1 **The APETALA2 transcription factor LsAP2 regulates seed shape in lettuce**

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3 **Running title:** LsAP2 regulates lettuce seed shape

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24 **Highlight**

25 The APETALA2 transcription factor LsAP2 regulates lettuce seed shape via
26 interacting with LsBP and repressing brassinosteroid biosynthesis and signaling.

27 Abstract

28 Seeds are major vehicles of propagation and dispersal in plants. A number of
 29 transcription factors, including APETALA2 (AP2), play crucial roles during the seed
 30 development process in various plant species. However, genes essential for seed
 31 development and the regulatory networks during seed development remain unclear in
 32 lettuce. Here, we identified a lettuce *AP2* (*LsAP2*) gene that was highly expressed at
 33 the early stages of seed development. *LsAP2* knockout plants obtained by the
 34 CRISPR/Cas9 system were used to explore the biological function of *LsAP2*.
 35 Compared with wild-type, the seeds of the *Lsap2* mutant plants had increased length
 36 and decreased width, and developed an extended tip at the seed top. After further
 37 investigating the seed structural characteristics of *Lsap2* mutant plants, we proposed a
 38 new function of *LsAP2* in seed dispersal. Moreover, we identified several interactors
 39 of *LsAP2*. Our results showed that *LsAP2* directly interacted with the lettuce homolog
 40 of BREVIPEDICELLUS (*LsBP*) and promoted the expression of *LsBP*.
 41 Transcriptome analysis revealed that *LsAP2* might also be involved in brassinosteroid
 42 biosynthesis and signaling pathways. Taken together, our data indicate that *LsAP2* has
 43 a significant function in regulating seed shape in lettuce.

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45 **Keywords:** lettuce, APETALA2, seed shape, *LsAP2*, CRISPR/Cas9, achene,
 46 regulatory networks

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55 Introduction

56 In angiosperms, a mature seed has three major components, embryo, endosperm,
57 and seed coat. The seeds that store essential substances and contain genetic materials
58 are the most important harvested organ in many plant species. Moreover, seed size
59 and seed shape are crucial for plants to adapt during evolution, and seed size is also an
60 important agronomic trait in crop domestication (Moles *et al.*, 2005).

61 Lettuce (*Lactuca sativa* L.) belongs to the Asteraceae family and is one of the most
62 important leafy vegetables worldwide. The seed structure of lettuce is different from
63 the model plant *Arabidopsis* as the lettuce seed is actually an achene fruit. Therefore,
64 the achene development integrates the processes of fruit and seed development.
65 Achene has four major components, pericarp, integuments, endosperm and embryo.
66 The pericarp is derived from the ovary walls, and the pericarp and integuments are
67 tightly integrated to constitute the seed coat (Steinbrecher and Leubner-Metzger,
68 2017). Lettuce seeds are the vehicles of propagation and dispersal, and many traits
69 have changed dramatically during lettuce domestication, including seed size, seed
70 shattering, and seed dispersal (Zhang *et al.*, 2017). A previous study showed that
71 some lettuce MADS-box genes were highly expressed in seeds, and these genes may
72 be involved in seed development (Ning *et al.*, 2019). However, few studies have
73 performed the gene function analysis and explored the regulatory networks associated
74 with seed development in lettuce.

75 Seed development is initiated by double fertilization (Jiang and Köhler, 2012). The
76 zygote, the fertilized central cell and the maternal integuments develop together into a
77 complete seed. Thus, the seed growth is coordinately controlled by the growth of
78 maternal and zygotic tissues (Sreenivasulu and Wobus, 2013). Seed development is a
79 complicated process modulated by complex networks. Recent studies have identified
80 various pathways that control seed development through maternal tissues, including
81 the ubiquitin-proteasome pathway, G-protein signaling pathway, mitogen-activated
82 protein kinase signaling pathway, phytohormone signaling and homeostasis pathway,
83 as well as various kinds of transcriptional regulatory factors (Li and Li, 2015; Li *et al.*,

2019). Many genes involved in the seed development process have been studied extensively, and many key regulators of seed size and seed shape have been identified (Sreenivasulu and Wobus, 2013; Li *et al.*, 2019).

APETALA2 (*AP2*) gene belongs to the euAP2 lineage, a subgroup of the large AP2/ERF gene family. Genes in the euAP2 lineage encode two highly conserved AP2 domains and contain a microRNA172 (miR172) binding site (Kim *et al.*, 2006; Zumajo-Cardona and Pabon-Mora, 2016). *AP2* gene encodes a transcription factor, and its roles in specifying floral organ identity and regulating the expression of floral homeotic genes are well known (Kunst *et al.*, 1989; Bowman *et al.*, 1991; Coen and Meyerowitz, 1991; Drews *et al.*, 1991). Meanwhile, miR172 regulates the expression of *AP2* gene primarily through translational inhibition (Aukerman and Sakai, 2003; Chen, 2004). The *AP2* gene was first isolated in *Arabidopsis*, and *AP2* activity was shown to be required during seed development (Jofuku *et al.*, 1994). A mutation in the *AP2* gene (*ap2-1*) in *Arabidopsis* had a pleiotropic effect on seed shape, which changed from the normal oblong shape to a number of aberrant shapes (Leon-Kloosterziel *et al.*, 1994). *AP2* was shown to act as an important negative regulator of seed development because *ap2* mutants produce large seeds. *AP2* acts maternally to determine seed weight and seed yield by coordinating the growth of embryo, endosperm, and maternal integuments (Jofuku *et al.*, 2005; Ohto *et al.*, 2005; Ohto *et al.*, 2009). *Petunia AP2A*, an ortholog of *Arabidopsis AP2*, is also expressed in seeds, and it was found to restore the structural defect of seed epidermal cells in *Arabidopsis ap2-1* mutant (Maes *et al.*, 2001). Grain yield is an important agronomic trait in crop domestication. In rice, *SUPERNUMERARY BRACT* (*SNB*) encodes an AP2-like transcription factor (Lee *et al.*, 2007), and *SUPPRESSION OF SHATTERING1* (*SSH1*) was recently found to be an allele of *SNB*. The rice *ssh1* mutant shows reduced shattering and has larger seeds and higher grain weight (Jiang *et al.*, 2019). A genome-wide association study of grain length and grain width in rice also identified *SNB* as a novel gene that negatively regulates grain size. The *SNB* knockout plants show increased grain length, width, and weight (Ma *et al.*, 2019).

Nowadays, the CRISPR/Cas systems have received widespread attention because

114 of its high specificity and efficiency in editing the genomes of humans, animals, and
 115 plants (Cong *et al.*, 2013; Shan *et al.*, 2013; Kumar and Jain, 2015), and scientists
 116 have applied the CRISPR/Cas9 system to precisely modify crop traits and accelerate
 117 the breeding process (Botella, 2019; Chen *et al.*, 2019). In this study, we identified a
 118 lettuce *AP2* (*LsAP2*) gene that was highly expressed at the early stages of seed
 119 development. Then, we used the CRISPR/Cas9 system to edit *LsAP2* and found that
 120 *LsAP2* regulates lettuce seed shape. We also showed *LsAP2*-related regulatory
 121 networks during seed development in lettuce. Transcriptome analysis identified the
 122 differentially expressed genes (DEGs) between WT and *Lsap2* mutant plants.
 123 Moreover, *LsAP2* might regulate seed shape through its effects on the brassinosteroid
 124 (BR) biosynthesis and signaling pathways.

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143 **Materials and methods**

144 **Plant materials and growth conditions**

145 Lettuce cultivar S39 was used as wild-type (WT) in this study. The lettuce seeds were
146 provided by Dr. Han from Beijing University of Agriculture (Han *et al.*, 2016). The
147 lettuce plants were grown in growth chambers under a photoperiod of 16-h light (200
148 $\mu\text{mol m}^{-2} \text{s}^{-1}$) at 25°C and 8-h dark at 18°C. When the fifth true leaf was fully
149 expanded, the lettuce plants were transplanted to a greenhouse in the Science Park of
150 China Agricultural University under standard greenhouse conditions.

151 **Phylogenetic analysis**

152 Lettuce *AP2* genes were identified using BLASTp from the lettuce genome
153 (Reyes-Chin-Wo *et al.*, 2017). Lettuce sequences were downloaded from the CoGe
154 genome database (<https://genomevolution.org/coge/>). *Arabidopsis* sequences were
155 downloaded from The *Arabidopsis* Information Resource (TAIR;
156 <https://www.arabidopsis.org/index.jsp>). Petunia sequences were downloaded from the
157 Sol Genomics Network (SGN;
158 https://solgenomics.net/organism/Petunia_axillaris/genome). Sequences of tomato and
159 rice were downloaded from the Phytozome genome database
160 (<https://phytozome.jgi.doe.gov/pz/portal.html>). Sequences of maize and wheat were
161 downloaded from the National Center for Biotechnology Information (NCBI;
162 <https://www.ncbi.nlm.nih.gov>). The amino acid sequences were aligned using
163 ClustalW (Larkin *et al.*, 2007). The phylogenetic tree was constructed in MEGA 5
164 program using the neighbor-joining method with 1000 bootstrap replicates (Tamura *et al.*,
165 2011). The accession numbers of the used sequences are listed in Supplementary
166 Table S1.

167 **RNA extraction, cDNA synthesis, and qRT-PCR**

168 Total RNA was extracted from different lettuce tissues using the Quick RNA isolation

169 Kit (Huayueyang, China). The cDNAs were synthesized using the FastKing-RT
170 SuperMix (Tiangen, China) according to the manufacturer's protocol. Subsequently,
171 quantitative real-time PCR (qRT-PCR) assays were performed in the QuantStudio 6
172 Flex Real-Time PCR System (Applied Biosystems, USA) using TB Green Premix Ex
173 Taq II (Takara, Japan). The qRT-PCR assays were performed with technical triplicates
174 of three biological replicates. The expression data were normalized using *LsPP2A-1*
175 and *LsTIP41* as reference genes (Sgamma *et al.*, 2016). Relative expression levels
176 were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Primers used
177 for qRT-PCR assays are listed in Supplementary Table S2.

178 **Construction of the lettuce transformation vectors**

179 To generate the *Lsap2* mutants, pKSE401-*LsAP2* was constructed using the
180 previously described method (Xing *et al.*, 2014). The single-guide RNAs (sgRNAs)
181 were designed using the Cas-Designer Tool (Park *et al.*, 2015; Liu *et al.*, 2017).
182 Cas-OFFinder was used to predict the potential off-target sites of Cas9 RNA-guided
183 endonucleases (Bae *et al.*, 2014; Liu *et al.*, 2017). The β -glucuronidase (*GUS*)
184 reporter gene under the control of *LsAP2* promoter was used to generate the
185 *pLsAP2:GUS* construct. Briefly, the 2402-bp genomic fragment upstream of the
186 *LsAP2* start codon was amplified by PCR using genomic DNA as the template. Then,
187 the fragment was inserted into the *HindIII*-*BamHI* sites of the pBI121 vector to drive
188 the *GUS* reporter gene. Primers used to generate the above constructs are listed in
189 Supplementary Table S2.

190 ***Agrobacterium*-mediated transformation of lettuce**

191 Lettuce transformation procedure was improved from the previously described
192 method (Chen *et al.*, 2017). The detailed steps are as follows. Seeds of lettuce cultivar
193 S39 were sterilized with 1.0% sodium hypochlorite for 10 min and rinsed with sterile
194 water, then sown on MS medium (MS supplemented with 3% sucrose and 0.7% agar).
195 The plates were incubated under a photoperiod of 16-h light ($200 \mu\text{mol m}^{-2} \text{s}^{-1}$) at

25°C and 8-h dark at 18°C for about 5 days. When the cotyledons were expanded, they were cut and incubated with *Agrobacterium* GV3101 carrying the desired plasmid for 10 min in MS liquid medium (1/2 MS supplemented with 3% sucrose and 200 µmol/L acetosyringone). After incubation, the cotyledons were transferred to sterile filter papers to remove excess *Agrobacterium*. Then, the cotyledons were placed on MS co-cultivation medium (MS supplemented with 3% sucrose, 0.7% agar, 0.1 mg/L 6-benzylaminopurine, 0.05 mg/L α-naphthaleneacetic acid, and 200 µmol/L acetosyringone) and incubated at 25°C in the dark for 2 days. Subsequently, explants were transferred to MS selection medium (MS supplemented with 3% sucrose, 0.7% agar, 0.1 mg/L 6-benzylaminopurine, 0.05 mg/L α-naphthaleneacetic acid, 300 mg/L timentin, and 40 mg/L kanamycin monosulfate) and incubated under a cycle of 16-h light (200 µmol m⁻² s⁻¹) at 25°C and 8-h dark at 18°C. About two weeks later, regenerated shoots grew on the calli, and they were cut into MS shoot-inducing medium (MS supplemented with 3% sucrose, 0.7% agar, and 300 mg/L timentin). After two weeks, the shoots were subcultured on fresh MS shoot-inducing medium. When the shoots were 2-cm long, they were transferred to MS rooting medium (1/2 MS supplemented with 3% sucrose, 0.7% agar, 0.02 mg/L α-naphthaleneacetic acid, and 200 mg/L timentin). When the shoots and roots of the regenerated plants were well developed, they were transferred to the soil under standard growth conditions.

215 **GUS staining**

Tissues from the *pLsAP2:GUS* plants were used for GUS staining assays. First, flowers and seeds at different developmental stages were collected and immersed in GUS staining buffer (containing 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide). Next, the samples were vacuum infiltrated for 10 min, and then stained in a 37°C incubator overnight. After incubation, the staining buffer was removed, and samples were washed with 70% ethanol for several times to remove the pigments. Finally, the samples were immersed in fresh 70% ethanol before observation. The samples were observed using a Leica stereomicroscope (Leica S8 APO, Germany).

224 **Phenotypic analysis**

225 Plant materials were photographed with a digital camera (Nikon D7000, Japan) and a
 226 stereomicroscope (Leica S8 APO, Germany). To evaluate seed size, the images were
 227 imported into ImageJ software to measure the seed length and seed width. The
 228 measured seed length and seed width were used to calculate the ratio of seed length to
 229 width. To obtain the scanning electron micrographs, the seeds were gold plated and
 230 observed using a scanning electron microscope (Hitachi S-3400N, Japan). To evaluate
 231 cell size, the images were imported into ImageJ software to measure the cell length
 232 and cell width. Mature seeds were evaluated for calculating 1,000-grain weight using
 233 an electronic analytical balance (METTLER TOLEDO, USA). For seed germination
 234 tests, 20 seeds were tested for each line with three replicates, then the seeds were
 235 moistened with water and incubated at 25°C for two days to calculate the germination
 236 rates. To measure the seed beak diameter, beaks were photographed using a
 237 microscope (Olympus IX71, Japan), then the images were imported into ImageJ
 238 software to calculate the beak diameter. To evaluate seed dispersal, the breaking
 239 tensile strength (BTS) between beak and seed was measured with a digital force
 240 gauge (SHSIWI, China) at the harvest stage.

241 **Yeast two-hybrid assays**

242 The full-length coding sequence (CDS) of *LsAP2* was cloned into pGADT7 vector.
 243 The full-length CDSs of *LsAS1*, *LsAS2*, *LsBP*, *LsKANI*, and *LsKAN2* were amplified
 244 and cloned into pGBKT7 vector. Yeast two-hybrid assays were performed as
 245 described previously (Ding *et al.*, 2015). Briefly, bait plasmids and prey plasmids
 246 were co-transformed into yeast strain AH109 according to the manufacturer's protocol
 247 of GAL4-based yeast two-hybrid system (Clontech, USA). Then, yeast transformants
 248 were grown on SD/-Leu-Trp plates for growth of the colonies and grown on
 249 SD/-Leu/-Trp/-His/-Ade plates for protein interaction selection. Primers used for yeast
 250 two-hybrid assays are listed in Supplementary Table S2.

251 **Firefly luciferase complementation imaging (LCI) assays**

252 The full-length CDSs without the stop codon of *LsAS2*, *LsBP*, *LsKANI*, and *LsKAN2*
 253 were cloned into the *35S:nLUC* vector. The full-length CDS of *LsAP2* was cloned into
 254 the *35S:cLUC* vector. Then, the above constructs were transformed into
 255 *Agrobacterium* strain GV3101. LCI assays were performed using the previously
 256 described method (Chen *et al.*, 2008). Briefly, different combinations were
 257 co-infiltrated into tobacco leaves using *Agrobacterium*-mediated transient
 258 transformation. After infiltration, the plants were grown under a 16-h light/8-h dark
 259 cycle for 2 days. Before imaging, the abaxial sides of the tobacco leaves were sprayed
 260 with luciferin, and the leaves were kept in the dark for 5 min. Then a cooled CCD
 261 camera (Roper 1300B, USA) was used to capture the fluorescence signal with 10 min
 262 exposures. Primers used for LCI assays are listed in Supplementary Table S2.

263 **Dual-luciferase reporter assays**

264 The *LsBP* promoter (1595-bp genomic fragment upstream the start codon) was cloned
 265 and inserted into the pGreenII 0800-LUC vector as the reporter. The full-length CDSs
 266 of *LsAP2* and *LsBP* were inserted into the pGreenII 62-SK vector as effectors. The
 267 empty vector pGreenII 62-SK was used as the negative control. Dual-luciferase
 268 reporter assays were performed as described previously (Hellens *et al.*, 2005). Briefly,
 269 *Agrobacterium* strain GV3101 carrying the reporter and effector constructs were
 270 infiltrated into tobacco leaves. After infiltration, the plants were grown under a 16-h
 271 light/8-h dark cycle for 2 days. Luciferase (LUC) and renilla luciferase (REN)
 272 activities were measured by a multiscan spectrum (Molecular Devices SpectraMax®
 273 i3x, USA) using the Dual-Luciferase Reporter Assay Kit (Promega, USA) according
 274 to the manufacturer's protocol. The ratio of LUC to REN was calculated as the final
 275 transcriptional activity. Primers used for dual-luciferase reporter assays are listed in
 276 Supplementary Table S2.

277 **Transcriptome analysis**

278 Total RNA was extracted from the seeds of WT and *Lsap2* mutant plants on the day of
 279 pollination, with three biological replicates. Paired-end libraries were constructed and
 280 sequenced using an Illumina NovaSeq 6000 platform at Biomarker Technologies
 281 (BioMarker, China). Analyses of the transcriptome data were performed on the
 282 BMKCloud platform (www.biocloud.net). Clean reads were mapped to the lettuce
 283 reference genome (Reyes-Chin-Wo *et al.*, 2017) using the HISAT2 software (Kim *et*
 284 *al.*, 2015). Differential expression analysis between WT and *Lsap2* mutant plants (fold
 285 change ≥ 2 , FDR < 0.01) was performed using the DESeq (Anders and Huber, 2010).
 286 Gene Ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and
 287 Genomes (KEGG) analysis of the DEGs were performed using Goseq R packages
 288 and KOBAS software (Mao *et al.*, 2005; Young *et al.*, 2010).

289 **Measurement of endogenous hormones**

290 Extraction and purification of the endogenous hormones were performed as described
 291 previously (Wang *et al.*, 2012). Briefly, about 0.2 g of the fresh seed samples from
 292 WT and *Lsap2* mutant plants were collected and homogenized in 4 mL of 80%
 293 methanol (containing antioxidant), with three biological replicates. After incubation at
 294 4°C for 48 h, the extract was centrifuged at 5000 rpm for 10 min at 4°C. Then, the
 295 supernatant was passed through the extraction cartridge, and the phytohormone
 296 fraction was eluted with methanol and then ether. Next, the eluate was dried down and
 297 dissolved in phosphate-buffered saline (PBS) buffer. Quantification of auxin (IAA)
 298 and BR were measured by enzyme-linked immunosorbent assay (ELISA) following
 299 the described protocol (Zhao *et al.*, 2006) at the Center of Crop Chemical Control,
 300 China Agricultural University.

301

302 **Results**

303 **Identification of *AP2* genes in lettuce**

304 To understand the seed development process in lettuce and identify the key
305 regulators during lettuce seed development, we continuously observed the developing
306 seeds after pollination. We found that the seed morphology was fully developed
307 within ten days after pollination (Fig. 1A). The seeds grew longer and wider in the
308 first three days. Then, the seeds began to expand, and obvious longitudinal ribs were
309 observed on the seed surface. One week later, the seed coat became darker, and the
310 longitudinal ribs were more pronounced. Ultimately, the seeds were ready to ripen.

311 Previous studies have identified many transcription factors as important regulators
312 of seed development (Li *et al.*, 2019), and *AP2* gene has been shown to play crucial
313 roles in regulating seed development in many species. To investigate whether lettuce
314 *AP2* gene plays a role in seed development, we performed BLAST searches at the
315 lettuce genome database and identified three lettuce *AP2* genes as the candidate genes.
316 The phylogenetic tree revealed that *LsAP2* was more closely related to *Arabidopsis*
317 *AP2* than the other two lettuce *AP2* homologs, namely *LsAP2-like1* and *LsAP2-like2*
318 (Supplementary Fig. S1).

319 To functionally characterize the lettuce *AP2* genes during seed development, we
320 examined their expression patterns by analyzing the FPKM values from a
321 transcriptome dataset. Strikingly, *LsAP2* was the most highly expressed gene among
322 the three lettuce *AP2* genes at different seed development stages, and the expression
323 of *LsAP2* gradually decreased between successive stages (Supplementary Table S3).
324 According to the homology and expression analysis, we speculated that of the three
325 lettuce *AP2* genes, *LsAP2* might be the one that plays a major role during lettuce seed
326 development. Therefore, we chose *LsAP2* for further characterization in this study.

327 **Expression pattern of *LsAP2***

328 To detect the expression pattern of *LsAP2* further, we analyzed its expression in

different lettuce tissues by qRT-PCR assays. We found that *LsAP2* was expressed in all lettuce tissues, and the expression level of *LsAP2* in flower was many times higher than in other tissues (Supplementary Fig. S2). Further, we examined the expression of *LsAP2* in floral organs. *LsAP2* was highly expressed in petal, pistil, and seed, while *LsAP2* displayed lower expression level in bract and stamen (Fig. 1B). To explore when *LsAP2* was expressed during seed development, we then analyzed the expression of *LsAP2* in seeds at different developmental stages. Significantly, *LsAP2* transcripts were strongly accumulated at the early stages of seed development, and its expression decreased gradually as the seed developed (Fig. 1C). These results confirmed that *LsAP2* was expressed in seeds, especially at the early stages of seed development.

Further, we analyzed the spatial and temporal expression pattern of *LsAP2* in flower using the *GUS* reporter gene under the control of *LsAP2* promoter. The observed *GUS* activities were generally consistent with the endogenous *LsAP2* mRNA level determined by qRT-PCR. *GUS* staining showed that *LsAP2* was mainly expressed in petal, pistil, and seed, while weaker or even no *GUS* signal was observed in bract and stamen (Fig. 1F-H). Then, we analyzed the expression of *LsAP2* at different seed development stages. Strong *GUS* activities were also detected in seeds at the corresponding stages (Fig. 1J-M). Further, we observed different sections of the stained seeds, and only strong *GUS* signal was found in the seed coat (Fig. 1O-Q).

CRISPR/Cas9-mediated editing of *LsAP2* alters seed shape

To investigate the biological function of *LsAP2* gene, we generated *LsAP2* knockout plants using the CRISPR/Cas9 system (Fig. 2A). Genotyping analysis of the ten T0 transgenic plants identified six plants with mutations in the target sites. There were significant differences in the editing efficiency of the two targets; Target 1 was mutated in six plants, whereas Target 2 was mutated in only one plant (Supplementary Figs. S3, S4). Therefore, it is crucial to assess the on-target efficiency when design sgRNAs. There were two types of mutations among the mutant plants; two plants

357 were homozygous, and four plants were biallelic that had two mutant alleles
358 (Supplementary Table S4). We also assessed the potential off-target sites using
359 Cas-OFFinder, and no mutation was detected in the predicted off-target sites
360 (Supplementary Table S5). Through the self-pollination of lettuce, homozygous and
361 non-transgenic T1 generation plants were obtained and confirmed by PCR genotyping
362 and sequencing. Three mutant lines were chosen for further exploration (Fig. 2B, C).

363 Given that the CRISPR/Cas9 system was used to edit the genomic sequence of
364 *LsAP2*, genome editing resulted in the non-sense transcripts of *LsAP2* gene. These
365 transcripts disrupted the normal translation of *LsAP2* mRNA with frame-shift
366 mutation and premature stop codons (Supplementary Data S1), which led to the
367 absence of functional *LsAP2* protein. *AP2* is an A-class gene that determines perianth
368 identity in flowers (Kunst *et al.*, 1989; Coen and Meyerowitz, 1991). However, we
369 did not observe any defect in the bract, petal, or pappus of *Lsap2* mutant plants.
370 Interestingly, we found that the seed shape was changed in all mutant lines because
371 the seeds were lanky, and there was an extended tip at the top of the seed (Fig. 2D).

372 In *Lsap2* mutant plants, the seed length increased significantly (Fig. 2E, H), and the
373 seed width decreased significantly (Fig. 2F, I), whereas the 1,000-grain weight did not
374 change significantly compared with WT (Fig. 2J). Seed germination tests showed that
375 almost all seeds germinated, and the roots developed normally after two days
376 incubation at 25°C (Fig. 2G, K), which indicated that the seed germination was not
377 affected in *Lsap2* mutant plants. The scanning electron micrographs revealed that the
378 seed surface of *Lsap2* mutant plants was smoother, and there were fewer longitudinal
379 ribs and striking spines on the seed surface of *Lsap2* mutant plants compared with WT
380 (Supplementary Fig. S5). Further, the length of the seed epidermal cells in *Lsap2*
381 mutant plants was slightly longer than in WT, while the cell width did not change
382 significantly (Supplementary Fig. S5), indicating that the elongated cell length might
383 lead to the increased seed length in *Lsap2* mutant plants.

384 **Important roles of *LsAP2* in regulating seed morphology**

385 As *LsAP2* was highly expressed at the early stages of seed development, we
 386 speculated that *LsAP2* might regulate seed shape at the early stages. To verify this
 387 idea, we observed the seeds of WT and *Lsap2* mutant plants at different seed
 388 development stages. Compared with WT, the seeds of *Lsap2* mutant plants were
 389 already longer on the day of pollination (Fig. 3A). Subsequently, the seeds of *Lsap2*
 390 mutant plants grew longer and thinner than those of the WT, while the seed beak of
 391 *Lsap2* mutant plants was shorter and thicker than that of the WT (Fig. 3B-D). Because
 392 the seeds of *Lsap2* mutant plants were lanky, we measured the length-width ratio of
 393 the mature seeds and showed that *Lsap2* mutant plants had a larger seed length-width
 394 ratio compared with WT (Fig. 3E). Interestingly, in *Lsap2* mutant seeds, the lower
 395 part of the beak and upper part of the seed seemed to be fused (Fig. 3B-D). After seed
 396 ripening, the beaks were broken and seeds were harvested, and an extended tip was
 397 left at the top of the *Lsap2* mutant seeds. These results confirmed that the seed shape
 398 of *Lsap2* mutant plants was altered at the early stages of seed development, and this
 399 phenotype corresponded to the high expression of *LsAP2* at these stages.

400 In *Lsap2* mutant seeds, the beak was thicker and closely connected with the seed,
 401 suggesting that this structure might resist stronger external force and contribute to
 402 seed dispersal. To verify this hypothesis, we first measured the diameter of the seed
 403 beak at the harvest stage, and our data showed that the seed beak diameter of *Lsap2*
 404 mutant plants was larger than that of the WT (Fig. 3F). Further, we measured the
 405 breaking tensile strength (BTS) between beak and seed, and the results showed that
 406 *Lsap2* mutant plants had stronger BTS than WT (Fig. 3G), implying that *LsAP2* might
 407 have a function in seed dispersal.

408 ***LsAP2*-related protein interaction networks during lettuce seed development**

409 Previous studies have shown that AP2 regulates genes related to fruit and seed
 410 development. However, whether AP2 could directly interact with these regulators to
 411 modulate fruit or seed development are largely unknown. To identify the proteins that

potentially interact with LsAP2, we performed yeast two-hybrid assays to test the interactions between LsAP2 and regulators associated with fruit and seed development. Our results showed that LsAP2 directly interacted with four proteins, namely the lettuce homologs of ASYMMETRIC LEAVES2 (AS2), BREVIPEDICELLUS (BP), KANADI1 (KAN1), and KANADI2 (KAN2) (Fig. 4A). AS2 encodes a LOB domain protein, and AS2 is required for leaf and fruit development in *Arabidopsis* (Iwakawa *et al.*, 2002; Alonso-Cantabrana *et al.*, 2007). BP is a class I KNOX gene (Venglat *et al.*, 2002), which modulates replum formation during fruit development in *Arabidopsis* (Alonso-Cantabrana *et al.*, 2007). KAN1 and KAN2 are members of the KANADI (KAN) family, and KAN1 and KAN2 have been shown to play important roles in the growth of ovule integuments (McAbee *et al.*, 2006).

To confirm these potential interactions *in planta*, we performed LCI assays in tobacco leaves. Co-expression of LsAP2 with LsAS2, LsBP, or LsKAN1 produced strong fluorescence activity (Fig. 4B), whereas co-expression of LsAP2 with LsKAN2 or control vectors showed only background levels of fluorescence activity (Fig. 4B). These results demonstrated that LsAP2 could interact with LsAS2, LsBP, and LsKAN1 *in planta*.

To determine whether the interactions of LsAP2 with LsAS2, LsBP, and LsKAN1 might be important for seed development, we performed expression analyses of LsAS2, LsBP, and LsKAN1. Our data showed that LsBP and LsKAN1 were expressed in seeds, while LsAS2 was not detected in seeds. Significantly, LsBP was strongly expressed in seeds (Supplementary Fig. S6). Further experiments were performed to examine the expression levels of LsBP and LsKAN1 at different seed development stages. The qRT-PCR analysis showed that LsBP was expressed at all stages and displayed higher expression level at the early stages, and the expression of LsBP decreased with the development of the seeds (Fig. 4C), which was overlapped temporally with the expression pattern of LsAP2. The transcripts of LsKAN1 could also be detected at different developmental stages, but LsKAN1 exhibited a different expression pattern from LsAP2 (Fig. 4C). The biological relevance between LsAP2

442 and *LsBP* indicated that *LsAP2* might cooperate with *LsBP* to regulate seed shape.

443 ***LsAP2* affects the expression of fruit development related genes**

444 During the fruit development of *Arabidopsis*, *AP2* negatively regulates *BP* and
445 *REPLUMLESS* (*RPL*) to prevent replum overgrowth. Meanwhile, *AP2* negatively
446 regulates valve margin formation by repressing the expression of valve margin
447 identity genes (Ripoll *et al.*, 2011). Since the seeds of lettuce are also fruits, and the
448 morphology of *Lsap2* mutant seeds was changed, we then examined the expression of
449 fruit development related genes in *Lsap2* mutant plants. Compared with WT, the
450 expression levels of *LsBP* and *LsRPL* were significantly decreased in the seeds of
451 *Lsap2* mutant plants (Fig. 5A). However, orthologs of *SHATTERPROOF1* (*SHP1*),
452 *SHATTERPROOF2* (*SHP2*), *INDEHISCENT* (*IND*), and *ALCATRAZ* (*ALC*), which
453 are required for valve margin formation and dehiscence, had not been found in lettuce.

454 The phylogenetic analysis revealed that *LsAP2* was closely related to *Arabidopsis*
455 *AP2*, and we demonstrated that *LsAP2* also functioned in lettuce seed and fruit
456 development. However, our results implied that *LsAP2* might positively regulate
457 *LsBP* and *LsRPL*, which was opposite from *Arabidopsis*. Actually, the fruit structure
458 of lettuce is different from that of *Arabidopsis*. The achenes of lettuce are indehiscent
459 fruits, whereas the siliques of *Arabidopsis* are dehiscent fruits. Similarly, the
460 caryopses of rice are also dry fruits with indehiscent, and the *AP2*-like transcription
461 factor *SNB* positively regulates the expression of two rice *RPL* orthologs, *qSH1* and
462 *SH5* (Jiang *et al.*, 2019). These results indicated that the *AP2* genes might exhibit
463 divergent regulatory patterns between different types of fruit because of the structural
464 differences.

465 Interestingly, *LsAP2* interacted with *LsBP* at the protein level, and the *LsBP* gene
466 was also down-regulated in *Lsap2* mutant plants. We speculated that *LsBP* or *LsAP2*
467 might promote *LsBP* expression, and the protein interaction between *LsAP2* and
468 *LsBP* would enhance the promotion. To test this regulatory network, we used the
469 *LsBP* promoter to drive *LUC* reporter gene and performed dual-luciferase reporter

assays. Compared with the control experiment, expression of *LsAP2* or *LsBP* alone had a minor effect on the promotion of LUC activity, while co-expression of *LsAP2* and *LsBP* significantly increased the LUC activity (Fig. 5C), indicating that *LsAP2* cooperated with *LsBP* to enhance the activity of *LsBP* promoter. These results demonstrated that the protein interaction between *LsAP2* and *LsBP* could promote the expression of *LsBP*, which might be important for seed development in lettuce.

Identification of differentially expressed genes between WT and *Lsap2* mutant plants

To dissect the molecular function of *LsAP2* in seed development further, we performed the transcriptome analysis of the seeds from WT and *Lsap2* mutant plants on the day of pollination. Many genes were differentially expressed between WT and *Lsap2* mutant plants, and we identified 586 up-regulated genes and 547 down-regulated genes among the transcriptomes (Supplementary Table S6). GO enrichment analysis of the DEGs revealed that they were involved in multiple processes. Significantly enriched GO terms included nucleosome assembly and its related cellular component. Moreover, some GO categories were related to photosynthesis (Fig. 6A). Nucleosome assembly is involved in DNA replication and cell division, and seed photosynthesis is essential for storage activity. These processes are necessary for normal seed development.

Further KEGG analysis of the DEGs indicated that some hormone-related genes were differentially expressed between WT and *Lsap2* mutant plants, such as cytokinin, auxin, and BR. Phytohormones have various functions in plant growth and development. Because auxin and BR have been shown to regulate seed growth (Jiang *et al.*, 2013; Figueiredo and Köhler, 2018), we measured the endogenous hormones in the developing seeds of WT and *Lsap2* mutant plants. We found that the BR level was significantly increased in the developing seeds of *Lsap2* mutant plants compared with WT. However, there was no significant difference in IAA level between WT and *Lsap2* mutant plants (Fig. 6B).

498 To explore the significance of the increased BR level in *Lsap2* mutant seeds, we
 499 examined the expression of BR-related genes that have been shown to control seed
 500 development. The transcriptome data indicated that a lettuce homolog of
 501 *BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED KINASE 1* (*BAK1*) was
 502 significantly up-regulated in *Lsap2* mutant plants (Fig. 6C), while the expression
 503 levels of other BR-related genes were not changed significantly. We also performed
 504 qRT-PCR assays to verify the transcription levels of some genes, and the results were
 505 generally consistent with the transcriptome data (Fig. 6D). *BAK1* is a receptor kinase
 506 involved in BR signal transduction (Li *et al.*, 2002), and it has been shown to regulate
 507 grain size in rice. Loss of function of *OsBAK1* exhibits decreased grain size (Yuan *et al.*, 2017). Thus, we speculated that the increased BR level in *Lsap2* mutant seeds
 508 might stimulate the BR perception and signaling, thus promoting cell elongation and
 509 increasing seed length.
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527 Discussion

528 Unique roles of *LsAP2* during seed development in lettuce

529 *AP2* gene encodes a transcription factor, and the broad roles of the *AP2* gene in
 530 plant growth and development have been shown in many species. Here, we identified
 531 a lettuce *AP2* gene and explored its biological function. Expression analysis revealed
 532 that *LsAP2* was highly expressed in seeds, especially at the early stages of seed
 533 development (Fig. 1C). We used the CRISPR/Cas9 system to edit *LsAP2* gene and
 534 found that *LsAP2* regulates lettuce seed shape (Fig. 2A-D). Although the loss of *AP2*
 535 function alters seed shape in both *Arabidopsis* and lettuce, there are some differences.
 536 In *Arabidopsis*, *ap2* mutants produce large seeds with aberrant shapes. Reducing *AP2*
 537 gene activity increases seed length and width and leads to increased seed mass
 538 (Jofuku *et al.*, 2005; Ohto *et al.*, 2005). Meanwhile, the epidermal cells of *ap2* mutant
 539 seeds lack the columella structure, and the epidermal cells are larger and more
 540 irregular in shape than WT seeds (Jofuku *et al.*, 1994). In lettuce, the seed shape of
 541 *Lsap2* mutants was affected as the seeds were lanky and developed an extended tip
 542 (Fig. 2D-F). In *Lsap2* mutant plants, the seed length increased, and the seed width
 543 decreased (Fig. 2H, I), while the seed mass did not change significantly compared
 544 with WT (Fig. 2J). Our scanning electron micrographs showed that the seed surface of
 545 *Lsap2* mutants was smoother than that of the WT due to decreased longitudinal ribs
 546 and fewer striking spines. Moreover, cell length of the seed epidermal cells increased
 547 slightly in *Lsap2* mutant plants, while the cell shape and cell structure did not show
 548 noticeable changes (Supplementary Fig. S5).

549 In the Asteraceae plant dandelion, the pappus attaches to the beak for seed dispersal,
 550 and the seed falls and stops dispersing when the junction between beak and seed is
 551 broken. Lettuce also belongs to the Asteraceae family and has the same seed structure
 552 as dandelion. In *Lsap2* mutant seeds, the beak was thicker than that of the WT (Fig.
 553 3A-D). Besides, the beak was closely connected with the seed top, and the BTS
 554 between beak and seed was stronger than WT (Fig. 3G), implying that this structure

555 may resist stronger external force and contribute to longer distance seed dispersal with
556 the help of the wind. This finding may also reveal a potential new function of *LsAP2*
557 in seed dispersal, which is radically divergent from *Arabidopsis* as the seed structure
558 of lettuce is different from *Arabidopsis*.

559 Since *AP2* is an A-class gene that determines the identity of perianth organs, the
560 sepals and petals transform into reproductive organs in *ap2* mutants (Kunst *et al.*,
561 1989; Coen and Meyerowitz, 1991). Although the expression analysis showed that
562 *LsAP2* was expressed in bract and petal (Fig. 1B), we did not observe any perianth
563 defect or transformation in the floral organs of *Lsap2* mutant plants. On one hand, the
564 petunia *AP2*-type *REPRESSOR OF B-FUNCTION (ROB)* genes have incomplete
565 A-function. The *ROB* genes are redundantly required for the normal development of
566 the perianth and are not required to antagonize the C-function in the perianth (Morel
567 *et al.*, 2017). Based on current understanding, it is also possible that the complete
568 A-function of *AP2* gene does not occur in lettuce. On the other hand, the lettuce
569 reference genome was published in 2017, and researchers found that some types of
570 genes, including *AP2*, were enriched in the triplicated regions (Reyes-Chin-Wo *et al.*,
571 2017). Indeed, there are three *AP2* genes in lettuce (Supplementary Fig. S1), and this
572 gene redundancy mechanism may explain the absence of perianth defect in *Lsap2*
573 mutant plants.

574 ***LsAP2* may regulate seed shape via interacting with *LsBP***

575 In *Arabidopsis*, the regulatory networks of fruit development have been studied
576 extensively, and many genetic regulators involved in fruit development were
577 identified. *BP* and *RPL* play direct roles in promoting replum identity during fruit
578 development. *AP2* cooperates with *AS1* and *AS2* to modulate replum development by
579 independently regulating the activity of *BP* and *RPL* (Ripoll *et al.*, 2011). Although
580 their interactions are known at the genetic level, the direct protein interaction
581 networks of *AP2* in fruit or seed development are largely unknown. In this study, we
582 identified several interactors of *LsAP2* using yeast two-hybrid and LCI assays. *LsAP2*

583 directly interacted with LsBP *in vivo* (Fig. 4A, B), and expression analysis revealed
584 that *LsBP* was highly expressed in seeds and showed a temporally overlapping
585 expression pattern with *LsAP2* (Fig. 4C; Supplementary Fig. S6).

586 As LsAP2 directly interacted with LsBP at the protein level, and the expression of
587 *LsBP* gene was also decreased in *Lsap2* mutant seeds (Fig. 5A), we further performed
588 dual-luciferase reporter assays to explore the regulatory network between LsAP2 and
589 LsBP. Our results indirectly demonstrated that the protein interaction between LsAP2
590 and LsBP increased the gene expression of *LsBP* (Fig. 5C). Together, the high
591 biological relevance between *LsAP2* and *LsBP* suggests that LsAP2 may cooperate
592 with LsBP to regulate seed shape in lettuce.

593 **LsAP2 may negatively regulate BR biosynthesis and signaling in seeds**

594 Previous studies showed that BR plays crucial roles in regulating seed size and seed
595 shape (Jiang *et al.*, 2013; Song, 2017). BR functions through the cell surface receptor
596 BRASSINOSTEROID INSENSITIVE1 (BRI1) and the transcription factor
597 BRASSINAZOLE-RESISTANT1 (BZR1) to control BR-responsive genes (Nolan *et al.*, 2020). In both *Arabidopsis* and rice, inactivation of BR biosynthesis genes or BR
598 receptors lead to reduced seed size and abnormal seed shape, while elevated BR
599 content or enhanced BR signaling results in increased seed size. In *Arabidopsis*, the
600 seeds of the BR-deficient mutant *de-etiolated2* (*det2*) and the BR-insensitive mutant
601 *bril-5* are smaller and less elongated than those of the WT (Jiang *et al.*, 2013). A rice
602 gain-of-function mutant, *slender grain Dominant* (*slg-D*), shows increased BR level
603 and longer grains (Feng *et al.*, 2016), while the loss of function of *OsBAK1* (Yuan *et al.*, 2017), a receptor kinase involved in BR signaling, results in reduced grain length.
604 In this study, we found that the BR content of the *Lsap2* mutant seeds was much
605 higher than that of the WT (Fig. 6B), and the transcription level of *LsBAK1* increased
606 significantly in *Lsap2* mutant seeds (Fig. 6C, D). Meanwhile, the seed length and seed
607 length-width ratio of *Lsap2* mutant plants were larger than those of the WT (Fig. 2H;
608 Fig. 3E). Our results are consistent with the reports that elevated BR content or
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611 enhanced BR signaling results in increased seed length. Thus, we speculate that
612 LsAP2 may negatively regulate BR biosynthesis and signaling to modulate seed
613 morphology.

614 Taken together, LsAP2 has a significant function during seed development in
615 lettuce, and we proposed a hypothetical working model in which LsAP2 may regulate
616 lettuce seed shape via interacting with LsBP and repressing BR biosynthesis and
617 signaling (Fig. 7). Applying the CRISPR/Cas9 system to generate the knockout plants
618 of the identified interactors or downstream genes of LsAP2 will shed light on the
619 unique regulatory networks during lettuce seed development.

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640 **Supplementary data**

641 Supplementary data are available at *JXB* online.

642 Fig. S1. Phylogenetic tree of the euAP2 subfamily.

643 Fig. S2. Expression of *LsAP2* in different tissues.

644 Fig. S3. Sanger sequencing analyses of the mutant alleles in T0 generation plants.

645 Fig. S4. Sequencing chromatogram analyses of the edited sites in T0 generation
646 plants.

647 Fig. S5. Scanning electron microscope observation of the seeds from WT and *Lsap2*
648 mutant plants.

649 Fig. S6. Expression analyses of the interactors of LsAP2.

650 Table S1. Gene information in this study.

651 Table S2. Primers used in this study.

652 Table S3. FPKM values of lettuce *AP2* genes at different seed development stages.

653 Table S4. Editing efficiency and mutation types of CRISPR/Cas9-mediated editing of
654 *LsAP2* in T0 generation plants.

655 Table S5. Summary of the putative off-target sites analyses.

656 Table S6. List of DEGs between WT and *Lsap2* mutant plants.

657 Data S1. Coding sequences and amino acid sequences of *LsAP2* gene in WT and three
658 mutant lines.

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677 **Author contributions**

678 QW and CL designed the project. CL, SW, KN, ZC, YW, JY and MQ performed the
 679 experiments. CL, SW and KN analyzed the experimental data. QW and CL wrote the
 680 manuscript with the help of SW, KN and ZC. All the authors read and approved the
 681 final manuscript.
 682

683 **Data availability statement**

684 All data supporting the findings of this study are available within the paper and within
 685 its supplementary data published online.

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Figure legends

Fig. 1. Expression pattern of *LsAP2*. (A) Different seed development stages. S0, the day of pollination; S1-S9, 1-9 days after pollination (DAP), respectively. (B) Expression of *LsAP2* in floral organs. br, bract; pe, petal; st, stamen; pi, pistil; se, seed. Values are means $\pm$ SD ($n = 3$). (C) Expression of *LsAP2* at different seed development stages. Values are means $\pm$ SD ($n = 3$). The expression data were normalized using *LsPP2A-1* and *LsTIP41* as reference genes. (D) The capitulum of WT at S0 stage. (E) A floret in D. pt, petal tube. (F) The capitulum of *pLsAP2:GUS* plant at S0 stage. (G) A floret in F. (H) The seed head of *pLsAP2:GUS* plant at S3 stage. pa, pappus; be, beak. (I) The seed of WT at S0 stage. (J-M) The seeds of *pLsAP2:GUS* plants at S0, S1, S2, and S3 stages, respectively. (N) Longitudinal section of the seed from WT. sc, seed coat (pericarp and integuments). (O) Longitudinal section of the seed from *pLsAP2:GUS* plant. (P) Magnified image of the longitudinal section in O. (Q) Transverse section of the seed from *pLsAP2:GUS* plant. Scale bars: 1 mm.

Fig. 2. CRISPR/Cas9-mediated editing of *LsAP2* alters seed shape. (A) Gene structure of *LsAP2*. The two target sites are indicated by arrows. Rectangles and lines represent exons and introns, respectively. Red regions indicate the two AP2 domains. Green region indicates the miR172 binding site. Blue region indicates the untranslated region. (B) Sanger sequencing analyses of the mutant alleles in T1 homozygous plants. The target sequences are indicated under the black line. The PAM sequences are depicted in red. The insertion is depicted in green. Numbers on the right side indicate the deletion (-) or insertion (+) compared with WT. (C) Sequencing chromatogram examples of the edited site in the mutant allele compared with WT. Arrow indicates the 1-bp insertion. (D-F) Seed phenotype of WT and three *Lsap2* mutant lines. Extended tips are marked with dotted boxes in D. (G) Seed germination tests of WT and *Lsap2* mutant plants. (H) Seed length of WT and *Lsap2* mutant plants. Values are means $\pm$ SD ($n = 10$). (I) Seed width of WT and *Lsap2* mutant plants. Values are

means $\pm$ SD ($n = 10$). (J) The 1,000-grain weight of WT and *Lsap2* mutant plants. Values are means $\pm$ SD ($n = 5$). (K) Seed germination rate of WT and *Lsap2* mutant plants. Values are means $\pm$ SD ($n = 3$). Significant difference analysis was conducted with two-tailed Student's *t* tests (ns, not significant; **, $P < 0.01$). Scale bars: 2 mm.

Fig. 3. Important roles of *LsAP2* in regulating seed morphology. (A-D) Seed phenotype of WT and three *Lsap2* mutant lines at different seed development stages. Upper and lower brackets indicate beaks and seeds, respectively. Asterisks indicate the junctions between beaks and seeds. (A) The seeds of WT and *Lsap2* mutant plants, 0 DAP. (B) The seeds of WT and *Lsap2* mutant plants, 1 DAP. (C) The seeds of WT and *Lsap2* mutant plants, 2 DAP. (D) The seeds of WT and *Lsap2* mutant plants, 20 DAP. (E) Seed length-width ratio of WT and *Lsap2* mutant plants. Values are means $\pm$ SD ($n = 10$). (F) Seed beak diameter of WT and *Lsap2* mutant plants. Values are means $\pm$ SD ($n = 10$). (G) The BTS between beak and seed of WT and *Lsap2* mutant plants. Values are means $\pm$ SD ($n = 10$). Significant difference analysis was conducted with two-tailed Student's *t* tests (**, $P < 0.01$). Scale bars: 1 mm.

Fig. 4. *LsAP2*-related protein interaction networks during lettuce seed development. (A) *LsAP2* interacts with *LsAS2*, *LsBP*, *LsKAN1*, and *LsKAN2* in yeast two-hybrid assays. AD, activation domain; BD, binding domain. (B) LCI assays show that *LsAP2* interacts with *LsAS2*, *LsBP*, and *LsKAN1* *in planta*. (C) Expression of *LsBP* and *LsKAN1* at different seed development stages. Values are means $\pm$ SD ($n = 3$). The expression data were normalized using *LsPP2A-1* and *LsTIP41* as reference genes.

Fig. 5. *LsAP2* affects the expression of fruit development related genes. (A) Expression of *LsBP* and *LsRPL* between WT and *Lsap2* mutant plants. Values are means $\pm$ SD ($n = 3$). The expression data were normalized using *LsPP2A-1* and *LsTIP41* as reference genes. (B) Schematic diagram of the reporter and effector constructs. (C) Dual-luciferase reporter assays show the interactions of *LsAP2* and

LsBP with the *LsBP* promoter. Values are means $\pm$ SD ($n = 6$). Significant difference analysis was conducted with two-tailed Student's *t* tests (ns, not significant; *, $P < 0.05$; **, $P < 0.01$).

Fig. 6. Identification of differentially expressed genes between WT and *Lsap2* mutant plants. (A) GO enrichment analysis of the DEGs. (B) The IAA and BR levels in the developing seeds of WT and *Lsap2* mutant plants. Values are means $\pm$ SD ($n = 3$). (C) Heat map for the expression levels of BR-related genes between WT and *Lsap2* mutant plants. Red boxes indicate up-regulation, and blue boxes indicate down-regulation. (D) Expression analyses of BR-related genes between WT and *Lsap2* mutant plants. Values are means $\pm$ SD ($n = 3$). The expression data were normalized using *LsPP2A-1* and *LsTIP41* as reference genes. Significant difference analysis was conducted with two-tailed Student's *t* tests (ns, not significant; *, $P < 0.05$; **, $P < 0.01$).

Fig. 7. A hypothetical working model of LsAP2 in regulating seed shape in lettuce. LsAP2 may regulate seed shape by directly interacting with LsBP and promoting the expression of *LsBP*. Meanwhile, LsAP2 may negatively regulate BR biosynthesis and signaling in lettuce seeds. The biological function of *LsBP* needs to be further confirmed in lettuce. Arrow, positive regulation; bar, negative regulation.

A **B****Reporter:** **Effector:** **C**