1 **Time-course transcriptome landscape of achene development in lettuce**

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9 **Conflict of interest**

10 The authors have no conflict of interest to declare.

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39 Abstract

40 Lettuce (*Lactuca sativa* L.), which belongs to the large Asteraceae (Compositae)
 41 family, breeds by sexual reproduction and produce seeds. Actually, lettuce seeds are
 42 achenes, which are defined as fruits. However, few studies have described the
 43 morphological characteristics of the lettuce achenes, and genes essential for achene
 44 development are largely unknown in lettuce. To investigate the gene activity during
 45 achene development and determine the possible mechanisms that influence achene
 46 development in lettuce, we performed a time-course transcriptome analysis of lettuce
 47 achenes. A total of 23,790 expressed genes were detected at the five achene
 48 development stages. We investigated the gene expression patterns during achene
 49 development and identified the enriched biological processes at the corresponding
 50 stages. Kyoto Encyclopedia of Genes and Genomes and Gene Ontology analyses
 51 revealed a variety of transcriptomic similarities and differentiation at different achene
 52 development stages. Further, transcription factors and phytohormones were found to
 53 play important roles during achene development. Finally, we proposed a working
 54 model to illustrate the gene expression modules and possible molecular mechanism
 55 underlying achene development. Our time-course transcriptome data also provides a
 56 foundation for future functional studies to reveal the genetic control of achene
 57 development in lettuce.

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59 **Keywords:** lettuce; achene development; transcriptome; fruit; seed; transcription
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64 1. Introduction

65 Lettuce (*Lactuca sativa* L.) supplies dietary fiber, vitamins, and minerals, and is
 66 one of the most important leafy vegetables consumed worldwide (Zhang *et al.*, 2017).
 67 Lettuce belongs to the large Asteraceae (Compositae) family (Reyes-Chin-Wo *et al.*,
 68 2017). It is a heat-promoted bolting vegetable, so the transition from vegetative
 69 growth to reproductive growth is accelerated under high temperatures (Han *et al.*,
 70 2016). Finally, the lettuce plants begin to bloom and bear fruit. Previously, we
 71 characterized the inflorescence development of lettuce (Chen *et al.*, 2017), and
 72 proposed a model for lettuce floral organ specification (Ning *et al.*, 2019). Lettuce
 73 seeds are achene fruits that are developed from the ovaries. However, gene activity
 74 during lettuce achene development and the general mechanisms influencing achene
 75 development are still largely unknown.

76 In angiosperms, the ovule develops into a seed upon fertilization, whereas the ovary
 77 differentiates into a fruit (Ruan *et al.*, 2012). Many fruit types have evolved as
 78 flowering plants have adapted to different conditions (Seymour *et al.*, 2013). Fruit
 79 types can be classified according to the following characteristics: carpel number, free
 80 or fused carpels, texture, dehiscence or indehiscence. Fruits derived from a mature
 81 ovary and containing seeds are defined as true fruits. However, some fruits are
 82 composed of a variety of tissues other than the ovary, such as bracts, sepals, petals,
 83 and receptacle, and these fruits are defined as false fruits. In general, fruits can be
 84 divided into two main categories: fleshy fruits and dry fruits (Dardick and Callahan,
 85 2014). Fleshy fruits include berries, pomes, drupes, pepos, and hesperidia. Dry fruits
 86 can be either dehiscent or indehiscent depending on whether or not the pericarp splits
 87 open at maturity. In dehiscent fruits, including siliques, capsules, and legumes, the
 88 fruits open to disperse the seeds during maturation. In indehiscent fruits, including
 89 achenes, caryopses, and nuts, the fruits do not open during maturation.

90 The fruit of *Arabidopsis* has been considered as a model to study fruit development.
 91 The fruit is mostly made up of an ovary with three distinct tissues, the valve, the
 92 replum, and the valve margin (Roeder and Yanofsky, 2006). Until now, crucial genetic

93 networks that regulate fruit development have been uncovered mainly in *Arabidopsis*
 94 (Pabon-Mora *et al.*, 2014). For example, *FRUITFULL* (*FUL*) plays a direct role in
 95 promoting valve development (Gu *et al.*, 1998); *REPLUMLESS* (*RPL*) and
 96 *BREVIPEDICELLUS* (*BP*) are necessary for replum identity (Venglat *et al.*, 2002;
 97 Roeder *et al.*, 2003), and *ASYMMETRIC LEAVES1* (*AS1*) and *ASYMMETRIC*
 98 *LEAVES2* (*AS2*) regulate replum development by repressing *BP* and *RPL*
 99 (Alonso-Cantabrana *et al.*, 2007). Furthermore, *INDEHISCENT* (*IND*) and
 100 *ALCATRAZ* (*ALC*) are necessary for correct valve margin formation in fruit
 101 development (Rajani and Sundaresan, 2001; Liljegren *et al.*, 2004), and
 102 *SHATTERPROOF1* (*SHP1*) and *SHATTERPROOF2* (*SHP2*) regulate valve margin
 103 formation by positively regulating *IND* and *ALC* (Liljegren *et al.*, 2000). Fruit
 104 development and seed development are highly synchronized (Robert, 2019). Fruits
 105 protect the developing seeds and contribute to seed dispersal. The seed is composed of
 106 seed coat, embryo, and endosperm (Sreenivasulu and Wobus, 2013). Seed
 107 development is a complex process that is controlled by multiple biological processes
 108 and signaling pathways, such as the ubiquitin-proteasome pathway, phytohormone
 109 biosynthesis and signal transduction, and transcriptional regulators (Li and Li, 2016).
 110 Many important regulators associated with seed development and seed size
 111 determination have been identified (Li *et al.*, 2019), including *DA1*, *DA1-related1*
 112 (*DAR1*), *AUXIN RESPONSE FACTOR2* (*ARF2*), *BRASSINOSTEROID*
 113 *INSENSITIVE1* (*BRI1*), *BRASSINAZOLE-RESISTANT1* (*BZR1*), *APETALA2* (*AP2*),
 114 *AINTEGUMENTA* (*ANT*), and *TRANSPARENT TESTA GLABRA2* (*TTG2*).

115 Many transcriptome profiling studies have been performed to elucidate the gene
 116 dynamic activity and biological processes during fruit or seed development in
 117 different plant species. In *Arabidopsis*, a time-course transcriptome analysis of the
 118 siliques identified genes that control fruit formation and maturation, and revealed the
 119 molecular mechanisms affecting silique development and maturation (Mizzotti *et al.*,
 120 2018). In kiwifruit, an integrated analysis of the fruit metabolome and transcriptome
 121 provided insights into the regulatory network of flavonoid biosynthesis during fruit
 122 development (Li *et al.*, 2018). In chickpea, a global transcriptome and co-expression

123 network analysis revealed the molecular mechanism underlying seed development,
124 and identified candidate genes associated with seed development and seed size/weight
125 determination (Garg *et al.*, 2017). In maize, a high temporal-resolution transcriptome
126 landscape of early maize seed development was reported. The high-density,
127 time-course transcriptome data provided a high-resolution gene expression profile and
128 demonstrated four key stages during early seed development in maize (Yi *et al.*,
129 2019).

130 Achenes are indehiscent dry fruits of, for example, strawberry, buckwheat, and
131 Asteraceae plants. Achenes are single-seeded fruits, and a mature achene has four
132 major components: a hard and relatively thick pericarp, a thin testa (integument), an
133 embryo, and an endosperm. However, few studies have analyzed the transcriptomes
134 of achenes to explore the gene activity and molecular mechanisms involved in achene
135 development, especially in Asteraceae plants. In this study, we identified
136 representative developmental stages of lettuce achenes and performed time-course
137 transcriptome analysis of the achenes at five developmental stages. We analyzed gene
138 activity during achene development and proposed a possible mechanism that may
139 influence achene development. Our time-course transcriptome data provide an
140 important resource for future functional studies and shed light on the genetic control
141 of achene development in lettuce.

142

143 **2. Materials and Methods**

144 **2.1 Plant materials and growth conditions**

145 The lettuce cultivar S39 was used in this study. In the vegetative growth stage, the
146 lettuce plants were grown in growth chambers under a cycle of 16-h light at 25°C and
147 8-h dark at 18°C. When the lettuce plants transitioned to reproductive growth, they
148 were transplanted to a greenhouse under standard conditions.

149 **2.2 Sample collection and RNA extraction**

150 Achene samples were collected at the S0, S1, S3, S6, and S9 stages for
151 RNA-sequencing (RNA-seq). The achenes were harvested from at least five
152 capitulum for each pooled sample. A total of 15 samples, from five developmental
153 stages with three biological replicates, were collected and stored in an ultra-low
154 temperature freeze at -80°C . Total RNA was extracted from each sample using a
155 Quick RNA isolation kit (Huayueyang, Beijing, China) according to the
156 manufacturer's protocol.

157 **2.3 RNA-seq and bioinformatic analysis**

158 Paired-end libraries were constructed and sequenced using an Illumina
159 Nova-PE150 platform at Novogene (Beijing, China). Bioinformatic analysis of the
160 RNA-seq data was performed on the BMKCloud platform (www.biocloud.net). Raw
161 reads were filtered to generate the clean reads, and the clean reads were mapped to the
162 lettuce reference genome using the HISAT2 software (Kim *et al.*, 2015). Transcript
163 abundances were determined by the fragments per kilobase of transcript per million
164 mapped reads (FPKM) values, and genes with FPKM values ≥ 0.1 were retained for
165 further analysis. Principle component analysis (PCA) was performed using the
166 prcomp function in R software (R Core Team, 2014).

167 **2.4 Gene expression pattern analysis**

168 To further explore the gene expression patterns during achene development,
169 clustering analysis was performed using the K-means method with the Pearson
170 correlation distance in the MeV software (<https://sourceforge.net/projects/mev-tm4/>).
171 The expression level of each gene was normalized by the average FPKM values of the
172 different achene development stages. Then, 12 clusters were generated, and the
173 heatmaps for each cluster were created using the normalized FPKM values of the
174 different achene development stages.

175 **2.5 Functional annotation**

176 Genes were functionally annotated with the NCBI non-redundant protein sequences
177 database using the BLAST software. Gene Ontology (GO) enrichment analysis was
178 performed using the GSeq R packages (Young *et al.*, 2010). GO enrichment heatmap
179 for the genes in five of the clusters (Cluster1–Cluster5) was created using the TBtools
180 software (Chen *et al.*, 2020). Kyoto Encyclopedia of Genes and Genomes (KEGG)
181 analysis was performed using the KOBAS software (Mao *et al.*, 2005).

182

183 **3. Results**

184 **3.1 Representative achene development stages of lettuce**

185 The lettuce inflorescence is a capitulum that consists of multiple ray florets, each of
186 which has five major components: petal, pappus, stamen, pistil, and ovary (Fig. 1A).
187 The ovary develops into an achene, often referred to as the seed in lettuce. We defined
188 the achene development stages according to the number of days after pollination, and
189 the representative stages are as follows: S0 stage, the day of pollination (the processes
190 of pollination and double fertilization take place, and the achene begins to develop);
191 S1 stage, 1 day after pollination (the achene elongates rapidly); S3 stage, 3 days after
192 pollination (the achene grows longer and wider); S6 stage, 6 days after pollination
193 (the achene expands and obvious longitudinal ribs appear on the achene surface); and
194 S9 stage, 9 days after pollination (the longitudinal ribs are more pronounced and the
195 achene coat becomes darker, and the achene is fully developed and ready to ripen)
196 (Fig. 1B).

197 **3.2 Global analysis of the time-course transcriptome data from different achene** 198 **samples**

199 To investigate the gene activity and analyze the molecular networks during achene
200 development in lettuce, we performed time-course transcriptome analysis of the

201 achenes from the five developmental stages, with three biological replicates for each
202 time point. A total of 730.58 million clean reads (109.59 Gb clean bases) were
203 generated from the 15 samples, with 39.31–57.45 million clean reads for each sample
204 (Table S1). Clean reads were mapped to the lettuce genome, and the unique mapped
205 reads ratios were 85.60%–93.08% (Table S1).

206 The principal component analysis (PCA) of the transcriptome datasets showed that
207 the 15 time-series samples formed four groups: group1 (S0 and S1), group2 (S3),
208 group3 (S6), group4 (S9) (Fig. 1C). The S0 and S1 stages were closer than the other
209 adjacent stages, indicating that the gene expression profiles for S0 and S1 were more
210 similar than those for the other stages. Transcript abundances were determined by
211 FPKM values (Fig. S1), and a total of 27,390 transcripts (FPKM >0.1) were found
212 among the five stages (Table S2). Among them, 18,226 genes were widely expressed
213 at the five developmental stages (Fig. 1D). A further 459, 349, 949, 302, and 313
214 genes were specially expressed at the S0, S1, S3, S6, and S9 stages, respectively (Fig.
215 1D).

216 **3.3 Gene expression during different achene development stages**

217 To investigate the gene expression patterns during achene development, we
218 performed K-means clustering analysis for the 27,390 expressed genes and generated
219 12 co-expression clusters (Fig. 2A). Genes grouped into the same cluster showed
220 similar expression patterns. The genes in five of the clusters (Cluster1–Cluster5) were
221 mainly highly expressed at only one of the five developmental stages (Fig. 2B),
222 indicating that they might have specific functions at the corresponding stages. These
223 genes were functionally annotated by GO enrichment analysis (Fig. 2C).

224 Genes in Cluster1 were highly expressed at the S0 stage, but their expression
225 decreased sharply at the later stages. Most of these genes were related to basic
226 functional processes, such as RNA binding (GO:0003723), structural constituent of
227 ribosome (GO:0003735), and translation (GO:0006412). Moreover, genes related to
228 embryo sac egg cell differentiation (GO:0009560) also were abundant in Cluster1. S0

stage is best represented by Cluster1 because pollination and fertilization take place in this stage, which also mark the beginning of achene development. After double fertilization, cell division and cell differentiation occur more frequently and biosynthesis processes proceed rapidly. Genes in Cluster2 were more highly expressed at the S1 stage than at the other stages. Some of these genes were related to cell wall, such as plant-type cell wall (GO:0009505) and pectin catabolic process (GO:0045490), indicating that they might be associated with primary cell wall formation during achene development. Genes in Cluster3 were highly expressed only at the S3 stage. Genes related to the anabolic processes of lignin and glucuronoxylan were abundant, and genes related to plant-type secondary cell wall biogenesis (GO:0009834) were enriched in Cluster3. Glucuronoxylan is the main component of hemi-cellulose, and secondary cell walls are composed of cellulose, hemi-cellulose, lignin, pectin, and some proteins (Zhong *et al.*, 2019). Because hardening of the pericarp occurs through secondary cell wall formation and lignification (Dardick and Callahan, 2014), reflecting that these enriched genes might be involved in pericarp hardening of lettuce achene. Genes in Cluster4 were more highly expressed at the S6 stage than at the other stages. These genes were related to nucleoside transmembrane transporter activity (GO:0005337), lipid metabolic process (GO:0006629), and oligopeptide transport (GO:0006857). Lipids include fatty acids and fats that are essential for energy storage and supply (Lim *et al.*, 2017). The high abundance of genes associated with lipid metabolic process indicated that biochemical reactions and pathways involving lipids were more active at the S6 stage. Genes in Cluster5 had low expression at the early stages, but their expression increased significantly at the S9 stage. Some of these genes were related to transcription from plastid promoter (GO:0042793), glyoxylate cycle (GO:0006097), and heat acclimation (GO:0010286), indicating that they may be involved in plastid transcription, energy and substance metabolism, and response to high temperatures during achene development.

The genes in Cluster6–Cluster12 were expressed at more than one of the five developmental stages, which indicated that common functional processes took place at the different developmental stages. For example, Cluster10 is best presented by

259 5050 genes, which displayed high expression levels at the S0, S1, and S3 stages.
 260 These genes were enriched in many basic functional processes (Fig. S2), including
 261 proteasome core complex assembly (GO:0080129), response to misfolded protein
 262 (GO:0051788), and intracellular protein transport (GO:0006886).

263 **3.4 Transcriptomic similarities at the different achene development stages**

264 To investigate the transcriptomic similarities at the five developmental stages, we
 265 performed KEGG analysis of the 18,226 widely expressed genes (Fig. 3A). The most
 266 significantly enriched pathways were ribosome (ko03010), mRNA surveillance
 267 pathway (ko03015), and RNA transport (ko03013), which are involved in mRNA
 268 translation to a polypeptide chain, indicating that protein biosynthesis played a
 269 dominant role during achene development. Other pathways involved in the
 270 biosynthesis and metabolism of other substances also were active during achene
 271 development. These results indicate that biosynthesis and metabolism processes are
 272 highly active to ensure the normal development of achenes.

273 The 18,266 widely expressed genes also were assigned terms under the three main
 274 GO categories (molecular function, cellular component, and biological process) (Fig.
 275 3B). Under molecular function, the most enriched GO terms were zinc ion binding
 276 (GO:0008270) and structural constituent of ribosome (GO:0003735). Under cellular
 277 component, the most enriched terms were cytosol (GO:0005829) and cytosolic small
 278 ribosomal subunit (GO:0022627). Under biological process, the most enriched terms
 279 were translation (GO:0006412) and RNA splicing, via endonucleolytic cleavage and
 280 ligation (GO:0000394). These genes, which were expressed at all five developmental
 281 stages, were enriched in many basic functional processes, and the results of the GO
 282 and KEGG analyses were generally consistent.

283 **3.5 Transcriptomic differentiation at the different achene development stages**

284 We analyzed the transcriptome profiling data to identify the specially expressed
 285 genes at particular stages of achene development. Then, we performed KEGG

analysis of these genes in each developmental stage. At the S0 stage, the most enriched pathways were plant-pathogen interaction (ko04626) and galactose metabolism (ko00052) (Fig. 4A), which are related to environmental adaptation and carbohydrate metabolism. At the S1 stage, the most enriched pathways were phenylpropanoid biosynthesis (ko00940) and carbon fixation in photosynthetic organisms (ko00710) (Fig. 4B). Phenylpropanoids are plant secondary metabolites that function as structural and signaling molecules (Liu *et al.*, 2015). Carbon fixation is essential for energy metabolism during achene development. Brassinosteroid (BR) biosynthesis (ko00905) also was active at the S1 stage (Fig. 4B), indicating that BR is an important regulator during achene development in lettuce. Genes related to flavonoid biosynthesis (ko00941) were more abundant at the S3 stage (Fig. 4C). Flavonoids are major secondary metabolites that are related to fruit flavor and color, and provide resistance against diseases and pests (Xu *et al.*, 2015). At the S6 stage, biosynthesis of unsaturated fatty acids (ko01040) and fatty acid metabolism (ko01212) were the most enriched pathways (Fig. 4D). Fatty acids have multitude functions including energy storage and heat supply (Lim *et al.*, 2017), which indicated that the rapid expansion of achene at this stage was accompanied with the biosynthesis and metabolism of fatty acids. Anthocyanins are common plant pigments (Jaakola, 2013), so the active anthocyanin biosynthesis (ko00942) process at the S9 stage was consistent with the color change of achene coat at this stage (Fig. 4E).

3.6 Identification of transcription factors during different achene development stages

Transcription factors (TFs) are important regulators of transcription and gene expression, and TFs play dominant roles in regulating the growth, development, and stress tolerance of plants (Riechmann *et al.*, 2000; Samad *et al.*, 2017). Members of many TF families have been shown to play a wide variety of roles in controlling fruit and seed development (Sun *et al.*, 2010). In this study, we identified 1579 genes that encode TFs from the transcriptome data (Table S3). Most of these TFs were expressed

at all five achene development stages, but were strongly expressed at the early stages, especially at the S1 stage, although a small number of genes were highly expressed at the later stages (Fig. 5A). The top 100 highly expressed TFs at each developmental stage were classified and counted. The significantly enriched TFs mainly belonged to the MYB, APETALA2/ETHYLENE RESPONSIVE FACTOR (AP2/ERF), Homeobox (HB), MADS-box, basic leucine zipper (bZIP), and NAM/ATAF/CUC (NAC) families (Fig. 5B).

MYB TFs play prominent roles in controlling development, signal transduction pathways, the biosynthesis of secondary metabolites, and response to biotic and abiotic stresses (Dubos *et al.*, 2010). We found that the MYB TFs accounted for 8%–10% of the top 100 TFs at all five developmental stages, indicating that MYB TFs functioned throughout the achene development process. The AP2/ERF TFs constitute one of the largest TF families in plants, and orchestrate the developmental processes and stress responses (Licausi *et al.*, 2013; Xie *et al.*, 2019). Here, we found that the AP2/ERF TFs accounted for about 9% of the top 100 TFs at the early stages, and 15% and 17% at the S6 and S9 stages respectively, reflecting the important role of AP2/ERF TFs in achene development. HB TFs, which belong to several subfamilies, regulate a large number of developmental processes in plants, including embryonic development, meristem maintenance, and organ formation (Bürglin and Affolter, 2015). Members of MADS-box TF family participate in numerous developmental process in plants, and their role in floral organ identity determination is conserved among divergent plant species. MADS-box TFs also play important roles in inflorescence architecture and fruit and seed development (Ng and Yanofsky, 2001; Smaczniak *et al.*, 2012). In this study, we found that there were more HB and MADS-box TFs highly expressed at the early stages than at the later stages, suggesting that they played crucial roles at the early stages of achene development. The bZIP TFs are involved in diverse biological processes, including environmental signaling, seed maturation, flower development, and stress response (Jakoby *et al.*, 2002; Droge-Laser *et al.*, 2018). Here, the bZIP TFs accounted for 6%–8% of the top 100 TFs at the different developmental stages. Previous studies have revealed that

344 NAC TFs function in developmental programs, response to environmental signals,
345 and defense (Olsen *et al.*, 2005; Puranik *et al.*, 2012; Kim *et al.*, 2016). Here, we
346 found that the NAC TFs were expressed throughout the five developmental stages but
347 had high proportions at the S3 and S6 stages, indicating that NAC TFs might have
348 important functions at these stages. Further, the C2H2 and Trihelix TFs accounted for
349 7% and 6% only at the S6 and S9 stages, respectively.

350 **3.7 Expression pattern of key genes associated with fruit and seed development** 351 **across the achene development stages**

352 The lettuce achene is developed from the ovary wall and ovule, thus the achene
353 development combines both fruit development and seed development. Here, we first
354 identified genes associated with fruit development across the development stages (Fig.
355 6A). Notably, the majority of these regulators are TFs. Most of the genes involved in
356 fruit development were highly expressed at the early stages, although some genes
357 were expressed at low levels at all five stages. We found that the lettuce homologs of
358 *AGAMOUS* (*AG*) and *SEEDSTICK* (*STK*), which belong to the MADS-box TF family
359 (Favaro *et al.*, 2003), were up-regulated at the S0, S1 and S3 stages, indicating that
360 they might be important for carpel development in lettuce. The
361 *APETALA1/FRUITFULL* (*API/FUL*) clade of lettuce had multiple gene duplications,
362 implying that these genes may display functional redundancy in valve development.
363 In *Arabidopsis*, *SPATULA* (*SPT*) plays a minor role in promoting separation layer
364 formation of the fruit (Heisler *et al.*, 2001; Groszmann *et al.*, 2011). We identified a
365 lettuce homolog of *SPT* that showed increased expression at the S3 stage then
366 decreased expression at the later stages. Furthermore, the expression levels of *BP* and
367 *ASI* were higher than those of other genes associated with replum development,
368 indicating that *BP* and *ASI* may contribute most to replum formation. Members of the
369 KANADI (*KAN*) and YABBY (*YAB*) TF families that play central roles in lateral
370 organ development also are crucial for ovule development in *Arabidopsis* (Kelley and
371 Gasser, 2009). Several lettuce homologs of *KANs* and *YABs* were identified, and a

372 *YAB1* gene (*Lsat_1_v5_gn_7_9041*) that was highly expressed across the five
373 developmental stages might play a major role in ovule development.

374 Seed development is a complex process, and many pathways involved in seed
375 development have been proposed (Li *et al.*, 2019). In this study, we identified genes
376 associated with seed development across the five developmental stages (Fig. 6B).
377 Genes related to the ubiquitin-proteasome pathway, G-protein signaling pathway, and
378 the mitogen-activated protein kinase signaling pathway were stably expressed with
379 normal expression levels at all stages. The genes related to the ubiquitin-proteasome
380 pathway had duplicate copies, which may have resulted in functional redundancy
381 among them. Genes related to phytohormone signaling and homeostasis and
382 transcriptional regulators were the most highly expressed genes at the early stages,
383 and the expression of most of them gradually decreased at the later stages. Previous
384 studies have shown that plant hormones regulate seed growth (Sun *et al.*, 2010), and
385 the BR receptor *BRI1* and the BR response TF *BZR1*, which are involved in BR
386 signal transduction, were shown to control seed development (He *et al.*, 2005; Kim *et al.*,
387 2009; Huang *et al.*, 2013). Here, we found that the lettuce homologs of *BRI1* and
388 *BZR1* were highly expressed at the S0, S1, and S3 stages, and their expression
389 decreased at the later stages. Besides, a homolog of *DE-ETIOLATED2* (*DET2*), which
390 is involved in BR biosynthesis (Fujioka *et al.*, 1997; Jiang *et al.*, 2013), was more
391 abundant at the S3 stage. These results revealed the importance of BR in regulating
392 achene development in lettuce. The expression patterns of lettuce AP2 TFs were
393 similar to those of the lettuce *BZR1* TFs. Homologs of *ANT* and *TTG2* displayed
394 higher expression at the S3 stage than at the other stages, indicating that *ANT* and
395 *TTG2* may have important functions at the S3 stage. Further, genes that influence
396 endosperm growth were highly expressed at the later stages of achene development,
397 especially the abscisic acid (ABA) response TF *ABA-INSENSITIVE5* (*ABI5*), which
398 was highly expressed at the S6 and S9 stages. ABA activates seed dormancy and
399 suppresses germination (Cutler *et al.*, 2010), which indicated that the lettuce achenes
400 were about to ripen and become dormant at the later stages.

401 **4. Discussion**

402 The lettuce seed is an achene fruit, and the development of a lettuce achene from
 403 the small ovary to its final size must involve extensive biological processes. In this
 404 study, we performed a time-course transcriptome analysis of lettuce achenes and
 405 revealed a possible mechanism that influenced achene development. To illustrate this
 406 idea, we proposed a working model of lettuce achene development (Fig. 7).

407 Overall, genes related to basic functional categories were abundant at almost every
 408 achene development stage, indicating that the basic biosynthesis and metabolism
 409 processes proceeded throughout the achene development, especially pathways
 410 associated with protein biosynthesis. These continuous and stable biological processes
 411 likely ensure the normal development of the achenes. We also found that genes that
 412 encode TFs and genes associated with phytohormone signaling and homeostasis play
 413 crucial roles during achene development. Most of these genes were highly expressed
 414 at the early stages of achene development, and their expression gradually decreased
 415 with the development of the achene.

416 The K-means clustering analysis identified the highly expressed genes at each
 417 developmental stage. Differential expression analysis identified genes that were
 418 particularly expressed at each stage. We also found that many biological processes,
 419 especially synthetic and metabolic activities, were significantly different at different
 420 developmental stages. At the S0 stage, the enriched pathways were RNA binding and
 421 translation, and genes related to galactose metabolism also were abundant in this stage,
 422 which reflected the rapid growth of achene after pollination and fertilization. At the
 423 S1 stage, genes related to cell wall and phenylpropanoid biosynthesis were enriched.
 424 The BR biosynthesis pathway also was active at the S1 stage, indicating the important
 425 role of BR during achene development. At the S3 stage, genes associated with the
 426 components and biogenesis process of the secondary cell wall were highly expressed,
 427 indicating that these genes may be involved in pericarp hardening of lettuce achene.
 428 The flavonoid biosynthesis pathway also was enriched at the S3 stage. Flavonoids are
 429 involved in multiple physiological functions, and many types of flavonoids in seeds

are found such as flavonols and anthocyanins (Lepiniec *et al.*, 2006), reflecting the important roles of flavonoids during achene development. At the S6 stage, the anabolic processes of lipids occurred more frequently, including fatty acid biosynthesis and metabolism. Lipids are essential for energy storage and supply (Lim *et al.*, 2017), indicating that the rapid expansion of achene was accompanied by the accumulation of energy substances at the S6 stage. At the S9 stage, the enriched pathways included plastid transcription and heat acclimation, and genes related to anthocyanin biosynthesis also were abundant. Anthocyanins are common plant pigments and temperatures affect the stability of anthocyanins (Marszałek *et al.*, 2017), so the high expression of these genes is consistent with the dark achene coat that develops at this stage.

Direct orthologs of *IND*, *ALC*, *SHPI*, and *SHP2* have not been detected in lettuce, which coincide with the report that these genes may be found only in Brassicaceae (Pabon-Mora *et al.*, 2014). The fruit structure of lettuce is different from that of *Arabidopsis*; the achenes of lettuce are indehiscent fruits, whereas the siliques of *Arabidopsis* are dehiscent fruits. The four genes, *IND*, *ALC*, *SHPI*, and *SHP2*, are required for the valve margin formation and dehiscence, but the achenes do not undergo dehiscence when the fruits are fully mature. The structural differences between the fruits of lettuce and *Arabidopsis* might explain the absence of genes associated with fruit valve margin formation in lettuce.

Taken together, our data provide a foundation for future functional studies. Many genes that were highly expressed in the achenes, such as *AG*, *STK*, *BZRI*, *AP2*, and *ABI5*, may be responsible for important traits in lettuce, such as achene size, achene shape, grain weight, seed shattering, seed dispersal, and oil concentration. The biological functions of these genes need to be further confirmed in lettuce. The lettuce genome has been sequenced and CRISPR/Cas9-mediated genome editing has been applied to lettuce (Woo *et al.*, 2015; Reyes-Chin-Wo *et al.*, 2017; Zhang *et al.*, 2018), so making good use of the genomics and biotechnologies will be helpful to reveal novel genetic and epigenetic variations in lettuce that can be applied to breed improved lettuce varieties in the future.

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465 **Supplemental Materials**

466 Fig. S1. Boxplot for the gene expression level distribution.

467 Fig. S2. GO enrichment analysis of the genes in Cluster10.

468 Table S1. Summary of the sequencing data.

469 Table S2. List of genes analyzed in this study.

470 Table S3. Expression patterns of identified transcription factors.

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656 **Figure legends**

657 **Fig. 1. Morphological characterization of the lettuce achenes and global analysis**
 658 **of the transcriptome data.** (A) Morphological characterization of the lettuce
 659 capitulum and floret. (B) Morphology of achenes at different developmental stages.
 660 (C) PCA of gene expression in different achene samples. (D) Venn diagram for gene
 661 expression analysis in different achene samples. Scale bars, 1 mm.

662
 663 **Fig. 2. Gene expression patterns and functional transition at different achene**
 664 **development stages.** (A) Twelve clusters for the gene expression patterns based on
 665 the normalized FPKM values. (B) Heatmaps for the gene expression patterns in
 666 different co-expression clusters. (C) GO functional enrichment analysis of the genes
 667 in different co-expression clusters.

668
 669 **Fig. 3. KEGG and GO analysis of the widely expressed genes at different achene**
 670 **development stages.** (A) KEGG enrichment analysis of the widely expressed genes.
 671 (B) Significantly enriched GO terms of the widely expressed genes.

672
 673 **Fig. 4. Analysis of the specially expressed genes at different achene development**
 674 **stages.** (A–E) KEGG enrichment analysis of the stage-specific genes at the S0 (A), S1
 675 (B), S3 (C), S6 (D), and S9 (E) stages.

676
 677 **Fig. 5. Identification of transcription factors at different achene development**
 678 **stages.** (A) Heatmap expression profile of the identified TFs. (B) Pie charts of the top
 679 100 highly expressed TFs at each developmental stage.

680
 681 **Fig. 6. Expression patterns of key genes associated with fruit and seed**
 682 **development at different achene development stages.** (A) Heatmap expression
 683 profile of the fruit development related genes. (B) Heatmap expression profile of the
 684 seed development related genes.

685

686 **Fig. 7. Proposed working model of achene development in lettuce.** The gene
 687 expression modules and possible molecular mechanism underlying achene
 688 development in lettuce are illustrated. Biological processes and components above the
 689 lines indicate that the related genes are expressed during the corresponding achene
 690 development stages.

A

B

Protein biosynthesis

Transcription factors

Phytohormone signaling and homeostasis

RNA binding

Translation

Galactose metabolism

Secondary cell wall biogenesis

Flavonoid biosynthesis

Plastid transcription

Heat acclimation

Anthocyanin biosynthesis

Cell wall

Phenylpropanoid biosynthesis

Brassinosteroid biosynthesis

Lipid biosynthesis and metabolism

S0

S1

S3

S6

S9