ABC model gene expression was analyzed in the flowers of 'Williams 82', *Gmufo2-1*, *Gmufo2-2*, and *Gmufo2-3*. All data presented are mean ± SD for three biological replicates. (**d**) *GmUFO1* and *GmUFO2* expression level in three mutant lines and the wild type. (**e**) CRISPR/Cas9 target site sequencing of *GmUFO1* and *GmUFO2* in the three selected lines.

2.6. Double Knockout of GmUFO1 and GmUFO2 Had More Serious Defects in Organ Number and Shape

Among the T1 knockout lines, three lines were double mutated and were selected for further analysis. In the case of the *GmUFO1* gene, line *Gmufo1ufo2-4* had a 4-bp deletion at the target site, *Gmufo1ufo2-17* had a 1-bp deletion at the target site, and *Gmufo1ufo2-18* showed a 1-bp deletion after the target site. However, in the case of the *GmUFO2* gene, all three selected mutant plants were heterozygotes at the target site (Figure 6). The vegetative appearance showed no obvious difference in the seedlings of double mutants compared with the wild type (Figure 3F), whereas the floral organ showed even more serious defects than *Gmufo1* single mutants compared with the wild type (Figure 4A and Figure 6A). We observed more mosaic organs formed with sepal-like petals and petal-like stamens, and the alteration of organ number and shape in the double mutant relative to the *Gmufo1* single mutant (Figure 6A).

The three selected double mutant lines showed obvious variations in the number of sepals, petals, and stamens (Figure 6A, B). The expression of the five ABC functional genes (*GmAP1a*, *GmAP2*, *GmAP3*, *GmPI04G*, and *GmAG*) in the three lines was detected using qPCR. The expression levels of all of the ABC genes, *GmAP1a*, *GmAP2*, *GmAP3*, *GmPI04G*, and *GmAG*, decreased in all three *Gmufo1ufo2* plants, and the decrease was greater than that in *Gmufo1* (Figure 6C). We also detected the expression of *GmUFO1* and *GmUFO2* gene transcripts in *Gmufo1ufo2* and found that they did not differ from the wild type (Figure 6D).

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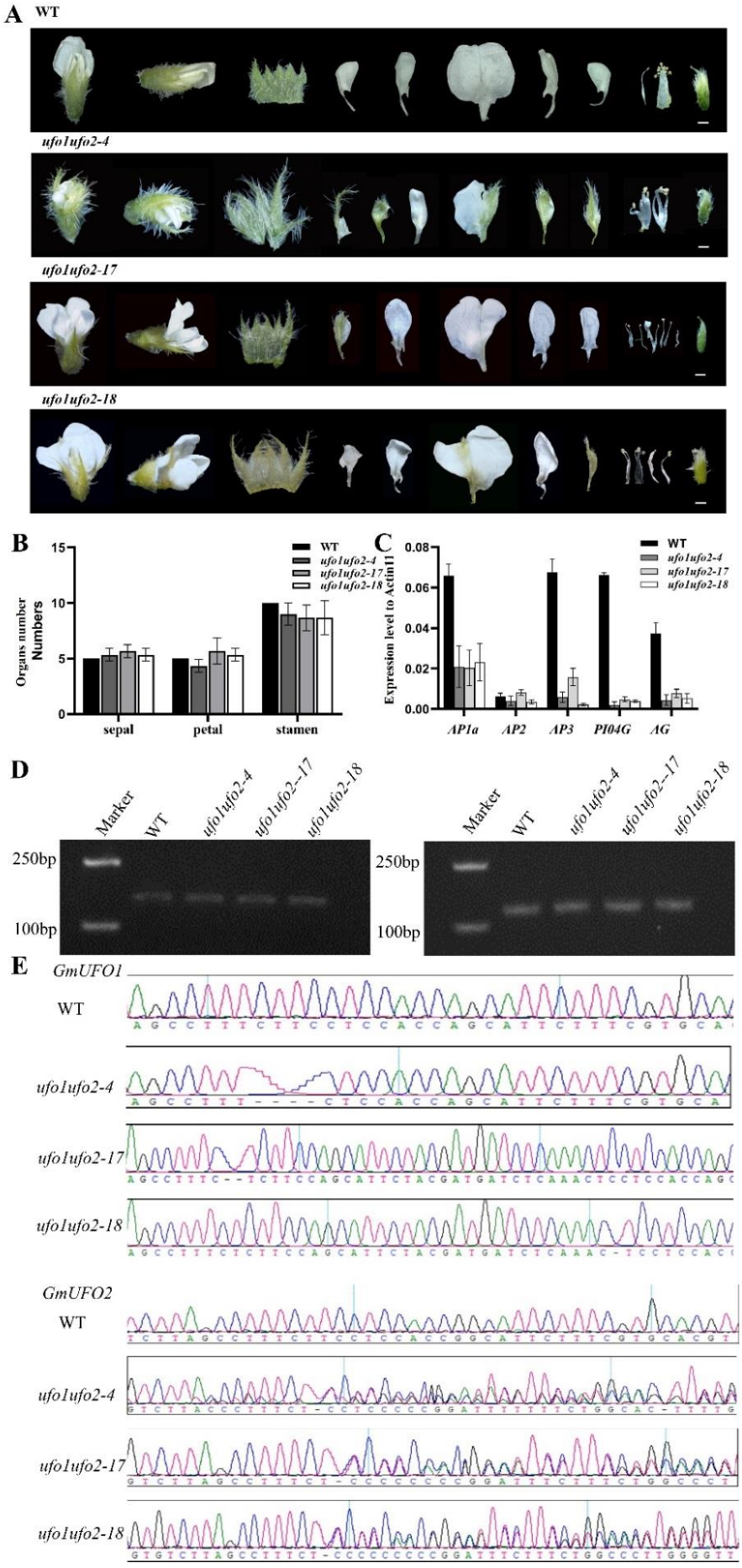

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Figure 6. (**a**) Floral organ phenotype of the three *Gmufo1ufo2* mutant lines compared with the wild type. Scale bar = 1 mm. (**b**) Statistics of the number of sepals, stamens, and petals in the *Gmufo1ufo2* mutant lines. (**c**) ABC model gene expression was analyzed in the flowers of 'Williams 82', *Gmufo1ufo2-4*, *Gmufo1ufo2-17*, and *Gmufo1ufo2-18*. All data presented are the mean ± SD for three biological replicates. (**d**) *GmUFO1* and *GmUFO2* expression level in three mutant lines and the wild type. (**e**) CRISPR/Cas9 target site sequencing of *GmUFO1* and *GmUFO2* in the three selected lines.

3. Discussion

In previous studies, the *ufo* mutant of different plant species has been observed to show the abnormal development of flower organs. However, there was a difference among the *ufo* mutants of various plants in regard to abnormal flower organ development, which might be due to some differences in the underlying molecular mechanism originating from plant evolution. For example, the single mutant of *UFO* in *Arabidopsis thaliana* (*Atufo*) showed a slight reduction in inflorescences at co-florescence, but the meristem of all primary and co-flowering buds in *Atufo* stopped growing after the appearance of structures fused with sepal-carpus organs. The attribute changes in *Atufo* flower organs were mainly concentrated in the second and third round organs, and the quantitative changes were reflected in all round organs. There are always deletions in the second round of early primordium development [20]. In the model legume *Lotus japonicus*, an orthologue of the *Arabidopsis UFO* gene mutation resulted in the *pfo* mutant [21]. In the *pfo* mutant, the flower primordium was unable to form normal flower organs, resulting in some sepal-like structures with filamentous structures, and petals failed to develop [21]. In our study, the changes in flower organs in soybean *ufo* mutants were mainly concentrated in the second whorl organ, and the increase in sepals and the decrease in stamens were also found in the first and third whorl organs. Even the most severe mutant phenotype of *Gmufo* did not show complete replacement of the second whorl organ with sepals, and sepal-like petals mostly appeared on the ventral part. Previous studies have documented that by controlling the interaction between UFO and LFY, the *UFO* gene can regulate the downstream targets of *AP3* and *PI*, thereby affecting the flower phenotype [8]. In this context, the interaction among the UFO, ABC, LFY, WUS, and CAL genes has been explored previously in different studies at the petal formation stage. For example, LFY interacts with UFO, CAL, and AP1, and UFO is expressed downstream of AP1 [22-24]. The interaction between LFY and UFO, as well as between LFY and AP1, is mutual and activates each other [23, 24]. LFY directly activates *AG*, and *AG* is also activated by WUS, but AG acts as a WUS antagonist [25-27]. In addition, AG directly activates *AP3* and *PI*. Moreover, AP2 is an AG antagonist, and AP2 activates *AP3* and *PI* directly [28]. UFO also directly activates *AP3* and *PI* [29]. Based on the above interactions of UFO with ABC and other genes, it is evident that the loss-of-function mutation of the *UFO* gene affects the expression of ABC genes, both directly and indirectly. For example, it indirectly reduces the expression of downstream *AP3* and *PI* by affecting *LFY* expression, which in turn affects *AG* and *AP1* expression [23, 30] and directly decreases the expression of downstream *AP3* and *PI* [29]. Our qPCR analysis is consistent with previous findings. We also demonstrated that knockout of *GmUFO* in *Gmufo1* and *Gmufo1ufo2* soybean mutants reduced the expression of ABC genes, *GmAP1a*, *GmAP2*, *GmAP3*, *GmPI04G*, and *GmAG* (Figure 4C, Figure 5C, and Figure 6C). Hence, *GmUFO* can be regarded as a key regulator of ABC function genes in soybean. The ABC model of flower organ development works as follows: A alone controls sepal development, A and B together regulate petal development, B and C together control stamen identity, and C regulates carpel development alone. In our study, we observed that the *Gmufo1ufo2* and *Gmufo1* mutants showed abnormal development of the sepals, petals, and stamens (increased sepals, decreased stamens, and petal looks like sepals), but carpel development was normal. Therefore, in agreement with the ABC model, our results also showed that knockout of *GmUFO* genes reduced the expression of ABC genes, viz., A (*GmAP1a*, *GmAP2*), B (*GmAP3*, *GmPI04G*), and C (*GmAG*) in the *ufo1ufo2* and *ufo1* mutants, which in turn led to the abnormal development of sepals, petals, and stamens. Although *GmAG* expression was reduced in the *ufo1ufo2* and *ufo1* mutants, carpel development was still normal in the mutant plants. This suggests that there might be some other interaction factors working together with *GmAG* in the regulation of carpel

development in soybean, which needs to be further explored. The *ufo2* mutant did not show any difference in flower development compared with the wild type. We also observed only the expression levels of *GmAP2* and *GmPI04G*, which decreased in the *ufo2* mutant, and the expression of the remaining ABC genes did not differ relative to the wild type. In addition, our study documented that the effect of the *ufo1ufo2* mutant on flower development was more severe than that of *ufo1*. These findings suggest the major role of *GmUFO1* in the regulation of flower organ formation in soybean. *GmUFO2* did not have any direct effect on flower development, but it might have an interaction role with *GmUFO1* in the regulation of flower development.

In conclusion, our work demonstrated that *GmUFO* genes regulated the development of flower organs, viz., soybean petals, stamens, and sepals, in soybean by controlling the expression of ABC genes. These findings will be the basis for further investigation of the in-depth mechanism involved in the *GmUFO*-mediated regulation of flower development in soybean. This, in turn, will have a significant effect on soybean hybrid breeding, leading to the development of high-yielding cultivars.

4. Materials and Methods

4.1. GmUFO Identification and Cloning

The *Arabidopsis thaliana UFO* gene (*AT1G30950.1*) sequence was downloaded from The Arabidopsis Information Resource (TAIR) database (https://www.arabidopsis.org/), and this gene was used as a query to identify putative orthologs of *UFO* genes in the Glycine max using BLASTp. The *GmUFO1* gene fragment was amplified from soybean cultivar 'Williams 82', and the primers were designed using Primer5 software (https://primer.software.informer.com/5.2/) (Table S1). PCR amplifications were performed using 2 × Phanta Max Master Mix (Dye Plus) DNA (Vazyme Biotech Co., Nanjing, Jiangsu, China) polymerase in a 50 μL mix containing 2 μmol L^{-1} of each primer, 25 units of 2 × Phanta Max Master Mix (Dye Plus), and 1 μL of cDNA. The PCR experiments were performed as follows: 95°C for 30 s, 32 cycles of 95°C for 15 s, 56°C for 15 s, 72°C for 2 min, and 72°C for 5 min. The PCR products were cloned using the TA cloning vector pGEM-Teasy (Promega Biotech Co., Beijing, China) and sequenced. After verifying the sequences, these products were used to construct the in situ hybridization and subcellular localization vector.

4.2. Phylogenetic Analysis and Physical Properties of UFO Genes

Phylogenetic analysis was performed using MEGA11 software [31] following the neighbor-joining method to analyze the evolutionary relationships among the 11 *UFO* genes from nine plants, including both monocots and dicots, namely *Arabidopsis*, soybean, *Medicago*, cowpea, kidney bean, cotton, rice, maize, and barley.

ProtParam (https://web.expasy.org/protparam/database) was used to determine the physical properties of the UFO proteins, such as the molecular weight, the length of each protein, and the protein isoelectric point (PI). Protein structure prediction was performed using an online website (https://robetta.bakerlab.org/domains/robetta/).

4.3. In Situ Hybridization

Tissue fixation for in situ hybridization was performed following the protocol of Feng et al [32]. The tissues were embedded in paraffin (Paraplast Plus, Sigma-Aldrich, Saint Louis, MO, USA) and sliced into 8 μm sections with a microtome (Leica, Wetzlar, Germany). The 3'-region of *GmUFO1* cDNA was subcloned and used as a template to generate sense and antisense RNA probes (Table S1). Digoxigenin-labeled RNA probes were prepared with a DIG RNA Labeling Kit (T7/SP6) (Cat. No. 11175025910, Roche) according to the manufacturer's instructions. The slides were observed under a bright field using a Leica microscope (DMI8, Leica, Wetzlar, Germany).

4.4. Subcellular Localization

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For the GmUFO protein subcellular experiment, the full-length CDS (removal terminator) sequence of *GmUFO1* was amplified and linked with a GFP fluorescent protein sequence and inserted into the *pUC19* vector using NdeI and SalI for double digestion. The subcellular expression vector *pUC19-GFP-GmUFO1* was constructed containing the 35S promotor controlling expression of the *GFP-GmUFO1* fusion construct. A modified polyethylene glycol (PEG) method was used for the transfection of protoplasts [33-35]. The *pUC19-GFP-GmUFO1* and *pUC19-AHL22-mCherry* plasmids were extracted by TIANprep Mini Plasmid Kit (TIANGEN Biotech Co., Beijing, China), and the two plasmids were transiently transformed into *Arabidopsis* protoplasts. The transformed protoplasts were subsequently cultured at room temperature in the dark for 16 h, and fluorescence observation and photography were performed using a Leica microscope (DMI8, Leica, Wetzlar, Germany).

4.5. Cytological Analysis

The floral primordium of wild-type 'Williams 82' during five developmental stages (stages 1–5) was observed by scanning electron microscopy. After removing the bracts, they were fixed in a formaldehyde alcohol acetic acid (FAA) fixation solution for at least 12 h. All materials were dehydrated with ethanol (30, 50, 70, 90, 95, 100%) and then dehydrated with ethanol-tert-butanol to 100% tert-butanol. The samples were dissected using a positive microscope and held in small Petri dishes with conductive tape. The floral primordium was scanned using InTouchScope Scanning Electron (JSM-IT500, Japan Electronics, Tokyo, Japan). The anatomical observation of flower organs was performed using an orthomicroscope (SZ2-ILST, Olympus Co., Tokyo, Japan) and photographed with cellSens Standard imaging software (Olympus cellSens Standard, Version 1.16, Olympus Co., Tokyo, Japan).

4.6. Gene Knockout and Material Identification

A Cas9/sgRNA plasmid construction kit (Viewsolid Biotech Co., Beijing, China) was used to construct the CRISPR/Cas9 plasmids. These plasmids contained a dicotyledon codon-optimized dpCas9 (under the *GmUbi3* promoter) and a gRNA scaffold (under the *GmU6-2* promoter). Construction of the CRISPR/Cas9 vector was performed following the manufacturer's instructions (Viewsolid Biotech Co., Beijing, China). The constructed CRISPR/Cas9-gDNA plasmid was transformed into *Agrobacterium* EHA105, and soybean genetic transformation was performed following Yamada et al [36]. Genomic DNA was extracted from the seedlings of wild-type and all positive transgenic plants, and DNA fragments spanning the target sites were amplified by PCR. The target site for *GmUFO1* was amplified with the primers GmUFO-F (5′-ATGGAAGGTTTTCACCCTTCTATGT-3′) and GmUFO1-R (5′-AGCATTGAAACTCTGGAAGGGC-3′). The target site for GmUFO2 was amplified with the primers GmUFO-F (5′-ATGGAAGGTTTTCACCCTTCTATGT-3′) and GmUFO2-R (5′-TTGGGCACGTTCAGCTTGCTCTTCTCA-3′).

4.7. Total RNA Extraction and qPCR

Total RNA was extracted using the Trizol method [37], and the cDNA was synthesized by the process of reverse transcription using the TransScript One-Step genomic DNA (gDNA) removal and cDNA synthesis Super-Mix kit (TransGen Biotech Co., Beijing, China), with gDNA removal in one step. The cDNA was diluted in sterile water to 100 ng/μl, with 2 μl diluent as a template for qPCR. qPCR was performed using 2 × RealStar Green Fast mix (GenStar Biosolutions Co., Beijing, China) on a Stratagene Mx3005P sequence detection system (Stratagene, La Jolla, CA, USA) following the manufacturer's instructions. Three biological replicates were used to quantify the expression levels. Differences between groups were calculated using the $2^{-\Delta\Delta Ct}$ method. The soybean *GmActin 11* (*Glyma.18G290800*) gene was used as an internal control, and the relative expression levels were calculated. Primers used for RT-qPCR analysis are shown in Supplemental Table S1.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Table S1: Primers used in this research; Table S2: Protein physical information of 11 UFO genes from nine species.

Author Contributions: H.Y performed the experiment and wrote manuscripts. X.Y, Z.Z and T.W helped to guide the experiment. J.F and Y.J helped to complete experiments. Y.Z and X.F discussed the results and wrote the manuscript. Y.Z, M.H.K, J.A.B and F.W revised the manuscript. X.F and Y.W designed the project and provided resources. All authors read and approved the final manuscript.

Funding: This research was funded by National Natural Science Foundation of China (U21A20215), Jilin Province Science and Technology Development Plan Project (20210302005NC), Zhejiang Lab (2021PE0AC04) and Hainan Yazhou Bay Seed Laboratory (B21HJ0101).

Data Availability Statement: The datasets used in the study are available from the corresponding author upon request.

Conflicts of Interest: The authors declare no conflict of interest.

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