1   RESEARCH ARTICLE

2   ABIOTIC STRESS GENE 1 mediates aroma volatiles
3   accumulation by activating MdLOX1a in apple

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17   **One-sentence Summary:** MdASG1 directly activates *MdLOX1a* expression to

18   promote aroma volatiles accumulation especially under moderate salt stress.

19   **ABSTRACT**

20   Fruit aroma is an important organoleptic quality, which influences consumer preference

21   and market competitiveness. Aroma compound synthesis pathways in plants have been

22   widely identified of which the lipoxygenase pathway is crucial for fatty acid catabolism

23   to form esters in apple. However, the regulatory mechanism of this pathway remains

24   elusive. In this study, linear regression analysis and transgene verification revealed that

25   the lipoxygenase MdLOX1a participates in ester biosynthesis. Yeast one-hybrid library

26   screening indicated that a novel abiotic stress gene, *MdASG1* (*ABIOTIC STRESS GENE*

27   *1*), was a positive regulator of the *MdLOX1a* promoter and ester production based on

28   yeast one-hybrid and dual-luciferase assays, and correlation analysis among eight apple

29   cultivars. Overexpression of *MdASG1* in apple and tomato stimulated the lipoxygenase

30   pathway and increased the fatty acid-derived volatile content, whereas the latter was

31   decreased by *MdASG1* silencing. Furthermore, *MdASG1* overexpression enhanced the

32   salt-stress tolerance of tomato and apple 'Orin' calli accompanied by a higher content

33   of fatty acid-derived volatiles compared with that of non-stressed transgenic tomato

34   fruit. Collectively, these findings indicate that *MdASG1* activates *MdLOX1a* expression

35   and participates in the lipoxygenase pathway, subsequently increasing the accumulation

36   of aroma compounds especially under moderate salt stress treatment. The results also

37   provide insight into the regulation of aroma production, and the potential strategy of

38   prudent development and utilization of saline-alkali land to produce high-quality fruit,

39   thereby reducing pressure on arable land and ensuring national food security.

40   **Key words:** Apple, Lipoxygenase, MdLOX1a, ABIOTIC STRESS GENE 1, Aroma,

41   Ester, Volatiles, Salt stress

42   **INTRODUCTION**

43   Plant volatile organic compounds are secondary metabolites that play important roles

44   in biotic and abiotic stress responses, and act as signals to attract or repel pests, confer

45   resistance to pathogens, and participate in seed dispersal (Rodriguez et al., 2013). Many

46   volatiles are produced by plants at different developmental stages, especially during

47   fruit ripening. A large number of volatile esters are produced by strawberry (*Fragaria*

48   *vesca*), banana (*Musa sapientum*), apple (*Malus domestica*), and peach (*Prunus persica*)

49   (Beekwilder et al., 2004; Souleyre et al., 2014; Cao et al., 2021). Fruit quality mainly

50   reflects fruit shape, size, color, aroma, acidity, sugar content, and nutritional content.

51   Among these traits, aroma is an important determinant of the commercial value of fruit.

52   However, breeders tend to focus on yield, disease resistance, and fruit color, and pay

53 little attention to flavor and thereby weaken customer motivation to buy apple fruit

54 (Klee and Tieman, 2018). Therefore, improvement in fruit flavor is desirable to meet

55 consumer demand.

56   The aroma compound synthesis pathway has been extensively studied in plants. Fruit

57 esters are produced mainly from the fatty acid pathway contributing to straight-chain

58 ester synthesis and the amino acid pathway contributing to branched-chain ester

59 formation. In tomato (*Solanum lycopersicum*), a number of fatty acid-derived chemicals,

60 including C5 or C6 aldehydes and alcohols, are formed in the lipoxygenase pathway

61 (Stone et al., 2010). In apple, the β-oxidase and lipoxygenase pathways are the two

62 main enzyme systems involved in fatty acid catabolism to form esters (Rowan et al.,

63 1999). In the lipoxygenase pathway, lipoxygenases (LOX) catalyze polyunsaturated

64 fatty acids, including linolenic and linoleic acid, to produce hydroperoxides (Feussner

65 and Wasternack, 2002). The hydroperoxides are then converted to short-chain

66 aldehydes and an oxo-acid by hydroperoxide lyase, which belongs to the cytochrome

67 P450 superfamily (Matsui, 1998; Schwab et al., 2008). The short-chain aldehydes are

68 further reduced to corresponding alcohols by alcohol dehydrogenase during fruit

69 ripening (Manriquez et al., 2006; Schwab et al., 2008). Lastly, alcohol acyl-transferases

70 (AATs) catalyze the acid donor, acyl-coenzyme A (acy-CoA), and alcohol acceptor to

71 synthesize esters (Dunemann et al., 2012). Lipoxygenases are a non-heme iron-

72 containing dioxygenase, which are classified as either 9-LOX or 13-LOX according to

73 the position of the carbon targeted for oxygenation in the polyunsaturated fatty acid

74 (Feussner et al., 2001). Lipoxygenases are also classified as type 1 or type 2 LOXs

75 according to the sequence similarity. Lipoxygenases of tomato, pepino (*Solanum*

76 *muricatum*), and kiwifruit (*Actinidia deliciosa*) are involved in aroma compound

77   synthesis (Chen et al., 2004; Zhang et al., 2006; Zhang et al., 2009; Contreras et al.,

78   2017).

79   Certain other factors affect the accumulation of fruit flavor compounds, including

80   genetic differences (Kakiuchi et al., 2007), crop management (Mpelasoka and

81   Behboudian, 2002), harvest date (Song and Bangerth, 1996), storage environment

82   (Harb et al., 2012), and the plant hormones ethylene, abscisic acid (ABA), and jasmonic

83   acid (JA) (Yang et al., 2016; Wu et al., 2018; Luo et al., 2021). Recently, transcriptional

84   regulation of specific genes involved in aroma synthesis has been investigated. The

85   ETHYLENE-INSENSITIVE3-LIKE (EIL) and NAC transcription factors activate

86   terpene synthase gene *AaTPS1* transcription to control monoterpene production in

87   kiwifruit (*Actinidia arguta*) (Nieuwenhuizen et al., 2015). NAC transcription factors

88   modulate ester biosynthesis by regulating expression of the structural genes *FAD1* and

89   *AAT10* in kiwifruit (Zhang et al., 2020; Wang et al., 2022) and activate *AAT* expression

90   in tomato, peach, and apple (Cao et al., 2021). The AP2/ERF transcription factors

91   EREB58, CitAP2.10, and CitERF71 may *trans*-activate the terpene synthase *TPS* to

92   promote the synthesis of terpenes (Li et al., 2015; Shen et al., 2016; Li et al., 2017).

93   Strawberry ethylene response factors FaERF9 and FaMYB98 form a protein complex,

94   which indirectly activates strawberry quinone oxidoreductase (FaQR) expression,

95   thereby promoting the synthesis of furanone (Zhang et al., 2018). The R2R3 MYB

96   transcription factors FaEOBII and FaDOF2 synergistically regulate the volatile

97   phenylpropanoid pathway in strawberry (Medina-Puche et al., 2015; Molina-Hidalgo

98   et al., 2017). In tomato, the MADS box transcription factor RIN and SlMYB75 directly

99   bind to the promoter of genes for aroma compound synthesis pathway-related enzymes

100   to activate their expression (Qin et al., 2012; Jian et al., 2019). In addition, other

101   transcription factors, including a basic helix-loop-helix transcription factor (MYC2),

102   *PRODUCTION OF ANTHOCYANIN PIGMENT 1* (*PAP1*), and basic leucine zipper

103   (bZIP), mediate aroma compound biosynthesis (Hong et al., 2012; Zvi et al., 2012; Guo

104   et al., 2018).

105   *ABIOTIC STRESS GENE 1* (*ASG1*) is an abiotic stress gene identified in *Solanum*

106   *tuberosum* and *Arabidopsis thaliana* inducible by stress via an ABA-dependent

107   pathway (Batelli et al., 2012). However, little information is available on whether *ASG1*

108   mediates other biological activities, including aroma regulation. Stress can induce the

109   production of secondary metabolites to improve fruit quality. Treatment with ABA

110   reduces tannin content and positively affects grape (*Vitis vinifera*) fruit quality

111   (Lacampagne et al., 2009). Abscisic acid drives the accumulation of secondary

112   metabolites contributing to fruit aroma in grape and strawberry (Ferrandino and

113   Lovisolo, 2014; Kadomura-Ishikawa et al., 2015). MdAREB2 is responsive to ABA

114   and promotes soluble sugar accumulation by activating the expression of amylase and

115   sugar transporter genes (Ma et al., 2017). Soil water stress can improve fruit quality by

116   increasing the content of soluble sugar in kiwifruit and apple fruit (Miller et al., 1998;

117   Wang et al., 2019). Drought treatment induces accumulation of flavonoids and

118   anthocyanins in apple (Wang et al., 2020). Recently, transcriptome analysis of apricot

119   fruit revealed that MYC and bHLH transcription factors may respond to stress and play

120   a crucial role in flavor formation (Zhang et al., 2019). However, the regulatory

121   mechanism of stress-mediated aroma accumulation remains unclear.

122   Apple (*Malus domestica*) is an economically important tree cultivated worldwide

123   (Duan et al., 2017; Cornille et al., 2019). Ripening apple fruit produce approximately

124   350 volatile compounds, including aldehydes, alcohols, esters, ketones, and terpenes

125   (Dimick and Hoskin, 1983; Song, 2007). Twenty types of volatile compounds are

126   characteristic of the apple fruit aroma, which include *trans*-2-hexenal, hexanol, butyl

127   acetate, hexyl acetate, and 2-methyl butyl acetate (Dixon and Hewett, 2000). With

128   ripening of the fruit, the abundance of esters increases significantly (Rowan et al., 1999;

129   Echeverr´a et al., 2004). In 'Golden Delicious' apple, esters account for 80% of the

130   total volatile aroma components (López et al., 2010). In 'Golden Delicious', 23

131   functional LOXs have been identified of which MdLOX1a and MdLOX5e might be

132   involved in volatile component production (Vogt et al., 2013). *LOX* genes play a crucial

133   role in the lipoxygenase pathway. However, little information is available on the

134   regulation of LOXs in apple.

135   In this study, we selected a ripening-related gene, *MdLOX1a*, to investigate ester

136   biosynthesis based on the results of a correlation analysis and overexpression of

137   *MdLOX1a* in apple calli. A novel abiotic stress gene, *MdASG1*, was identified by yeast

138   one-hybrid library screening. *MdASG1* responded to salt stress, directly bound to the

139   promoter of *MdLOX1a* and activated its transcript, and, subsequently, enhanced the

140   synthesis of aroma compounds. Overexpression of *MdASG1* in tomato fruit increased

141   the production of volatile aroma compounds under salt stress. Overall, the findings

142   provide new insights into the regulation of aroma compound production and a potential

143   strategy to develop and utilize saline-alkali land to produce high-quality fruit, thereby

144   reducing pressure on arable land and ensuring national food security.

145   **RESULTS**

146   ***MdLOX1a* is involved in ester formation in apple and phylogenetic analysis of**

147   **LOXs**

148   We sampled apple fruit at four developmental and ripening stages (Fig. 1A) for gas

149   chromatography–mass spectrometry (GC-MS) analysis. With progression of ripening,

150   large amounts of esters were produced. In ripe fruit, the ester content attained about 14

151 $\mu g \cdot g^{-1}$ fresh weight, which was almost seven times that of immature fruit at 57 days

152 after full bloom (DAFB) (Fig. 1B). The lipoxygenase pathway is one pathway for the

153 synthesis of esters and the crucial participating enzyme is lipoxygenase. In apple, eight

154 groups of LOXs are involved in the lipoxygenase pathway (Vogt et al., 2013). To clarify

155 the key lipoxygenase genes in fruit ripening, we analyzed eight lipoxygenase genes

156 from each group by quantitative real-time PCR analysis during fruit development and

157 ripening (Fig. 1C, Supplemental Fig. S1). As the fruit matured, the transcript level of

158 *MdLOX1a* increased significantly, consistent with the rate of ethylene release; in

159 particular, *MdLOX1a* transcript abundance increased about 122-fold at the ripening

160 stage compared with that of immature fruit (57 DAFB) (Fig. 1, C and D). A significant

161 positive correlation was observed between the expression profile of *MdLOX1a* and ester

162 content during apple fruit development and ripening ($r = 0.989$, $P < 0.05$) (Fig. 1E,

163 Supplemental Fig. S2), which indicated that *MdLOX1a* may be a maturity-related gene.

164 To further analyze the relationship between *MdLOX1a* and ester synthesis, we

165 quantified *MdLOX1a* transcript levels (Fig. 1F), lipoxygenase activity (Fig. 1G), and

166 ester content (Fig. 1H) in ripe fruit of eight popular apple cultivars (Supplemental Fig.

167 S3). The transcript levels of *MdLOX1a* were positively correlated with lipoxygenase

168 activity ($r = 0.9464$, $P < 0.01$; Fig. 1I). In addition, *MdLOX1a* transcript levels were

169 positively correlated with ester content ($r = 0.7408$, $P < 0.05$) (Fig. 1J). These results

170 indicated that *MdLOX1a* may be a critical gene involved in volatile ester biosynthesis.

171 Functional LOXs have been identified in many plant species, including common

172 bean (*Phaseolus vulgaris*), tomato, kiwifruit, Arabidopsis, grape, rice (*Oryza sativa*),

173 persimmon (*Diospyros kaki*), and oriental melon (*Cucumis melo*) (Porta et al., 1999;

174 Chen et al., 2004; Zhang et al., 2006; Bannenberg et al., 2009; Podolyan et al., 2010;

175 Umate, 2011; Hou et al., 2015; Xing et al., 2020). Furthermore, 23 functional LOXs in
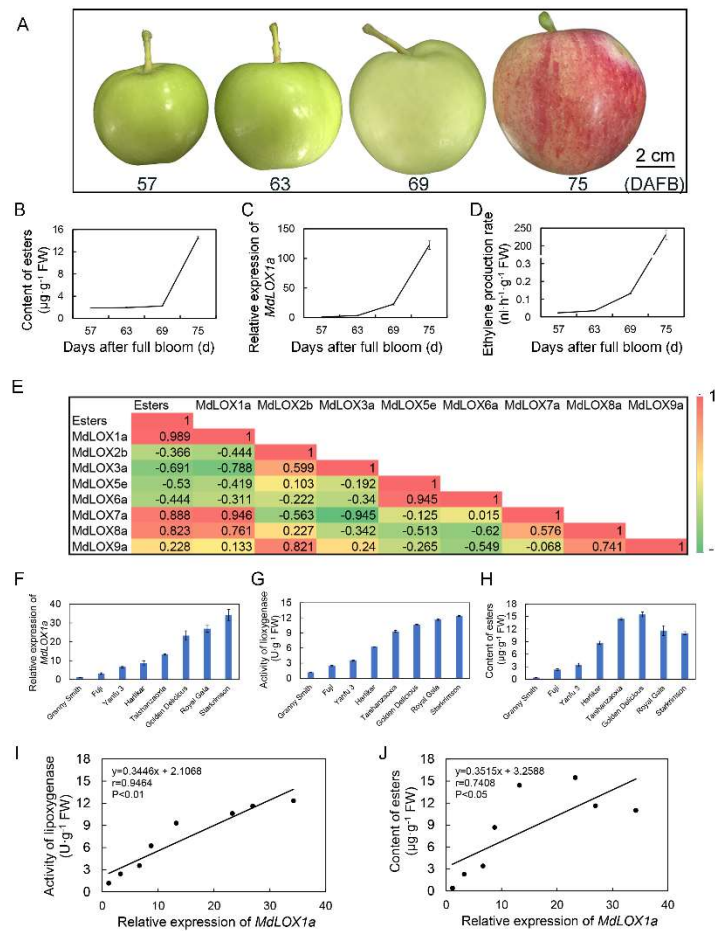
Figure 1. *MdLOX1a* is involved in ester formation in apple. A, Apple 'Taishanzaoxia' fruit were harvested at 57, 63, 69, and 75 days after full bloom (DAFB). Bar = 2 cm. B, Ester content during apple fruit development and ripening. C, Transcript level of *MdLOX1a* during apple fruit development and ripening quantified by qRT-PCR. *MdActin* was used as an internal control gene. D, Ethylene release rate during apple fruit development and ripening. E, Correlation analysis of *MdLOX* expression and ester content in apple fruit at the ripening stage. F, Relative expression of *MdLOX1a* in fruit of eight apple cultivars at the ripening stage. *MdActin* was used as an internal control gene. G, Lipoxygenase activity in fruit of eight apple cultivars at the ripening stage. H, Ester content in fruit of eight apple cultivars at the ripening stage. I, Linear regression analysis between *MdLOX1a* expression and lipoxygenase activity in fruit of eight apple cultivars. J, Linear regression analysis between *MdLOX1a* expression and ester content in fruit of eight apple cultivars. Error bars represent the standard deviation of three independent biological replicates. FW, Fresh weight. Significant differences were determined using Tukey one-way analysis of variance (ANOVA) with SPSS Statistics 22 (*$P < 0.05$ and **$P < 0.01$).

176

177 the lipoxygenase pathway have been identified in the genome of 'Golden Delicious'

178 apple (Vogt et al., 2013). In the present study, the amino acid sequence of 58 LOXs

179 from 14 plant species was analyzed. The LOXs were mainly divided into two groups

180 comprising 9-LOXs and 13-LOXs, respectively. *MdLOX1a* and *MdLXO7a*, which

181 showed similar expression trends during apple fruit development and ripening, were

182   grouped with 9-LOXs (Supplemental Fig. S4), which are classified as type 1 LOXs

183   (Vogt et al., 2013).
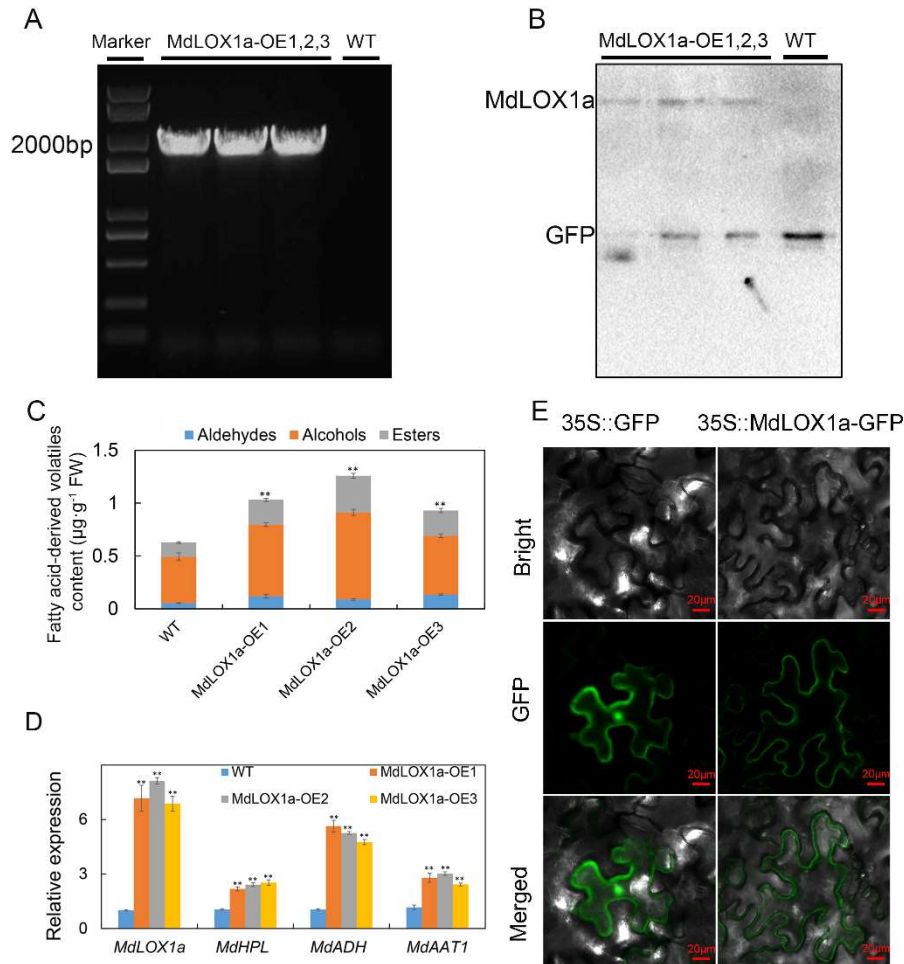
Figure 2. Overexpression of *MdLOX1a* increased fatty acid-derived volatile content in apple calli and subcellar localization of MdLOX1a. A and B, *MdLOX-1a* overexpression in 'Orin' calli verified by PCR amplification (A) and western blotting (B). The 35S and MdLOX1a-PRI101-R primers were used for verification of transformants. C, Fatty acid-derived volatile contents in wild-type apple calli (WT) and *MdLOX1a*-overexpression apple calli (MdLOX1a-OE). D, Relative expression of fatty acid-derived volatile biosynthesis genes in WT and Md-LOX1a-OE transgenic apple calli. *MdActin* was used as an internal control gene. E, Subcellular localization of MdLOX1a in tobacco leaves. MdLOX1a was mainly expressed in the cytoplasm of tobacco leaves. Bars = 20 μm. Error bars represent the standard deviation of three independent biological replicates. Asterisks indicate statistical significance (**P < 0.01 and *P < 0.05).

184

185 **Overexpression of *MdLOX1a* increases fatty acid-derived volatile content in apple**

186 **calli and subcellar localization of MdLOX1a**

187   To analyze the function of *MdLOX1a* in volatile aroma biosynthesis, we generated

188 *MdLOX1a*-overexpressing transgenic 'Orin' calli (Fig. 2, A and B). The content of fatty

189 acid-derived volatiles, including esters, was significantly increased compared with that

190 of the wild type (WT) (Fig. 2C). In addition, the corresponding synthetic genes in the

191 lipoxygenase pathway were up-regulated (Fig. 2D). Taken together, these results

192 indicated that *MdLOX1a* was associated with ester content. The construct

193 35S::MdLOX1a-GFP was generated to determine the subcellular localization of

194 MdLOX1a. Strong green fluorescence signal was detected in the cytoplasm of tobacco

195 (*Nicotiana benthamiana*) leaves (Fig. 2E), consistent with the subcellular localization

196 predicted using Cell-PLoc 2.0.

197 **A novel gene, *MdASG1*, is a direct regulator of the *MdLOX1a* promoter and**

198 **activates its expression**

199 Given that *MdLOX1a* is a crucial gene in volatile ester biosynthesis, we used the

200 *MdLOX1a* promoter as bait to conduct yeast one-hybrid library screening. We

201 identified a novel gene, designated *MdASG1* (accession number: XM_029093686), that

202 was capable of binding to the promoter of *MdLOX1a* in the presence of 400 ng·mL^{-1}

203 aureobasidin A (AbA) (Fig. 3A, Supplemental Fig. S5). The amino acid sequence of

204 MdASG1 showed 70% and 73% similarity with Arabidopsis AtASG1 and potato

205 (*Solanum commersonii*) ScASG1, respectively (Supplemental Fig. S6). The latter two

206 genes both respond to stress treatment (Batelli et al., 2012). To determine the specific

207 binding site of *MdASG1*, the promoter of *MdLOX1a* was divided into four fragments.

208 Yeast one-hybrid assays showed that *MdASG1* bound to the *p4MdLOX1a* fragment (Fig.

209   3B).
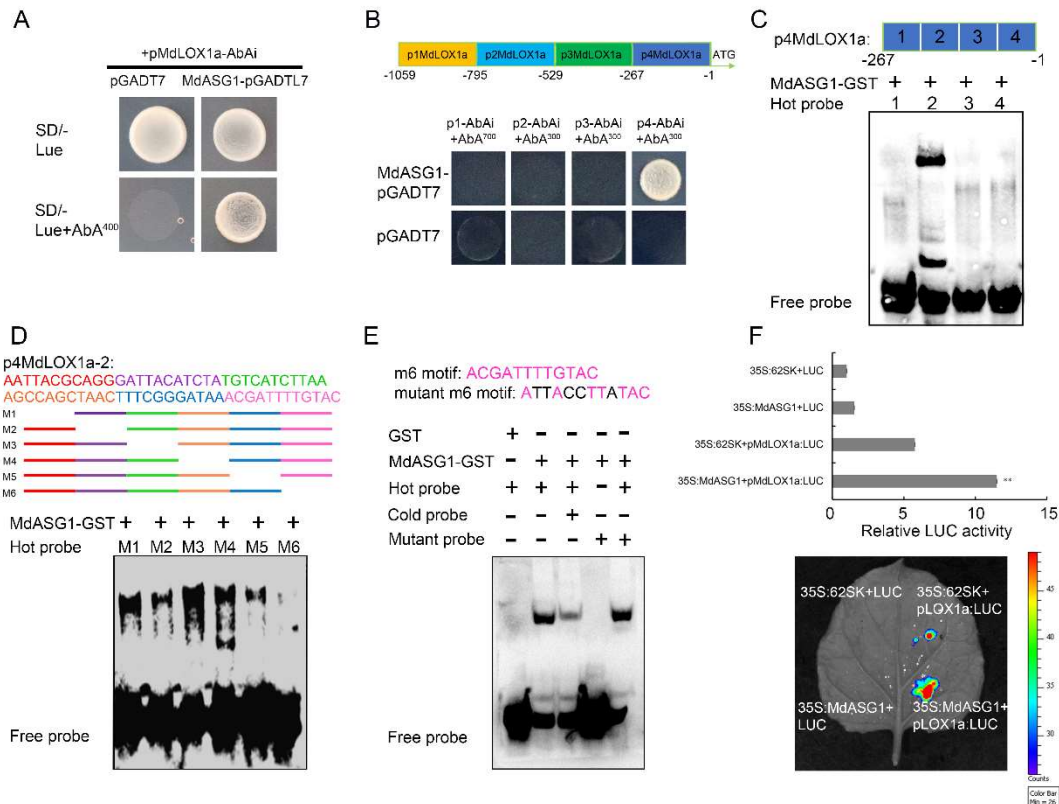
Figure 3. MdASG1 binds to the promoter of *MdLOX1a* and activates its expression. A, Yeast one-hybrid assays showing binding between MdASG1 and the promoter of *MdLOX1a*. B, Yeast one-hybrid assays showing binding between MdASG1 and the fourth segment of the promoter of *MdLOX1a* (*p4MdLOX1a*). C, Four segments of *p4MdLOX1a*. Electrophoretic mobility shift assay (EMSA) showing binding of MdASG1 to the -795~-529bp segment of *p4MdLOX1a* (*p4MdLOX1a-2*). D, Design of biotin-labeled probes (M1–M6) for partial deletion of the fragment *p4MdLOX1a-2*. The M6 fragment showed no binding with MdASG1. E, EMSA showing the binding of MdASG1 to the m6 motif in *MdLOX1a*. The hot probe was a biotin-labeled fragment. The cold probe was a nonlabeled fragment. The mutant probe contained five nucleotide mutations. The symbol + or − indicates the presence or absence of specific probes. F, Dual-luciferase assay verifying that MdASG1 transformation activated the *MdLOX1a* promoter. Error bars represent the standard deviation of three independent biological replicates. Asterisks indicate statistical significance (**P < 0.01 and *P < 0.05).

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211   An electrophoretic mobility shift assay (EMSA) demonstrated that only -795~-529bp

212   of *p4MdLOX1a* (*p4MdLOX1a-2*) contained a binding site (Fig. 3C). Partial deletion of

213   the fragment *p4MdLOX1a-2* was performed to generate six individual fragments.

214   Interestingly, binding was not observed in the absence of the m6 region (Fig. 3D).

215   Therefore, we concluded that the specific binding motif of *MdASG1* was located in the

216   m6 region. Addition of a cold probe weakened the binding. When the binding sites were

217   mutated, binding was eliminated (Fig. 3E). A dual-luciferase reporter assay, performed

218 to clarify the transcriptional regulation of *MdASG1* on *MdLOX1a*, indicated that

219 MdASG1 targeted *MdLOX1a* as a transcriptional activator (Fig. 3F).

220 **Correlation of *MdASG1* expression with *MdLOX1a* transcript level and ester**

221 **content**
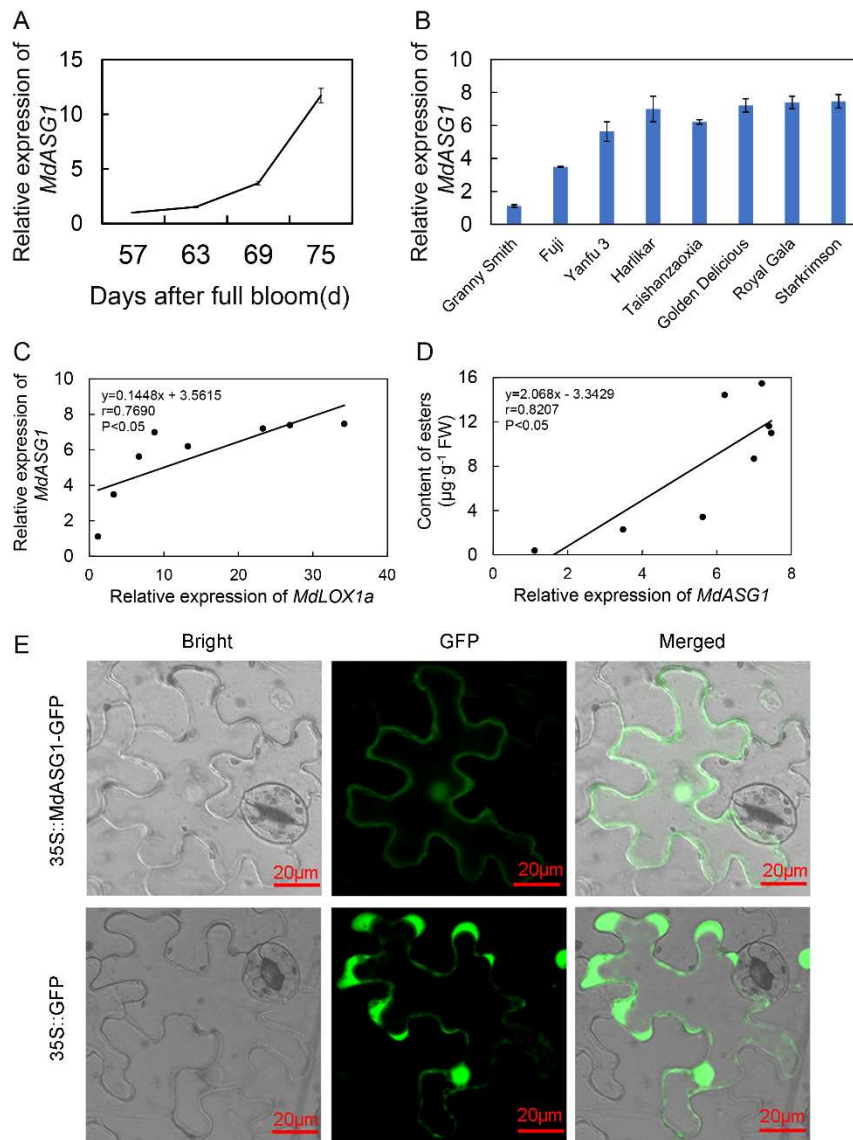
Figure 4. *MdASG1* is involved in ester biosynthesis in apple. A and B, Transcript levels of *MdASG1* during apple fruit development and ripening (A), and in fruit of eight popular apple cultivars at ripening (B). *MdActin* was used as an internal control gene. C, Linear regression analysis between *MdLOX1a* expression and *MdASG1* expression in fruit of eight apple cultivars. D, Linear regression analysis between *MdASG1* expression and ester content in fruit of eight apple cultivars. E, Subcellular localization of MdASG1 in tobacco leaves. Bars = 20 µm. Error bars represent the standard deviation of three independent biological replicates. Significant differences were determined using Tukey one-way analysis of variance (ANOVA) with SPSS Statistics 22 (*P < 0.05 and **P < 0.01).

222

223   To further explore the relationship between *MdASG1* expression and aromatic

224   compound synthesis, we analyzed the transcript level of *MdASG1* during apple fruit

225   development and ripening (Fig. 4A), which was consistent with the changes in ester

226   content. Subsequently, the expression profile of *MdASG1* among eight apple cultivars

227   was examined (Fig. 4B). Correlation analysis among the cultivars revealed that

228   *MdASG1* expression was positively correlated with *MdLOX1a* expression ($r = 0.7690$,

229    $P < 0.05$) (Fig. 4C). Furthermore, the expression profile of *MdASG1* was correlated

230   with ester content among the cultivars ($r = 0.8207$, $P < 0.05$) (Fig. 4D). Taken together,

231   these results suggested that *MdASG1* was a candidate gene involved in the lipoxygenase

232   biosynthesis pathway. Subcellular localization showed that *MdASG1* was uniformly

233   distributed in all subcellular compartments (Fig. 4E).

234   **Changes in fatty acid-derived volatile content caused by transient overexpression**

235   **of *MdASG1* or silencing of *MdASG1* in apple**

236   Given the positive correlation between the expression of *MdASG1* and *MdLOX1a*, as

237   well as the ester content (Fig. 4), we hypothesized that *MdASG1* plays a role in

238   regulating aroma compound biosynthesis. To test this hypothesis, we transiently

239   overexpressed *MdASG1* in 'Yinv' apple by injecting *Agrobacterium tumefaciens*

240   infiltration buffer containing the target gene or the empty vector (Fig. 5A). An

241   approximately 2-fold increase in *MdASG1* transcript levels and then a about 6-fold

242   increase in *MdLOX1a* transcript levels were observed (Fig. 5B). These changes were

243   accompanied by higher contents of fatty acid-derived volatiles, including 1-hexanol,

244   hexyl acetate, and 2-hexen-1-ol, acetate, (Z), compared with transient expression of the

245   empty vector 35S::GFP (Fig. 5, C–E). In addition, we transiently silenced *MdASG1*

246   (Fig. 5F). The opposite results were observed in *MdASG1*-silenced fruits and fatty acid-

247   derived volatile contents were significantly inhibited at the TRV-MdASG1 injection

248   sites (Fig. 5G).
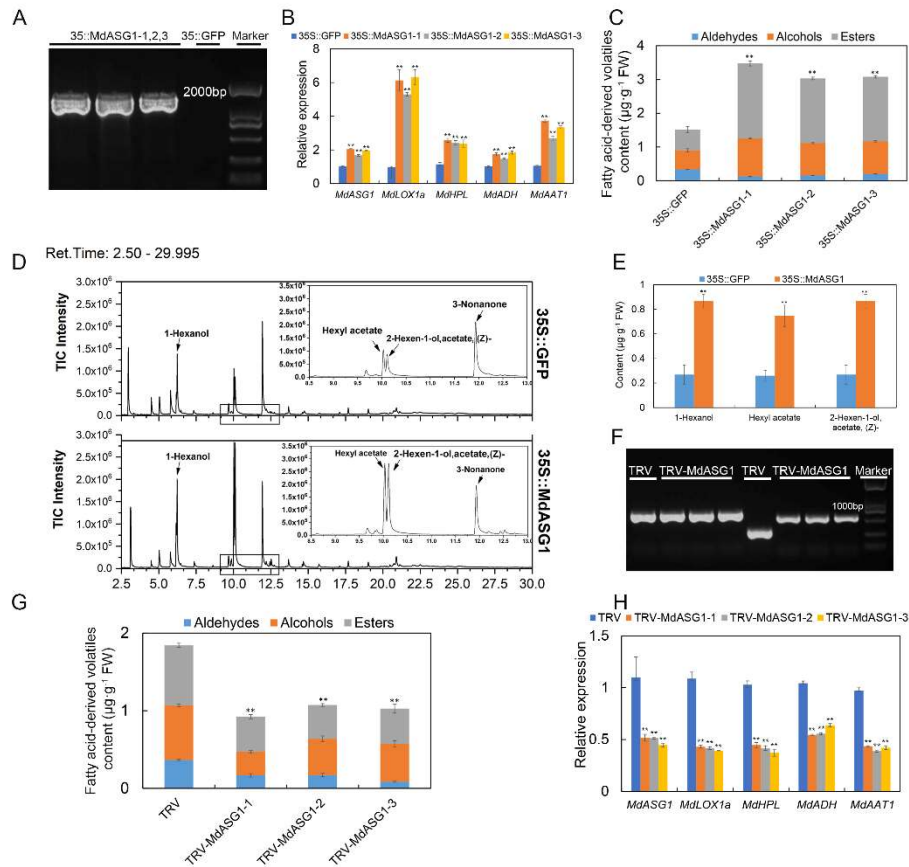
Figure 5. Transient overexpression or silencing of *MdASG1* in apple fruit. A, Transient overexpression of *MdASG1* was confirmed by PCR amplification. The GFP-F and MdASG-PHB-R primers were used for verification of transformants. B, Relative expression of *MdASG1* and fatty acid-derived volatile biosynthesis genes in apple fruit with transient overexpression of *MdASG1* (35S::MdASG1) and the empty vector (35S::GFP). *MdActin* was used as an internal control gene. C, Fatty acid-derived volatile content in 35S::GFP and 35S::MdASG1 transgenic apple fruit. D, Mass spectra of 35S::GFP and 35S::MdASG1 transgenic apple fruit. E, Contents of 1-hexanol, hexyl acetate, and 2-hexen-1-ol, acetate, (Z) in 35S::GFP and 35S::MdASG1 transgenic apple fruit. F, Transient silencing of *MdASG1* was confirmed by PCR amplification. The TRV1-F and TRV1-R primers were used in lanes 1–4 from the left, and the TRV2-F and TRV2-R primers were used in lanes 5–8 from the left. G, Fatty acid-derived volatiles content in apple fruit with transient silencing of *MdASG1* (TRV-MdASG1) and the empty vector (TRV). H, Relative expression of *MdASG1* and fatty acid-derived volatile biosynthesis genes in TRV and TRV-MdASG1 transgenic apple fruit. *MdActin* was used as an internal control gene. Error bars represent the standard deviation of three independent biological replicates. Asterisks indicate statistical significance (**$P < 0.01$ and *$P < 0.05$).

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250   Silencing of *MdASG1* led to a corresponding decrease in the transcript level of genes

251   associated with the lipoxygenase pathway (Fig. 5H).

252 **Changes in fatty acid-derived volatile content caused by stable overexpression of**

253 ***MdASG1***

254 To provide further evidence of *MdASG1*-mediated fatty acid-derived volatile

255 production, we generated *MdASG1*-overexpression 'Orin' calli (Fig. 6, A and B).
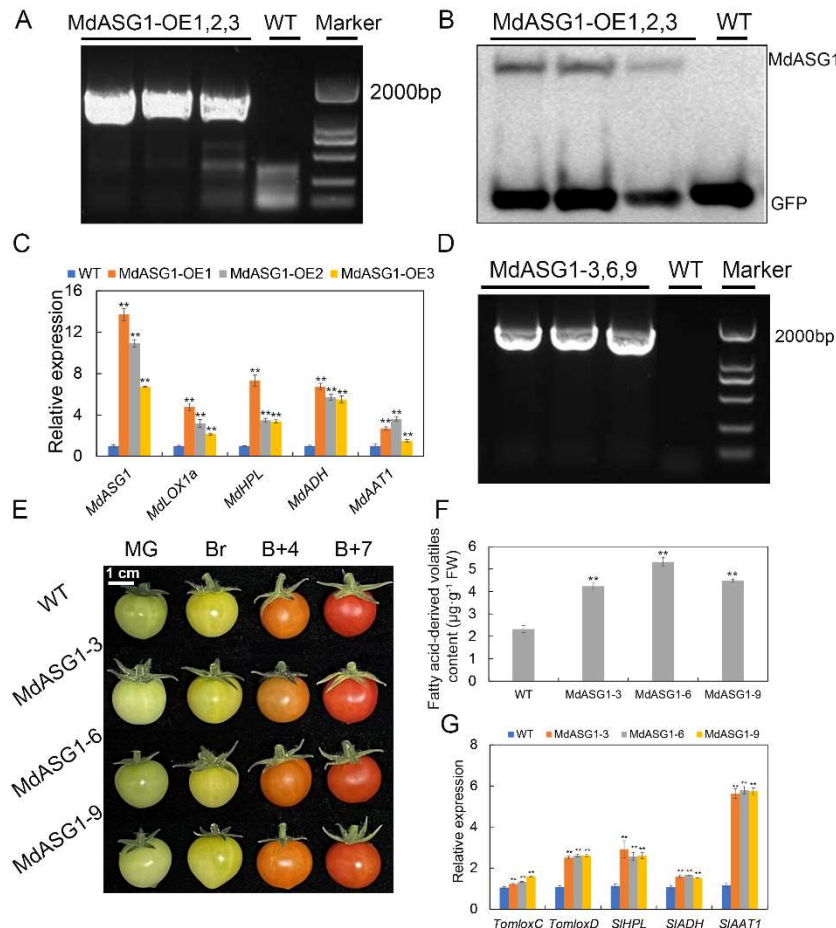
Figure 6. Stable overexpression of *MdASG1* in apple and tomato fruit. A and B, *MdASG1* overexpression in apple 'Orin' calli verified by PCR amplification (A) and western blotting (B). The 35S and MdASG1-PRI101-R primers were used for verification of transformants. C, Relative expression of *MdASG1* and fatty acid-derived volatile biosynthesis genes in *MdASG1*-overexpressing 'Orin' (MdASG1-OE) and wild-type (WT) calli. *MdActin* was used as an internal control gene. D, *MdASG1* overexpression in tomato verified by PCR amplification. The 188F and MdASG1-PCB302-R primers were used for verification of transformants. E, Fruit of tomato 'Micro-Tom' overexpressing *MdASG1*. F, Fatty acid-derived volatile content in fruit of wild-type Micro-Tom (WT) and *MdASG1*-overexpression tomato (MdASG1-3,6,9). G, Relative expression of fatty acid-derived volatile biosynthesis genes in fruit of WT and MdASG1-3,6,9 transgenic tomato. *SlActin* was used as an internal control gene. Error bars represent the standard deviation of three independent biological replicates. Asterisks indicate statistical significance (**$P < 0.01$ and *$P < 0.05$).

256

257 Overexpression of *MdASG1* caused upregulation in *MdLOX1a* expression and that of

258 other genes in the lipoxygenase pathway compared with the control calli (WT) (Fig.

259 6C). To rapidly generate transgenic fruit, we overexpressed *MdASG1* in tomato 'Micro-

260 Tom' and obtained the lines MdASG1-3, MdASG1-6, and MdASG1-9 (Fig. 6, D and

261 E). Ripening fruit of these overexpression lines accumulated higher contents of

262 volatiles than the WT (Fig. 6F). The transcript levels of the corresponding synthase

263 genes involved in the lipoxygenase pathway in transgenic tomato fruit were

264 significantly higher than those of WT tomato (Fig. 6G). To summarize, these results

265 suggested that *MdASG1* promotes fatty acid-derived volatile biosynthesis by activating

266 the transcript of *MdLOX1a* in the lipoxygenase pathway.

267 **Overexpression of *MdASG1* confers enhanced salt tolerance and accumulation of**

268 **higher contents of fatty acid-derived volatiles under salt treatment**

269 *MdASG1* showed high homology with *AtASG1*. Therefore, we speculated that *MdASG1*

270 may respond to abiotic stress similar to *AtASG1*. As expected, *MdASG1* transcript levels

271 were higher in response to NaCl treatment in tissue-cultured plantlets of 'Royal Gala'

272 (Fig. 7A) and 'Orin' calli (Fig. 7B), and especially in *MdASG1*-overexpression 'Orin'

273 calli (Fig. 7B). Similarly, *MdASG1*-overexpression 'Orin' calli were more tolerant to

274 salt stress than the control (Fig. 7C) and the transcription of stress-related genes was

275 up-regulated (Supplemental Fig. S7). Interestingly, the transcript levels of genes in the

276 lipoxygenase pathway were up-regulated in response to 50 mM NaCl treatment for 20

277 d, especially in calli overexpressing *MdASG1* (Fig. 7D). Similar results were observed

278 in tomato; the transcript levels of the tomato homolog *SlASG1* (Fig. 7E) and genes in

279 the lipoxygenase pathway were up-regulated with 200 mM NaCl treatment in WT and

280 transgenic tomato fruit (Supplemental Fig. S8). The contents of fatty acid-derived

281 volatiles increased accordingly under the salt treatment in WT and transgenic tomato

282   fruit (Fig. 7F). The transgenic tomato plants exhibited a significant increase in tolerance

283   to salt stress (Fig. 7G), higher photosynthesis capacity (Fig. 7H), and reduced oxidative

284   stress (Fig. 7I) compared with the WT.
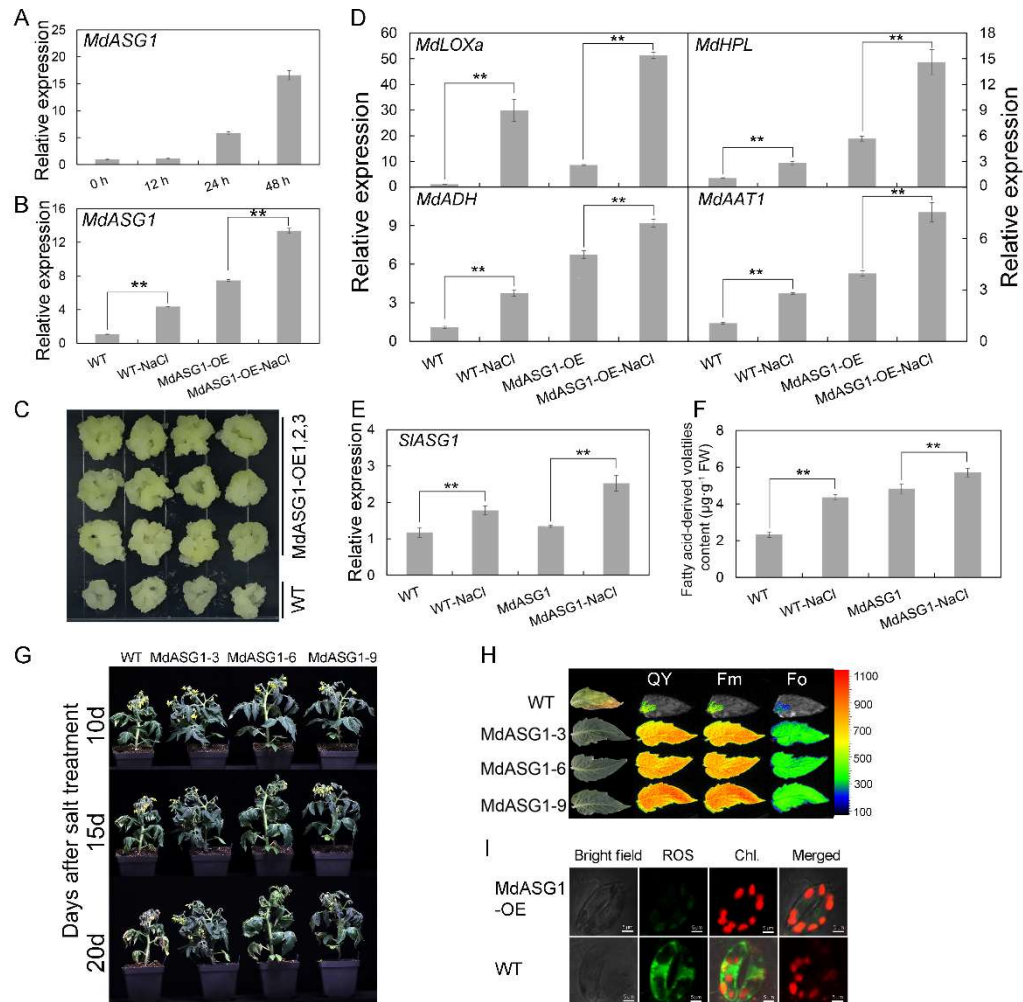
Figure 7. *MdASG1* enhances plant salt tolerance and mediates enhanced accumulation of fatty acid-derived volatiles under salt stress. A, Relative expression of *MdASG1* in wild-type tissue-cultured apple plantlets under 200 mM NaCl treatment. *MdActin* was used as an internal control gene. B, Transcriptional changes in *MdASG1* in response to 50 mM NaCl treatment in wild-type 'Orin' calli (WT) and *MdASG1*-overexpressing transgenic lines (MdASG1-OE). *MdActin* was used as an internal control gene. C, WT and MdASG1-OE transgenic 'Orin' calli treated with 50 mM NaCl. D, Transcriptional changes in fatty acid-derived volatile biosynthesis genes under 50 mM NaCl treatment in WT and MdASG1-OE transgenic 'Orin' calli. *MdActin* was used as an internal control gene. E, Transcriptional changes in *SlASG1* under 200 mM NaCl treatment in ripening fruit of WT and *MdASG1*-overexpression (MdASG1) tomato. *SlActin* was used as an internal control gene. F, Changes in fatty acid-derived volatile content under 200 mM NaCl treatment in ripening fruit of WT and MdASG1 transgenic tomato. G, Phenotype of WT and MdASG1-3,6,9 transgenic tomato plants subjected to 200 mM NaCl treatment for 10, 15, and 20 d. H, Chlorophyll fluorescence in tomato leaves after NaCl treatment for 20 d. I, Fluorescence of reactive oxygen species in tomato leaf cells after NaCl treatment for 20 d. Bars = 5 μm. Error bars represent the standard deviation of three independent biological replicates. Asterisks indicate statistical significance (**$P <$ 0.01 and *$P <$ 0.05).

285

286   The expression of stress-related genes was up-regulated in transgenic tomato plants

287   (Supplemental Fig. S9). In summary, these results suggest that *ASG1* is involved in the

288   synthesis of volatile aroma compounds in apple and tomato, and higher contents of

289   aroma compounds accumulate under salt stress.
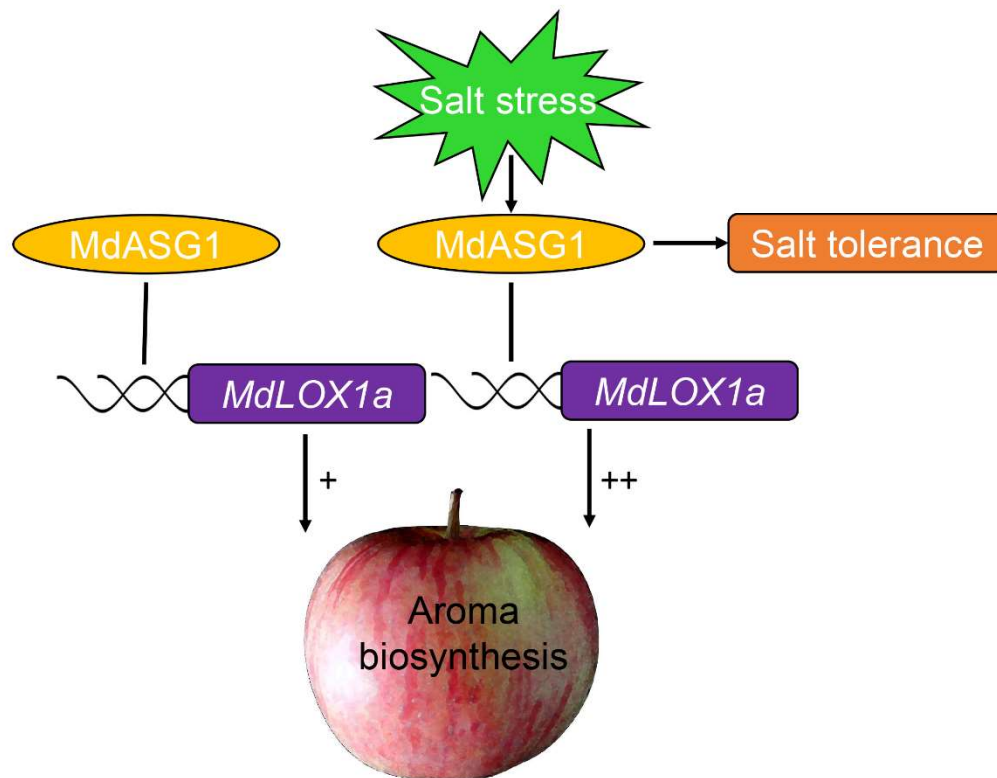
Figure 8. Proposed model for MdASG1 modulation of aroma compound biosynthesis in apple. MdASG1 can increase aroma compound biosynthesis by binding to the promoter of *MdLOX-1a*. Under moderate salt stress, MdASG1 enhances tolerance to salt stress and promotes accumulation of aroma compounds in fruit.

290

291   **DISCUSSION**

292   Fruit flavor comprises a complex set of interactions between taste and aroma (Brückner

293   and Wyllie, 2008). Aroma is a mixture of various volatile compounds and is an

294   important quality trait that influences consumer acceptance. The synthesis and

295   accumulation of aroma compounds are increased in ripening apple fruit of which esters

296   account for 80% of the volatiles (López et al., 1998; Lavilla et al., 1999; López et al.,

297   2010). Lipoxygenase is an important contributor to fruit ester production. In pepino

298   fruit during ripening, three LOX genes responsible for aroma compound biosynthesis,

299   namely *SmLOXD*, *SmLOXB*, and *SmLOX5-like2*, are up-regulated (Contreras et al.,

300   2017). In kiwifruit, *AdLox1* and *AdLox5* are up-regulated during ripening and are

301   involved in fruity aroma ester synthesis (Zhang et al., 2009). *MdLOX1a* is associated

302   with a quantitative trait locus for volatile esters in apple (Schiller et al., 2015). However,

303   the specific function of *MdLOX1a* in ester synthesis requires further study. In the

304   present study, *MdLOX1a* and *MdLOX7a* were up-regulated during fruit ripening,

305   consistent with results reported by Schiller et al. (2015). We determined that *MdLOX1a*

306   is involved in ester biosynthesis based on the significant positive correlations between

307   *MdLOX1a* expression and ester content. In addition, *MdLOX1a* overexpression in apple

308   'Orin' calli increased the ester content. Therefore, we speculated that *MdLOX1a* is a

309   crucial gene in the lipoxygenase pathway. Plant LOXs are localized in the cytoplasm

310   or chloroplasts. Tomato TomloxC is involved in the synthesis of C6 flavor compounds,

311   which are localised in the chloroplasts (Chen et al., 2004). MdLOX1a is localized in

312   the cytoplasm to participate in ester synthesis. Similarly, in kiwifruit, AdLox5

313   participates in fruity aroma ester synthesis in the cytoplasm (Zhang et al., 2006; Zhang

314   et al., 2009). Phylogenetic analysis revealed that MdLOX1a can be classified as a 9-

315   LOX. However, MdLOX1 is reported to have a dual positional specific function

316   generating 9- and 13-hydroperoxides (Schiller et al., 2015).

317   Transcriptional regulation of fruit aroma components has been widely reported in

318   plants. However, previous research has mainly focused on terpene biosynthesis. For

319   instance, multiple transcription factors of the MYC2, NAC, EIL, AP2/ERF, and MYB

320   families (Hong et al., 2012; Li et al., 2015; Nieuwenhuizen et al., 2015; Shen et al.,

321 2016; Li et al., 2017; Jian et al., 2019) are involved in terpene synthesis by directly

322 activating the terpene synthase *TPS*. Recently, transcription factors of the bZIP, NAC,

323 and Dof families have been reported to play important roles in ester biosynthesis by

324 regulating expression of the structural gene *AAT* in the lipoxygenase pathway (Guo et

325 al., 2018; Zhang et al., 2020; Cao et al., 2021; Wang et al., 2022). *LOX* is a crucial

326 structural gene in the lipoxygenase pathway, but the regulation of *LOX* is rarely

327 reported. Given the observation that *MdLOX1a* mediates fruit ester biosynthesis, we

328 used *MdLOX1a* as a candidate gene and identified a novel abiotic stress gene, *MdASG1*,

329 which activated *MdLOX1a* expression by directly binding to its promoter. Furthermore,

330 overexpression of *MdASG1* in apple fruit increased aroma compound production,

331 whereas synthesis of these compounds was decreased by *MdASG1* silencing. In

332 *Saccharomyces cerevisiae*, the zinc cluster transcriptional regulator Asg1 is an activator

333 of stress-responsive genes, which involves fatty acid utilization (Jansuriyakul et al.,

334 2016). However, MdASG1 and Asg1 of *S. cerevisiae* are entirely unrelated proteins.

335 The function of ASG (ScASG1 and AtASG1) was first identified in *Solanum*

336 *tuberosum* and *Arabidopsis thaliana*, and is a positive regulator of stress responses via

337 an ABA-dependent pathway (Batelli et al., 2012). Amino acid sequence analysis

338 revealed that MdASG1 showed high homology with Arabidopsis AtASG1 and potato

339 ScASG1. In the current study, we observed a novel function for *ASG* in apple in

340 mediating aroma compound biosynthesis. In addition, we observed that *MdASG1*

341 performed similar functions to those of *ScASG1* and *AtASG1* in response to NaCl

342 treatment (Batelli et al., 2012). Transgenic apple calli and tomato plants (MdASG1-

343 3,6,9) exhibited significantly enhanced tolerance to salt stress, and higher

344 photosynthesis capacity and lower oxidative stress in transgenic tomato plants

345 compared with the WT. We cloned *MdASG1* into the PHB vector and observed that

346   MdASG1 was uniformly distributed in all subcellular compartments. In contrast, potato

347   ScASG1 is localized to the plasma membrane (Batelli et al., 2012). Overexpression of

348   *DkLOX3* and *CaLOX1* in Arabidopsis plant confer increased tolerance to high salinity

349   and drought stress by modulating stress-related genes and reactive oxygen species

350   production (Hou et al., 2015; Lim et al., 2015). In oriental melon, *CmLOX10* positively

351   regulates drought tolerance through JA-mediated stomatal closure (Xing et al., 2020).

352   In tomato, overexpression of ω-3 fatty acid desaturases (FAD), which catalyze the

353   conversion of linoleic acid (18:2) to linolenic acid (18:3) in the lipoxygenase pathway,

354   enhance tolerance to cold stress (Dominguez et al., 2010). Therefore, we speculated

355   that *MdASG1* might function by mediating the lipoxygenase pathway in the response

356   to abiotic stress.

357   Abiotic stress strongly affects plant growth. However, the observation that moderate

358   stress may improve fruit quality is usually overlooked. Some previous studies have

359   examined stress-mediated fruit quality but were mainly focused on sweetness and

360   anthocyanin production, and less frequently on fruit aroma. For example, mild salt

361   stress improves strawberry fruit quality by increased accumulation of sucrose and the

362   antioxidant compounds anthocyanins and catechins (Casierra-Posada and Riaño, 2006;

363   Keutgen and Pawelzik, 2007; Galli et al., 2016). Similarly, in tomato, NaCl treatment

364   increases the concentration of soluble solids not only as a result of reduction in water

365   transport (Sato et al., 2006; Saito et al., 2008; Johkan et al., 2014). In grape, moderate

366   salinity increases anthocyanin and soluble solid contents, but decreases aroma quality

367   (Li et al., 2013). Conversely, in the present study, moderate salt stress increased the

368   expression of lipoxygenase pathway-related genes in apple calli and tomato fruit,

369   accompanied by increased accumulation of aroma compounds. Especially in *MdASG1*-

370   overexpressing apple calli and tomato, *MdASG1* further improved fatty acid-derived

371   volatile content under moderate salt stress. At the same time, tomato *SlASG1*, which is

372   a homolog of apple *MdASG1*, was significantly up-regulated under moderate salt stress,

373   accompanied by the increase in aroma compound synthesis. These results collectively

374   indicate that *ASG1* is involved in salt-induced aroma biosynthesis via enhanced

375   expression of genes in the lipoxygenase pathway.

376   The present results provide a theoretical foundation for exploitation of moderate salt

377   stress to improve fruit quality, and may enable the prudent development and utilization

378   of saline-alkali land to produce high-quality fruit. Rice and wheat (*Triticum aestivum*

379   L.) are major crops grown worldwide, but their growth and yield are frequently

380   restrained by salinity stress (Castro-Llanos et al., 2019; Yan et al., 2020). It is estimated

381   that at least 20% of all irrigated lands are impacted by salinity stress (Pitman and

382   Läuchli, 2002). Given that irrigated lands may be adversely affected by salinity, our

383   findings may contribute to improved utilization of saline-alkali land for crop production.

384   Ultimately, this would relieve pressure on arable land, thereby ensuring global food

385   security.

386   In summary, in this study we observed that the lipoxygenase gene *MdLOX1a* is a

387   crucial gene involved in ester biosynthesis. We identified an abiotic stress gene,

388   *MdASG1*, that directly bound to the promoter of *MdLOX1a* and activated its

389   transcription, and thus participated in the biosynthesis of fatty acid-derived volatiles in

390   apple fruit. In addition, *MdASG1* expression was enhanced by NaCl stress. Transgenic

391   apple calli and tomato plants (MdASG1-3,6,9) were more tolerant of salt stress than the

392   WT, and transcript levels of genes in the lipoxygenase pathway were higher under salt

393   stress compared with the non-stress condition, which may explain how moderate stress

394   improves fruit quality. The present results provide insight into the regulatory

395   mechanism by which *MdASG1* directly activates *MdLOX1a* expression to promote

396 accumulation of aroma volatiles especially under moderate salt stress. Our findings

397 establish a theoretical strategy for production of improved-quality apple fruit on

398 moderately saline soil to meet the needs of consumers.

399 **MATERIALS AND METHODS**

400 **Plant material and culture conditions**

401 Apple 'Taishanzaoxia' fruit were harvested at 57, 63, 69, and 75 DAFB from Liaocheng,

402 Shandong province, China. Fruit of eight apple cultivars were sampled at the ripening

403 stage from Liaocheng, Shandong province, China. The 'Orin' apple calli used for

404 genetic transformation were cultured on Murashige and Skoog (MS) medium

405 supplemented with 0.8 mg·L^{-1} 6-benzylaminopurine (6-BA) and 1.5 mg·L^{-1} 2,4-

406 dichlorophenoxyacetic acid (2,4-D) in the dark at 24℃. Tissue-cultured plantlets of

407 'Royal Gala' were subcultured on MS medium supplemented with 0.2 mg·L^{-1}

408 indoleacetic acid and 0.5 mg·L^{-1} 6-BA under a 16 h/8 h (light/dark) photoperiod at

409 24℃. Tomato 'Micro-Tom' plants were grown in a greenhouse at 24℃ under a 16 h/8

410 h (light/dark) photoperiod. Fruit of 'Yinv' apple used in the transient transformation

411 assays were harvested before coloring from trees in the germplasm nursery of the

412 Shandong Institute of Pomology. Tobacco (*Nicotiana benthamiana*) plants used for

413 subcellular localization and dual-luciferase assays were grown in a plant growth

414 chamber at 24℃ under a 16 h/8 h (light/dark) photoperiod.

415 **Stress treatment**

416 The shoot tip of 25-day-old 'Royal Gala' tissue-cultured plantlets was excised and

417 transferred to liquid MS medium supplemented with 200 mM NaCl. After 12, 24, and

418 48 h treatment, sampled shoots were immediately frozen in liquid nitrogen and stored

419   at −80°C until use. Transgenic (MdASG1-OE) and control (WT) calli of uniform

420   growth status were cultured on MS medium supplemented with 50 µM NaCl for salt

421   stress treatment for 20 d. Tomato plants grown in a square plastic pot (10 cm diameter

422   at the top, 7.5 cm diameter at the bottom, and 8.5 cm in height) were well watered

423   before salt treatment. One-month-old tomato plants (WT and T_3 transformants) of

424   uniform growth were watered with 200 mM NaCl solution at 4-day intervals until the

425   fruit were ripe. After 20 d salt treatment, the leaves were sampled for observation of

426   chlorophyll fluorescence, ROS, and RNA extraction. The ripe tomato fruit were

427   collected for GC-MS analysis. All of the above-mentioned treatments were applied with

428   three biological replicates.

429   **Volatile collection and GC-MS analysis**

430   Headspace solid-phase microextraction was used to collect fruit volatile compounds

431   following the method of Lu et al. (2021). Fresh fruit tissue (5 g) cut into pieces or 5 g

432   apple calli was transferred to a 50 mL conical flask, then 10 µL 3-nonanone (0.4

433   mg·mL^{-1}) was added as an internal standard, sealed, and extracted at 45°C for 40 min.

434   Tomato fruit required an additional 10 mL saturated NaCl to extract volatile compounds.

435   The   SPME   fiber   coated   with   a   50/30   µm   layer   of

436   divinylbenzene/carboxen/polydimethylsiloxane   (DVB/CAR/PDMS)   (Supelco,

437   Bellefonte, PA, USA) was used for volatile collection. The gas chromatograph–mass

438   spectrometer (GCMS-QP2010, Shimadzu, Kyoto, Japan) was equipped with a Rtx-

439   5MS capillary column (30 m × 0.25 mm i.d. × 0.25 µm film thickness) (Restek Co.,

440   Bellefonte, PA, USA). High-purity helium was employed as the carrier gas with a

441   constant flow rate of 2 mL·min^{-1}. The GC started at 35°C for 2 min, increased to 120°C

442   at 6°C·min^{-1}, then increased to 180°C at 10°C·min^{-1}, and finally increased to 250°C at

443   20°C·min^{-1} for 5 min. The transfer, MS source, and interface temperature were 250°C,

444   200℃, and 230℃, respectively. The mass spectra were acquired with 70 eV electron

445   ionization energy. The volatile compounds were identified by matching with the NIST

446   2017 mass spectral library and compared with the linear retention index (LRI) values.

447   The relative content of a volatile compound was determined using the peak area of the

448   internal standard as a reference according to the total ion chromatogram (TIC). For each

449   sample three biological replicates were analyzed.

450   **RNA extraction and quantitative real-time PCR**

451   Total RNA was extracted from plant tissue using the FastPure® Plant Total RNA

452   Isolation Kit (Vazyme, Nanjing, China). The synthesis of cDNA was performed using

453   HiScript® II Reverse Transcriptase (Vazyme). The qRT-PCR analysis was performed

454   with the ChamQ SYBR qPCR Master Mix (Vazyme) using a CFX Connect instrument

455   (Bio-Rad, Hercules, CA, USA). The *MdActin* gene was used as an internal control for

456   apple and the *SlActin* gene was used as an internal control for tomato. Gene relative

457   expression analysis used the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The primers

458   used for qRT-PCR in this study are listed in Supplemental Table S1.

459   **Determination of lipoxygenase activity**

460   Lipoxygenase activity was determined using a lipoxygenase assay kit (mlbio, Shanghai,

461   China). Briefly, fruit tissue was ground into powder in liquid nitrogen. The powder (0.1

462   g) was shaken and resuspended in 1 mL extract buffer, and then centrifuged for 20 min

463   at 16000 ×*g* at 4℃. The supernatant was the enzyme extract. The determination was

464   performed by adding 20 μL enzyme, 160 μL buffer solution reagent, and 20 μL

465   substrate solution. Lipoxygenase activity was measured as the increase in absorbance

466   at 234 nm over 1 minute. One unit of enzyme activity was defined as the change in

467   absorbance of 0.01 at 25°C per minute per gram of tissue. Each sample was analyzed

468   with three biological replicates.

Phylogenetic analysis

470   A phylogenetic tree was constructed from a multiple alignment of 58 LOX amino acid

471   sequences from 14 plant species. The sequences were downloaded from the Genome

472   Database for Rosaceae (https://www.rosaceae.org/) or the National Center for

473   Biotechnology Information database (http://www.ncbi.nlm.nih.gov/). The phylogenetic

474   tree was constructed using the neighbor-joining method with MEGA X. A bootstrap

475   analysis with 1000 replicates was performed to evaluate the reliability of the tree

476   topology. The accession numbers of the LOX sequences are listed in Supplemental

477   Table S4.

Subcellular localization of MdLOX1a and MdASG1

479   The full-length coding sequence (CDS) of *MdLOX1a* or *MdASG1* was inserted into the

480   pHB vector carrying the 35S promoter and then introduced into *A. tumefaciens* strain

481   GV3101 using the freeze–thaw method. *Agrobacterium* containing the target gene was

482   resuspended in infiltration buffer (10 mM MES, 10 mM $MgCl_2$, and 150 μM

483   acetosyringone, pH 5.5–5.7) and injected into 1-month-old tobacco leaves. The

484   fluorescence signal was imaged after infiltration for 2 d using a confocal laser

485   microscope (LSM880, Carl Zeiss, Oberkochen, Germany). The primers used for vector

486   construction are listed in Supplemental Table S2.

Yeast one-hybrid assay

488   To screen for proteins that potentially bind to the promoter of *MdLOX1a*, we used the

489   Matchmaker® Gold Yeast One-Hybrid Library Screening System (Clontech, Mountain

490   View, CA, USA) following the manufacturer's instructions. The *MdLOX1a* promoter

491   (fragment length 1059 bp) was cloned into the pAbAi vector and the linearized plasmid

492   was transformed into the yeast strain Y1H Gold. The optimal AbA screening

493   concentration was determined in accordance with the manufacturer's instructions. Total

494   RNA extracted from apple 'Taishanzaoxia' fruit at different developmental stages was

495   used to construct the prey cDNA library. The library plasmid (10 µL) was transformed

496   into the MdLOX1a-pAbAi Y1H Gold strain to screen the novel protein. In addition, the

497   identified protein MdASG1 was cloned into the pGADT7 vector to confirm the result.

498   The promoter of *MdLOX1a* was divided into four fragments (*p1MdLOX1a* to

499   *p4MdLOX1a*) to identify the binding site. The corresponding primers used for vector

500   construction are listed in Supplemental Table S2.

501   **Dual-luciferase reporter assay**

502   The full-length CDS of *MdASG1* was inserted into the pGreenII62-SK vector. The

503   *MdLOX1a* promoter was inserted into the pGreenII0800-Luc vector. The recombinant

504   plasmids were expressed transiently in tobacco leaves by *A. tumefaciens*-mediated

505   genetic transformation using the same method as described for the subcellar localization

506   assay. The In Vivo Imaging System (Xenogen, Alameda, CA, US) was used to detect

507   luminescence. The luciferase activities were measured using the Dual-Luciferase®

508   Reporter Assay System (Promega, Madison, WI, US).

509   **EMSA**

510   The EMSA was conducted using the Lightshift Chemiluminescent EMSA kit (Thermo,

511   New York, NY, USA) in accordance with the manufacturer's instructions as described

512   previously by Zhang et al. (2018). The full length CDS of *MdASG1* was inserted into

513   the pGEX-4T vector. The constructed vector was introduced into *Escherichia coli* strain

514   BL21 to induce protein production, and then was purified using the GST-tag Protein

515   Purification Kit (Beyotime, Shanghai, China) following the manufacturer's instructions.

516   The biotin-labeled probe and MdASG1-GST protein were mixed in the binding buffer

517   and incubated at 24℃ for 15 min. The GST protein was used as the control. The

518   unlabeled probes were used as competitors. The probes used in the EMSA assay are

519   listed in Supplemental Table S3.

Fluorescence detection of reactive oxygen species

521   Reactive oxygen species were detected with fluorescent probes using a previously

522   described method with slight modification (Zhuang et al., 2019; Wang et al., 2020).

523   Leaf discs were collected from transgenic and WT tomato plants after salt treatment for

524   20 d. The leaf discs were soaked in 0.01 mM PBS for 20 min, then placed in 10 μM

525   2′,7′-dichlorodihydrofluorescein diacetate (Invitrogen, Carlsbad, CA, USA) and

526   incubated under vacuum for 30 min. A confocal laser microscope LSM880 (Carl Zeiss,

527   Oberkochen, Germany) was used to observe the fluorescence signal.

Transient expression of *MdASG1* in apple fruit

529   Overexpression vector construction and infiltration of *MdASG1* were conducted as

530   described for the subcellar localization assay. Virus-induced gene silencing was used

531   to silence *MdASG1* in apple fruit. A partial CDS fragment for pTRV2-*MdASG1* (369

532   bp) was cloned by PCR with specific primers (Supplemental Table S2). *Agrobacterium*

533   *tumefaciens* containing the target genes was injected into the epidermis of 'Yinv' apple

534   fruit with a syringe. *Agrobacterium tumefaciens* carrying the empty vector (PHB or

535   TRV) was used as a control. After infiltration, the fruit were placed in an incubator at

536   24℃ under a 16 h/8 h (light/dark) photoperiod. After 3 d, the fruit injection sites were

537   sampled for transgene verification and qRT-PCR analysis. After 7 d, the fruit injection

538   sites were sampled for volatile compound analysis using GC-MS. Three biological

539   replicates with at least 15 fruits per group were analyzed.

Stable overexpression in apple calli and tomato

541   The full-length CDS of *MdLOX1a* or *MdASG1* was cloned into the PRI 101-AN vector

542   using the primers listed in Supplemental Table S2. The recombinant plasmid was

543   introduced into *A. tumefaciens* strain LBA4404 using the freeze–thaw method.

544   Transformation of apple calli was conducted as described by Zhang et al. (2018). The

545   transgenic calli were used for further analysis. The full-length CDS of *MdASG1* was

546   introduced into the PCB302 vector carrying the CaMV35S promoter and transformed

547   into *A. tumefaciens* strain LBA4404. The primers used for the transformation are listed

548   in Supplemental Table S2. *Agrobacterium* infection solution with $OD_{600} = 0.6$ was used

549   to infiltrate tomato cotyledons for 15 min. The infiltrated cotyledons were placed on

550   MS medium supplemented with 50 mg·L^{-1} kanamycin to screen for resistant buds.

551   Three lines were confirmed to be transgenic. Tomato fruit harvested at B+7 days from

552   T$_3$ transgenic and WT plants were sampled for aroma compound analysis. Three

553   biological replicates with 15 fruits per replicate were analyzed.

Chlorophyll fluorescence analysis

555   Chlorophyll fluorescence parameters were measured using a Closed FluorCam FC800

556   chlorophyll fluorescence imaging system (Photon Systems Instruments, Brno, Czech

557   Republic). Before measurement, the leaves were dark-adapted for 30 min, then

558   analyzed to determine F_0 (minimum fluorescence) and F_M (maximum fluorescence).

Statistical analysis

560 Student's *t*-test ($^*P < 0.05$, $^{**}P < 0.01$) was used to determine the significance of

561 differences of two samples in this study. Figures were generated using Microsoft Excel.

562 Linear regression analysis was performed using Microsoft Excel and significance of

563 multiple groups was analyzed using Tukey one-way analysis of variance (ANOVA)

564 with SPSS Statistics 22 (IBM Corporation, Armonk, NY, USA).

565 **Supplemental Data**

566 **Supplemental Figure S1**. Relative expression of *MdLOX* genes during apple fruit
567 development and ripening.

568 **Supplemental Figure S2**. Linear regression analysis between *MdLOX* expression and
569 the ester content in apple fruit at the ripening stage.

570 **Supplemental Figure S3**. Fruit of eight apple cultivars harvested at ripening.

571 **Supplemental Figure S4**. Phylogenetic analysis of plant LOX proteins.

572 **Supplemental Figure S5**. Background AbA^r expression of the yeast Y1H Gold strain
573 containing specific promoters.

574 **Supplemental Figure S6**. Protein sequence alignment of MdASG1 with AtASG1 and
575 ScASG1.

576 **Supplemental Figure S7**. Relative expression of stress-related genes in apple calli of
577 the wild-type 'Orin' (WT) and *MdASG1*-overexpressing transgenic lines (MdASG1-
578 OE) in response to 50 mM NaCl treatment for 20 d.

579 **Supplemental Figure S8**. Relative expression of stress-related genes in wild-type (WT)
580 and *MdASG1*-overexpressing (MdASG1-3,6,9) tomato plants in response to 200 mM
581 NaCl treatment for 20 d.

582 **Supplemental Figure S9**. Transcriptional changes in fatty acid-derived volatile
583 biosynthesis genes in response to 200 mM NaCl treatment in ripening fruit of wild-type
584 (WT) and *MdASG1*-overexpressing (MdASG1) tomato.

585 **Supplemental Table S1**. Primers used for qRT-PCR analysis in this study.

586 **Supplemental Table S2**. Primers used to construct or verify vectors in this study.

587 **Supplemental Table S3**. Probes used for EMSA in this study.

588 **Supplemental Table S4**. Accession numbers for LOX proteins in the phylogenetic
589 analysis.

590 **ACKNOWLEDGMENTS**

596   **CONFLICT OF INTEREST**

597   The authors declare no conflict of interests.

598   **FIGURE LEGENDS**

599   **Figure 1.** *MdLOX1a* is involved in ester formation in apple. A, Apple 'Taishanzaoxia'

600   fruit were harvested at 57, 63, 69, and 75 days after full bloom (DAFB). Bar = 2 cm. B,

601   Ester content during apple fruit development and ripening. C, Transcript level of

602   *MdLOX1a* during apple fruit development and ripening quantified by qRT-PCR.

603   *MdActin* was used as an internal control gene. D, Ethylene release rate during apple

604   fruit development and ripening. E, Correlation analysis of *MdLOX* expression and ester

605   content in apple fruit at the ripening stage. F, Relative expression of *MdLOX1a* in fruit

606   of eight apple cultivars at the ripening stage. *MdActin* was used as an internal control

607   gene. G, Lipoxygenase activity in fruit of eight apple cultivars at the ripening stage. H,

608   Ester content in fruit of eight apple cultivars at the ripening stage. I, Linear regression

609   analysis between *MdLOX1a* expression and lipoxygenase activity in fruit of eight apple

610   cultivars. J, Linear regression analysis between *MdLOX1a* expression and ester content

611   in fruit of eight apple cultivars. Error bars represent the standard deviation of three

612   independent biological replicates. FW, Fresh weight. Significant differences were

613   determined using Tukey one-way analysis of variance (ANOVA) with SPSS Statistics

614   22 (*$P < 0.05$ and **$P < 0.01$).

615   **Figure 2.** Overexpression of *MdLOX1a* increased fatty acid-derived volatile content in

616   apple calli and subcellar localization of MdLOX1a. A and B, *MdLOX1a* overexpression

617   in 'Orin' calli verified by PCR amplification (A) and western blotting (B). The 35S and

618   MdLOX1a-PRI101-R primers were used for verification of transformants. C, Fatty

619   acid-derived volatile contents in wild-type apple calli (WT) and *MdLOX1a*-

620   overexpression apple calli (MdLOX1a-OE). D, Relative expression of fatty acid-

621   derived volatile biosynthesis genes in WT and MdLOX1a-OE transgenic apple calli.

622   *MdActin* was used as an internal control gene. E, Subcellular localization of MdLOX1a

623   in tobacco leaves. MdLOX1a was mainly expressed in the cytoplasm of tobacco leaves.

624   Bars = 20 μm. Error bars represent the standard deviation of three independent

625   biological replicates. Asterisks indicate statistical significance (**$P < 0.01$ and *$P <$

626   0.05).

627   **Figure 3.** MdASG1 binds to the promoter of *MdLOX1a* and activates its expression. A,

628   Yeast one-hybrid assays showing binding between MdASG1 and the promoter of

629   *MdLOX1a*. B, Yeast one-hybrid assays showing binding between MdASG1 and the

630   fourth segment of the promoter of *MdLOX1a* (*p4MdLOX1a*). C, Four segments of

631   *p4MdLOX1a*. Electrophoretic mobility shift assay (EMSA) showing binding of

632   MdASG1 to the -795~-529bp segment of *p4MdLOX1a* (*p4MdLOX1a-2*). D, Design of

633   biotin-labeled probes (M1–M6) for partial deletion of the fragment *p4MdLOX1a-2*. The

634   M6 fragment showed no binding with MdASG1. E, EMSA showing the binding of

635   MdASG1 to the m6 motif in *MdLOX1a*. The hot probe was a biotin-labeled fragment.

636   The cold probe was a nonlabeled fragment. The mutant probe contained five nucleotide

637   mutations. The symbol + or – indicates the presence or absence of specific probes. F,

638   Dual-luciferase assay verifying that MdASG1 transformation activated the *MdLOX1a*

639   promoter. Error bars represent the standard deviation of three independent biological

640   replicates. Asterisks indicate statistical significance (**$P < 0.01$ and *$P < 0.05$).

641   **Figure 4.** *MdASG1* is involved in ester biosynthesis in apple. A and B, Transcript levels

642   of *MdASG1* during apple fruit development and ripening (A), and in fruit of eight

643   popular apple cultivars at ripening (B). *MdActin* was used as an internal control gene.

644   C, Linear regression analysis between *MdLOX1a* expression and *MdASG1* expression

645   in fruit of eight apple cultivars. D, Linear regression analysis between *MdASG1*

646   expression and ester content in fruit of eight apple cultivars. E, Subcellular localization

647   of MdASG1 in tobacco leaves. Bars = 20 μm. Error bars represent the standard

648   deviation of three independent biological replicates. Significant differences were

649   determined using Tukey one-way analysis of variance (ANOVA) with SPSS Statistics

650   22 (*$P < 0.05$ and **$P < 0.01$).

651   **Figure 5.** Transient overexpression or silencing of *MdASG1* in apple fruit. A, Transient

652   overexpression of *MdASG1* was confirmed by PCR amplification. The GFP-F and

653   MdASG-PHB-R primers were used for verification of transformants. B, Relative

654   expression of *MdASG1* and fatty acid-derived volatile biosynthesis genes in apple fruit

655   with transient overexpression of *MdASG1* (35::MdASG1) and the empty vector

656   (35S::GFP). *MdActin* was used as an internal control gene. C, Fatty acid-derived

657   volatile content in 35S::GFP and 35::MdASG1 transgenic apple fruit. D, Mass spectra

658   of 35S::GFP and 35::MdASG1 transgenic apple fruit. E, Contents of 1-hexanol, hexyl

659   acetate, and 2-hexen-1-ol, acetate, (Z) in 35S::GFP and 35::MdASG1 transgenic apple

660   fruit. F, Transient silencing of *MdASG1* was confirmed by PCR amplification. The

661   TRV1-F and TRV1-R primers were used in lanes 1–4 from the left, and the TRV2-F

662   and TRV2-R primers were used in lanes 5–8 from the left. G, Fatty acid-derived

663   volatiles content in apple fruit with transient silencing of *MdASG1* (TRV-MdASG1)

664   and the empty vector (TRV). H, Relative expression of *MdASG1* and fatty acid-derived

665   volatile biosynthesis genes in TRV and TRV-MdASG1 transgenic apple fruit. *MdActin*

666   was used as an internal control gene. Error bars represent the standard deviation of three

667   independent biological replicates. Asterisks indicate statistical significance (**P < 0.01

668   and *P < 0.05).

669   **Figure 6.** Stable overexpression of *MdASG1* in apple and tomato fruit. A and B,

670   *MdASG1* overexpression in apple 'Orin' calli verified by PCR amplification (A) and

671   western blotting (B). The 35S and MdASG1-PRI101-R primers were used for

672   verification of transformants. C, Relative expression of *MdASG1* and fatty acid-derived

673   volatile biosynthesis genes in *MdASG1*-overexpressing 'Orin' (MdASG1-OE) and

674   wild-type (WT) calli. *MdActin* was used as an internal control gene. D, *MdASG1*

675   overexpression in tomato verified by PCR amplification. The 188F and MdASG1-

676   PCB302-R primers were used for verification of transformants. E, Fruit of tomato

677   'Micro-Tom' overexpressing *MdASG1*. F, Fatty acid-derived volatile content in fruit of

678   wild-type Micro-Tom (WT) and *MdASG1*-overexpression tomato (MdASG1-3,6,9). G,

679   Relative expression of fatty acid-derived volatile biosynthesis genes in fruit of WT and

680   MdASG1-3,6,9 transgenic tomato. *SlActin* was used as an internal control gene. Error

681   bars represent the standard deviation of three independent biological replicates.

682   Asterisks indicate statistical significance (**P < 0.01 and *P < 0.05).

683   **Figure 7.** *MdASG1* enhances plant salt tolerance and mediates enhanced accumulation

684   of fatty acid-derived volatiles under salt stress. A, Relative expression of *MdASG1* in

685   wild-type tissue-cultured apple plantlets under 200 mM NaCl treatment. *MdActin* was

686 used as an internal control gene. B, Transcriptional changes in *MdASG1* in response to

687 50 mM NaCl treatment in wild-type 'Orin' calli (WT) and *MdASG1*-overexpressing

688 transgenic lines (MdASG1-OE). *MdActin* was used as an internal control gene. C, WT

689 and MdASG1-OE transgenic 'Orin' calli treated with 50 mM NaCl. D, Transcriptional

690 changes in fatty acid-derived volatile biosynthesis genes under 50 mM NaCl treatment

691 in WT and MdASG1-OE transgenic 'Orin' calli. *MdActin* was used as an internal

692 control gene. E, Transcriptional changes in *SlASG1* under 200 mM NaCl treatment in

693 ripening fruit of WT and *MdASG1*-overexpression (MdASG1) tomato. *SlActin* was

694 used as an internal control gene. F, Changes in fatty acid-derived volatile content under

695 200 mM NaCl treatment in ripening fruit of WT and MdASG1 transgenic tomato. G,

696 Phenotype of WT and MdASG1-3,6,9 transgenic tomato plants subjected to 200 mM

697 NaCl treatment for 10, 15, and 20 d. H, Chlorophyll fluorescence in tomato leaves after

698 NaCl treatment for 20 d. I, Fluorescence of reactive oxygen species in tomato leaf cells

699 after NaCl treatment for 20 d. Bars = 5 µm. Error bars represent the standard deviation

700 of three independent biological replicates. Asterisks indicate statistical significance

701 (**$P < 0.01$ and *$P < 0.05$).

702 **Figure 8.** Proposed model for MdASG1 modulation of aroma compound biosynthesis

703 in apple. MdASG1 can increase aroma compound biosynthesis by binding to the

704 promoter of *MdLOX1a*. Under moderate salt stress, MdASG1 enhances tolerance to salt

705 stress and promotes accumulation of aroma compounds in fruit.

706

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