# The role and proteomic analysis of ethylene in hydrogen gas-induced adventitious rooting development in cucumber (*Cucumis sativus* L.) explants

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Previous studies have shown that both hydrogen gas ($H_2$) and ethylene (ETH) play positive roles in plant adventitious rooting. However, the relationship between $H_2$ and ETH during this process has not been explored and remains insufficiently understood. In this study, cucumber (*Cucumis sativus* L.) was used to explore the proteomic changes in ETH-$H_2$-induced rooting. Our results show that hydrogen-rich water (HRW) and ethylene-releasing compound (ethephon) at proper concentrations promote adventitious rooting, with maximal biological responses occurring at 50% HRW or 0.5 μM ethephon. ETH inhibitors aminoethoxyvinylglycine (AVG) and $AgNO_3$ cause partial inhibition of adventitious rooting induced by $H_2$, suggesting that ETH might be involved in $H_2$-induced adventitious rooting. According to two-dimensional electrophoresis (2-DE) and mass spectrometric analyses, compared with the control, 9 proteins were up-regulatedwhile 15 proteins were down-regulatedin HRW treatment; 4 proteins were up-regulated while 10 proteins were down-regulated in ethephon treatment; and 1 protein was up-regulated while 9 proteins were down-regulated in HRW+AVG treatment. Six of these differentially accumulated proteins were further analyzed, including photosynthesis -related proteins [ ribulose-1,5-bisphosphate carall boxylase smsubunit (Rubisco), sedoheptulose-1,7-bisphosphatase (SBPase), oxygen-evolving enhancer protein (OEE1 )], amino and metabolism-related protein [ threonine dehydratase (TDH)], stress response-related protein [cytosolic ascorbate peroxidase (CAPX)], and folding, modification and degradation-related protein [ protein disulfide-isomerase (PDI)]. Moreover, the results of real-time PCR about the mRNA levels of these genes in various treatments were consistent with the 2-DE results. Therefore, ETH may be the downstream signaling molecule during $H_2$- induced adventitious rooting and proteins Rubisco, SBPase, OEE1, TDH, CAPX and PDI may play important roles during the process.

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## Abstract

18  Previous studies have shown that both hydrogen gas ($H_2$) and ethylene (ETH) play positive roles
19  in plant adventitious rooting. However, the relationship between $H_2$ and ETH during this process
20  has not been explored and remains insufficiently understood. In this study, cucumber (*Cucumis*
21  *sativus* L.) was used to explore the proteomic changes in ETH-$H_2$-induced rooting. Our results
22  show that hydrogen-rich water (HRW) and ethylene-releasing compound (ethephon) at proper
23  concentrations promote adventitious rooting, with maximal biological responses occurring at 50%
24  HRW or 0.5 μM ethephon. ETH inhibitors aminoethoxyvinylglycine (AVG) and $AgNO_3$ cause
25  partial inhibition of adventitious rooting induced by $H_2$, suggesting that ETH might be involved
26  in $H_2$-induced adventitious rooting. According to two-dimensional electrophoresis (2-DE) and
27  mass spectrometric analyses, compared with the control, 9 proteins were up-regulated while 15
28  proteins were down-regulated in HRW treatment; 4 proteins were up-regulated while 10 proteins
29  were down-regulated in ethephon treatment; and 1 protein was up-regulated while 9 proteins
30  were down-regulated in HRW+AVG treatment. Six of these differentially accumulated proteins
31  were further analyzed, including photosynthesis-related proteins [ribulose-1,5-bisphosphate
32  carall boxylase smsubunit (Rubisco), sedoheptulose-1,7-bisphosphatase (SBPase), oxygen-
33  evolving enhancer protein (OEE1)], amino and metabolism-related protein [threonine
34  dehydratase (TDH)], stress response-related protein [cytosolic ascorbate peroxidase (CAPX)],
35  and folding, modification and degradation-related protein [protein disulfide-isomerase (PDI)].
36  Moreover, the results of real-time PCR about the mRNA levels of these genes in various
37  treatments were consistent with the 2-DE results. Therefore, ETH may be the downstream
38  signaling molecule during $H_2$- induced adventitious rooting and proteins Rubisco, SBPase, OEE1,
39  TDH, CAPX and PDI may play important roles during the process.

40

41  **Keywords:** Cucumber, Adventitious root formation, Hydrogen gas, Ethylene, Proteomics

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## Introduction

45  Adventitious roots are formed from non-root tissue such as leaves, stems and hypocotyls.
46  Adventitious roots usually increase the area of roots, heightening their abilities to absorb
47  nutrients and support plants. Additionally, adventitious roots play an important role in promoting
48  the vegetative propagation of fine varieties and the agriculture and forestry industries. Thus,
49  exploring the mechanism of adventitious root formation is both necessary and meaningful. The
50  roles of signal molecules in adventitious root development have recently become a hot topic of
51  research. Some signal molecules including auxin (Bai et al. 2012), ethylene (Pan et al. 2002),
52  carbon monoxide (CO; Xuan et al. 2008), nitric oxide (NO), hydrogen peroxide ($H_2O_2$), $Ca^{2+}$
53  ions, calmodulin (Liao et al. 2012), hydrogen sulfide ($H_2S$; Lin et al. 2012), hydrogen ($H_2$; Lin et
54  al. 2014) and methane (Cui et al. 2015) have been associated with the initiation and development
55  of adventitious roots. More studies about signal transduction will help us understand the

56    mechanisms involved in adventitious rooting.

57

58    Despite its simple two-carbon structure, ethylene (ETH) plays an important role in regulating
59    plant growth and development. ETH may induce diverse effects throughout the plant life cycle,
60    including seed germination, flower senescence and fruit ripening (Khan et al. 2017). ETH is also
61    involved in the plant's defense reactions against pathogens or elicitor attacks, and in its
62    responses to abiotic stresses such as wounding, hypoxia, chilling, freezing and flooding (Khan et
63    al. 2017), indicating that ETH might be a regulator of plant growth, development and stress
64    responses.

65

66    Since it was firstly discovered that ethylene stimulates adventitious root formation, there have
67    been a great number of studies on the topic. Riov and Yang (1989) investigated the role of ETH
68    in adventitious root formation in auxin-induced cuttings of *Vigna radiata* (L.) and found that
69    ETH increased the number of adventitious roots while aminoethoxyvinylglycine (AVG, a kind
70    of ETH inhibitor) significantly inhibited rooting. Pitts et al. (1998) used the mutations of ETH
71    and auxin to prove that auxin and ETH are required for normal root hair elongation. Clark et al.
72    (1999) found that aboveground adventitious roots were produced in few ETH-insensitive never-
73    ripe tomato plants and ETH- insensitive transgenic petunia plants compared to wild type plants,
74    and proved the positive role of ETH in adventitious root formation. Similar results were
75    obtained from sunflower cuttings (Liu et al. 1990), mung bean hypocotyl cuttings (Pan et al.
76    2002), Panax ginseng C.A. Meyer (Bae et al. 2006), and globe artichoke seedlings (Shinohara et
77    al. 2016). However, some researchers have indicated that ETH can inhibit adventitious root
78    formation. Biondi et al (1990) showed that in *Prunus avium* shoot cultures, the addition of
79    ethylene precursor1-aminocyclopropane-1-carboxylic acid (ACC) decreased the rooting
80    percentage while AVG caused roots to form. Some years later, the studies of Ma et al. (1998)
81    demonstrated that $AgNO_3$ (another kind of ETH inhibitor) and AVG increased the number of
82    roots in apple shoots.

83

84    Hydrogen gas ($H_2$), the lightest gas, is a colorless, odorless, and tasteless diatomic gas in nature.
85    Ohsawa et al. (2007) found that $H_2$ can be used as an effective antioxidant therapy in the partial
86    brain of rat, and since then, the use of $H_2$ in the prevention and control of various diseases has
87    been a hot topic of research (Hong et al. 2010). More recently, the physiological functions of $H_2$
88    in higher plants have also been studied. $H_2$ has often been shown to respond to stresses like salt
89    stress, drought stress, heavy metal toxicity, oxidative stress and UV-A irradiation (Jin et al. 2013,
90    Zeng et al. 2014, Su et al. 2014), and delaying fruit senescence (Hu et al. 2014) and inducing
91    stomatal closure (Xie et al. 2014). Recently, another function of $H_2$ was discovered: the
92    promotion of adventitious root occurrence. The results of Lin et al. (2014) demonstrated that 50%
93    HRW could mimic hemin function and restore cucumber adventitious root formation by
94    mediating the expression of auxin signaling-related and adventitious root formation-related genes.
95    Zhu et al. (2016) has reported that HRW treatment increases the content of nitric oxide (NO) and
96    up-regulated cell cycle activation during adventitious root formation. Moreover, Cao et al. (2017)

97   suggested that HRW is involved in regulating auxin-induced lateral root formation by
98   modulating NR-dependent NO synthesis.
99
100  Based on the results of the studies summarized above, ETH and $H_2$ are involved in adventitious
101  root formation. However, the precise mechanism remains unclear. In this study, we hypothesized
102  the exists of a relationship between ETH and $H_2$ during adventitious rooting in cucumber
103  (*Cucumis sativus* L.).
104  To prove this hypothesis, ETH inhibitors were used. Proteomic methods have been widely
105  applied to determine genetic and cellular function at the protein level. Many plant proteomic
106  studies using 2-DE have found differential proteins during somatic embryogenesis, seed
107  germination, adventitious root formation, flowering transition, and stem dormancy. Proteomic
108  analysis has also proven useful in the identification of proteins associated with a range of biotic
109  and abiotic stress responses (Cui et al. 2015, Xu et al. 2016). Therefore, 2-DE and matrix-
110  assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-TOFTOF) were used
111  to compare quantitative and qualitative changes that occurred during ethylene-$H_2$-induced
112  adventitious rooting in the proteome. Through the comprehensive analysis of the function and
113  content of differential proteins under different treatments and the expression of genes related to
114  these proteins, we speculate that ETH possibly affect the expression of some proteins and the
115  expression of their related genes. The results provide new insights into the physiological and
116  molecular mechanisms associated with adventitious root development.
117

## Materials and Methods

### Plant materials and treatments

120  Cucumber (*Cucumis sativus* L. var. 'Xinchun 4') seeds were purchased from Gansu Agricultural
121  Institute (Lanzhou, China). The seeds were surface-sterilized in 5% sodium hypochlorite for 10
122  min and washed with water. The seeds were germinated on filter paper with distilled water in
123  Petri dishes (15 cm-diameter, 2.5 cm-deep) and maintained at $25\pm1^oC$ for 7 d with a 14-h
124  photoperiod (photosynthetically active radiation = 200 $\mu$mol m$^{-2}$s$^{-1}$). The 7-day-old seedlings
125  with primary roots removed were used as explants and were maintained under the same
126  temperature and photoperiod conditions for another 7 days in the presence of different media as
127  indicated below. We described these different treatments as (I) HRW (0, 1%, 10%, 50% and
128  100%); (II) ethephon (0, 0.1, 0.5, 1, 10 and 50 $\mu$mol·L$^{-1}$); (III) AVG (aminoethoxyvinylglycine,
129  0, 0.1, 1, 5 and 10 $\mu$mol·L$^{-1}$); (IV) AgNO$_3$ and NaNO$_3$ (0, 0.1, 0.3 and 0.5 $\mu$mol·L$^{-1}$); and (V) 50%
130  HRW + 0.5 $\mu$mol·L$^{-1}$ ethephon, 1 $\mu$mol·L$^{-1}$AVG, or 0.1 $\mu$mol·L$^{-1}$ AgNO$_3$. After applying
131  treatment explants were sampled to determine root number per explants. The hypocotyls (5-mm-
132  long segments of the hypocotyl base where the adventitious root develops) of the explants after
133  48 h of treatment were immediately used or frozen in liquid nitrogen for further analysis.
134

### Preparation of hydrogen-rich water (HRW)

136  Purified $H_2$ gas (99.99 %, v/v) generated from a hydrogen gas generator (QL-300, Saikesaisi

137   Hydrogen Energy Co., Ltd., China) was bubbled into distilled water at a rate of 300 mL min⁻¹ for

138   30 min. The corresponding HRW was rapidly diluted to the required saturations (Zhu et al. 2016).

139   The $H_2$ concentration analyzed by gas chromatography in freshly prepared HRW was 0.68 mM,

140   which was defined as 100% HRW, and was maintained at a relatively constant level in 25 °C for

141   at least 12 h.

142

143   **Quantification of ethylene**

144   ETH was detected by a gas chromatographic analyzer (SP-3420A, Beifenruili Inc., China). Five

145   fresh cucumber explants were placed in 20 mL vials, stoppered with secure rubber caps, and

146   containing 1 mL culture solution. These vials had the same conditions as before when the

147   seedlings grew for 12 h. A gas sample (2.5 mL) was extracted and injected into a gas

148   chromatograph, which was fitted with a GDX-502 column (2 m×3.2 mm) and a flame ionization

149   detector. The temperature of the column, inlet and detector were 50, 140 and 240 °C,

150   respectively. The flow rate of nitrogen gas, the carrier gas, was 30 mL·min⁻¹. ETH production

151   was expressed as µL·h⁻¹·kg⁻¹FW.

152

153   **Protein extraction**

154   The trichloroacetic acid (TCA)/acetone method was used to extract the total protein (Damerval et

155   al. 1986). Approximately 2.0 g of the sample was manually ground to a fine powder in liquid

156   nitrogen with 0.04 g crosslinking polyvingypyrrolidone (PVPP) and split into 6 tubes. The

157   powder was suspended in 2 mL of acetone containing 10% w/v TCA and 0.07% v/v β-

158   mercaptoethanol (β-ME). After precipitation at -20°C for at least 8 h or overnight, the pellet was

159   collected by centrifuging at 12,000×$g$ for 30 min. Then the powder was suspended in 2 mL of ice

160   cold acetone (-20°C) containing 0.07% (v/v) β-ME at 4°C for 1 h, then centrifuged at 12,000×$g$

161   for 20 min, and washed two times. The powder was then suspended in 2 mL of 80% acetone

162   containing 0.07% (v/v) β-ME at 4°C for 30 min, centrifuged at 12,000×$g$ for 15 min, and washed

163   two times. The supernatant was removed and the pellets were dried in a freezer dryer. After air-

164   drying, the powder was dissolved in a hydration solution [7 M urea, 2 M thiourea, 4% (w/v) 3-

165   [(3-Cholamidopropyl)dimethylammonio]propanesulfonate (CHAPS), 1% (w/v) DL-

166   Dithiothreitol (DTT)] at a normal temperature for 2 h, then centrifuged at 12,000×$g$ for 30 min.

167   The supernatant was the total protein. The protein content was determined calorimetrically using

168   bovine serum albumin as a standard. Protein samples were stored at -80°C for further analysis.

169

170   **2-DE and staining**

171   Sample aliquots containing protein were applied to 17 cm pH 4–7 immobilized pH gradient (IPG)

172   strips, and small volumes of lysis buffer were added to the sample aliquots. Isoelectric focusing

173   was performed on a PROTEAN IEF Cell system (Bio-Rad) for a total of 76 kVh at 20°C. The

174   voltage was set at 50 V for 14 h, 250 V for 1.5 h, 1000 V for 2.5 h, 9000 V for 5 h, and were run

175   at 90000 V and then 500 V for a maximum of 24 h. Next, the gels were run using the PROTEAN

176   II xi cell system (Bio-Rad) at 100 V for 1 h, followed by 250 V until the bromophenol blue

177   nearly reached the bottom of the gel. After electrophoresis, staining was performed by placing

178     the gels into Coomassie brilliant blue solution for 17 h. To provide support for the cucumber
179     proteomics, we performed system optimization of the pH IPG strips, loading quantity and
180     separation gel concentration. The protein spots on the pH 3-10 gels show that the acidic side (pH
181     3-5) in the region of the protein particle distribution was less than alkaline side (pH 9-10) area,
182     forming a longitudinal tail (*Fig. S1*). Most of the explant protein spots were focused in the pH 4-
183     7 region. The results showed that 800 μg of loading quantity was optimal (*Fig. S2*), and 12%
184     (w/v) gel concentration separation was the best choice to obtain the good resolving effect (*Fig.*
185     *S3*). The results also showed that pH 4-7 IPG strips (17 cm, nonlinear; Bio-Rad), 800 μg loading
186     quantity and 12% (w/v) separation gel concentration was the most suitable combination
187     for isoelectric focusing of the explants' total protein in cucumber.
188

189     **Image analysis and protein identification**
190     The proteins in the gels were visualized by CBB-G250 staining. The gel images were obtained at
191     a resolution of 300 dpi and then imported into PDQuest 8.0 (Bio-Rad). The protein spots were
192     excised from CBB-stained preparative polyacrylamide gels. The MS and MS/MS data for protein
193     identification were obtained with a MALDI-TOFTOF instrument (Zhang et al. 2015). The
194     database NCBI (https://www.ncbi.nlm.nih.gov/) were used to match and identified the protein
195     spots. According to Gene Ontology and UniProt Protein Knowledgebase
196     (http://www.uniprot.org/), the gene ontology (GO) analysis was performed on the proteins
197     identified by mass spectrometry. The proteomic data have been uploaded to the Supplemental
198     Files.
199

200     **Total RNA extraction and real time PCR**
201     Total RNA from each sample was isolated using a MiniBEST Plant RNA Extraction Kit
202     (TaKaRa, Beijing, China). Total RNA was reverse-transcribed by using the PrimeScript™RT
203     Master Mix (Perfect Real Time) according to the manufacturer's manual (Takara, Beijing,
204     China). Quantitative real-time PCR was performed with a LightCycler 96 Real- Time PCR

205     System (Roche, Switzerland) using SYBR® *Premix Ex* Taq™ Ⅱ (TaKaRa, Beijing, China).

206     Every PCR reaction was performed twice, with three independent replicates. The relative
207     expression level of each gene was acquired using a comparative Ct method followed by internal
208     control normalization. PCR was carried out in 20 μL volumes using the following amplification
209     protocol: 10 s at 95°C, followed by 40 cycles of 15 s at 95°C, and then annealing at 60°C for 30 s.
210     The relative quantization of gene expression was calculated and normalized to Actin. The
211     expression change was calculated using the $2^{-\Delta\Delta ct}$ method. The primers used for PCR analysis
212     are listed in *Table* 1.
213

214     # Results

215     **Effects of different concentrations of HRW and ethephon on adventitious root development**

216   To understand the effect of $H_2$ on adventitious root development, cucumber explants were treated
217   with different concentrations of HRW (0, 1%, 10%, 50% and 100%). As shown in *Fig. 1*,
218   different concentrations of HRW affected the development of adventitious roots. The cucumber
219   explants treated with 50% and 100% HRW produced more adventitious roots than the control
220   explants, but the root number under the 1% HRW treatment was significantly lower than that of
221   the control. The maximum root number was observed with 50% HRW treatment (*Fig. 1*) and this
222   was used for further study.
223
224   Different concentrations of ethephon, ETH-releasing compound, also had significant effects on
225   the development of adventitious roots (*Fig. 2*). Lower concentrations of ethephon (0.1, 0.5, 1,
226   and 10 $\mu mol \cdot L^{-1}$) increased adventitious root number, while the high concentration (50 $\mu mol \cdot L^{-1}$)
227   significantly inhibited adventitious roots. The maximum inducible response was observed in 0.5
228   $\mu mol \cdot L^{-1}$ ethephon-treated explants (*Fig. 2*). Thus, we used 0.5 $\mu mol \cdot L^{-1}$ to further investigate
229   the role of ethylene in rooting.
230
231   **Effects of ethylene inhibitors AVG and AgNO$_3$ on H$_2$-induced adventitious root**
232   **development**
233   To further study the function of ETH in adventitious root formation, ethylene inhibitors were
234   used. Results shown in *Fig. S4* and *Fig. S5* indicate that various concentrations of ETH synthesis
235   inhibitors AVG and AgNO$_3$ have negative effects on rooting. We used 1 $\mu mol \cdot L^{-1}$ AVG and 0.1
236   $\mu mol \cdot L^{-1}$ AgNO$_3$ for further study. As seen in *Fig. 3*, explants treated with either HRW or
237   ethephon alone showed improved adventitious rooting when compared with the control.
238   Although explants treated with HRW plus ethephon had higher root number, they were not
239   significantly higher than explants treated with either HRW or ethephon alone. When AVG
240   (inhibitor of ETH synthesis) or AgNO$_3$ (an ETH action inhibitor) was added to the HRW, the
241   inducing effects of ethephon on adventitious rooting were reversed (*Fig. 3*). Thus, we deduce
242   that ETH might be involved in H$_2$-induced adventitious root development.
243
244   **Effect of H$_2$ on ETH production and ETH-related genes' expression**
245   As shown in *Fig.4A*, the production of ETH in 50% HRW treatment was about double that of the
246   control. Moreover, the results in F*ig.4B* indicate that HRW treatment significantly enhances the
247   gene expression of *CsACS3*. When compared with the control, the expression of *CsACO1* was
248   noticeably improved by HRW. These consistent results suggested that ETH might function in the
249   downstream of H$_2$-induced adventitious root formation.
250
251   **Comparison of 2-DE gels and protein identification during adventitious root development**
252   All 70 protein spots from the 2-DE gels showed statistical differences. However, due to limited
253   genomic information about *C. sativus* in the NCBI database, only 48 differentially expressed
254   protein spots could be identified by MALDI TOF/TOF MS/MS with a significant search score,
255   shown in *Table 2* and *Fig. 5*. Out of the 48 differential proteins selected by analysis, 15 proteins
256   were down-regulated while 9 (spot 203, 230, 858, 158, 975, 1116, 1132, 626 and 1140) were up-

257  regulated in the HRW treatment; 10 proteins were down-regulated and 4 (spot 2129, 1908, 1561,
258  1284) were up-regulated in the ethephon treatment; 9 proteins were down-regulated whiles 1
259  (spot 239) was up-regulated in the HRW + AVG treatment. *Table 2* shows a list of the putative
260  names of proteins, matching species, protein score, accession number, molecular weights (MW),
261  isoelectric point (PI), peptides count and Up/Down regulation. The results indicate that matching
262  species listed above originated from the cucumber database, with molecular weights ranging
263  from 9.4 to 75.3 KDa, PI of spots ranging from 4.31 to 8.6, protein scores ranging from 62 to
264  905, and matched peptide counts between 2 and 27.

265
266  **Functional analysis of the six identified proteins**
267  Forty-eight protein spots were identified using the annotated NCBI database, and these were
268  designated as 41 known proteins and seven 7 unknown proteins. Using the Gene Ontology and
269  UniProt Protein Knowledgebase, all of the identified proteins were categorized into eight 8 major
270  groups (*Fig. 6*). Among these, Seventeen (17) photosynthesis-related proteins constituted the
271  largest percentage (35.4%), followed by six energy metabolism-related proteins (12.5%), six
272  translation and transcription-related proteins (12.5%), six protein folding, modification and
273  degradation-related proteins (12.5%), three stress response-related proteins (6.3%), two amino
274  and metabolism-related proteins (4.2%), one cellular cytoskeleton-related proteins (2.1%), and
275  seven unknown proteins (14.6%).

276
277  The differentially expressed proteins were identified using mass spectrometry and GO ontology
278  analysis. These proteins were distributed in 3 categories: "biological process" (the largest group),
279  "cellular component" and "molecular function" (the smallest group). As shown in *Fig. 7,* 4
280  categories of proteins were identified in the biological process, comparing cellular process,
281  metabolic process, response to stimulus and single-organism process; 3 categories of proteins
282  were identified in molecular function, comparing: catalytic activity, transporter activity and
283  binding; and 6 categories of proteins were identified in the cellular components, including
284  organelles, cell parts and so on.
285  Out of the 41 proteins with known functions, 5 proteins were up-regulated in HRW or ethephon
286  treatment, and were down-regulated in HRW + AVG treatment. these included ribulose-1,5-
287  bisphosphate carboxylase small subunit (Rubisco), sedoheptulose-1,7-bisphosphatase (SBPase),
288  threonine dehydratase (TDH), cytosolic ascorbate peroxidase (CAPX), and protein disulfide-
289  isomerase (PDI) (*Table 3*). The oxygen-evolving enhancer protein (OEE1) was down-regulated
290  under HRW or ethephon treatment and up-regulated under HRW + AVG treatment.

291
292  **Validation of the identified proteins at the mRNA level during adventitious root**
293  **development**
294  The mRNA levels of Rubisco, SBPase, TDH, CAPX and PDI genes under HRW or ethephon
295  treatment were significantly higher than those of the control (*Fig. 8*). However, HRW + AVG
296  treatment caused significant reduction in the expression of these genes. The mRNA levels of
297  OEE1 increased significantly in the explants treated with HRW or ethephon, and decreased

298  significantly in the explants treated with HRW+AVG. The changes of the mRNA levels of 6
299  genes were consistent with protein levels observed in the 2-DE results.
300

## Discussion

302  Since Renwick et al. (1964) first reported that $H_2$ could promote germination, the function of $H_2$
303  in plants has been extensively investigated. Recently, $H_2$ has been linked to a wide variety of
304  physiological processes in plants, ranging from the control of developmental processes to the
305  regulation responses to abiotic stresses (Xie et al. 2012, Hu et al. 2014, Wu et al. 2015, Chen et
306  al. 2017). Lin et al. (2014) and Zhu et al. (2017) reported that $H_2$ plays positive roles in
307  adventitious root development. In our study, suitable concentrations of exogenous $H_2$
308  significantly promoted the formation of adventitious roots in cucumber. Moreover, ETH was also
309  found to be involved in adventitious rooting. Previous studies showed that ETH stimulated
310  adventitious root formation in some crops including mung bean (Pan et al. 2002), sunflower (Liu
311  et al. 1990) and marigold (Jin et al. 2017). Xu et al. (2017) revealed that ETH increased the
312  formation of adventitious root in dose-dependent experiments. Our results suggested that
313  exogenous ETH and 0.5 $\mu mol \cdot L^{-1}$ ethephon significantly enhanced adventitious rooting. Evidently,
314  both $H_2$ and ETH can induce adventitious rooting, but the interaction between $H_2$ and ETH
315  during that process remains poorly understood. The results in this study suggest that ETH is
316  involved in the $H_2$-induced adventitious rooting process and might function at the downstream of
317  $H_2$.
318

319  We found that the selected ethylene inhibitors AVG and $AgNO_3$, caused partial inhibition of $H_2$-
320  induced adventitious root development. When comparing HRW and the control in the production
321  of ETH and the expression of two ETH-related genes, the HRW treatment was obviously higher
322  than the control. These results suggest that ETH may be a downstream signal molecule in $H_2$-
323  induced adventitious root development.
324

325  To further prove that ETH is involved in $H_2$-induced adventitious root formation, we used
326  proteomic analysis, which allowed us to identify proteins whose expression was altered during
327  adventitious rooting particularly in regard to $H_2$ and ETH treatments. The results of proteomic
328  analysis showed that there were 41 differential proteins with known functions successfully
329  identified in the adventitious root formation of cucumber.They were distributed among 3
330  categories. We also found 6 proteins, Rubisco, SBPase, TDH, CAPX, PDI and OEE1, to be used
331  for further study.
332

333  It is known that Rubisco, SBPase and OEE1 proteins are related to the photosynthetic process.
334  Rubisco is a vital enzyme associated with carbon fixation (Xu et al. 1994) and its catalytic
335  inefficiencies often limit plant productivity (Andersson et al. 2008, Gunn et al. 2017). SBPase
336  has been shown to increase photosynthetic capacity and plant yield and it also promotes the
337  growth of plants at the early growth stage (Ogawa et al. 2015). Our results show that Rubisco

338   and SBPase expression were up-regulated under HRW or ethephon treatment, suggesting that $H_2$
339   and ETH may increase the efficiency of photosynthesis in explants by enhancing Rubisco and
340   SBPase expression during rooting. ETH inhibitor AVG inhibited the inducing effects of HRW on
341   Rubisco and SBPase, further indicating that ETH may play important roles in the regulation of
342   $H_2$ during $H_2$-induced adventitious root formation.
343
344   OEE1 has been suggested to be involved in photosynthesis and PS II activities (Yabuta et al.
345   2008). Chen et al. (2017) reported that OEE1 protein is necessary for oxygen evolving activity
346   and is vital to maintain the stability of photosystem II. Sugihara et al. (2000) demonstrated that
347   the level of OEE1 increased under NaCl treatment, and similar results were obtained at the
348   transcription level. Fatehi et al. (2012) proposed that OEE1 was up-regulated in response to
349   salinity, although in their study, $H_2$ and ETH treatment down-regulated OEE1 expression. This
350   was similar to the results of Bai et al. (2017) who reported that the protein levels of OEE1 in oat
351   leaves decreased in response to salinity. The possible explanation for this discrepancy is that
352   OEE1 may change under different transcription, translation or treatment.
353
354   Amino acid metabolism has been found to play an important role in plant protein synthesis,
355   photosynthesis, and development (He et al. 2013). TDH, a key enzyme in amino acid metabolism,
356   contribute to both carbon skeleton supply and ammonium assimilation in plants and also plays an
357   important role in metabolic acclimation (Möckel et al. 1992). Our study suggests that $H_2$ up-
358   regulated TDH expression and ETH inhibitor AVG suppress the inducing effects. Proteomic
359   analysis showed higher levels of TDH accumulation in wild ginseng than in cultivated ginseng
360   (Sun et al. 2016). Moreover, proteomic analysis of rice and wheat coleoptiles revealed the
361   potential role of amino acid biosynthesis in cellular anoxia tolerance (Shingakiwells et al. 2011).
362   Thus, $H_2$ may increase carbon skeleton supply and metabolic acclimation in explants by
363   enhancing TDH expression, which promotes adventitious root development. ETH is essential
364   during this process. CAPX plays an essential role in the antioxidant defense mechanism (Wu et
365   al. 2014) regulates plant growth, and induces organ formation (Zhang et al. 2013). The
366   involvement of APX in fruit ripening and senescence has been proposed (Torres et al. 2003;
367   Giribaldi et al. 2007), and Caverzan et al. (2012) demonstrated that APX genes can regulate both
368   plant response to stresses and plant development. Additionally, *APX* mutant plant genes showed
369   alterations in growth, physiology and antioxidant metabolism, suggesting the function of APX in
370   plant development. Our results also show that CAPX expression was up-regulated by HRW or
371   ethephon treatment, suggesting that $H_2$ may increase the reduction of oxidative damage and
372   increase the productivity of explants by enhancing CAPX expression. However, ETH inhibitor
373   AVG depressed the promotive effects of HRW on CAPX. PDI, related to protein folding,
374   modification and degradation, is a multifunctional protein in cells that assists many protein
375   maturation processes (Wilkinson et al. 2004). It may catalyze protein thiol oxidation and restore
376   and catalyze transformation disulfide bonds (Houston et al. 2005). PDI is also mainly associated
377   with the protein secretory pathway in plants (Irsigler et al. 2007), and has been found to be
378   down-regulated in rice under freezing stress (Hashimoto and Komatsu 2007) and in soybean

379  leaves under drought stress (Irsigler et al. 2007). In our study, PDI was up-regulated under HRW
380  treatment and down-regulated under HRW+AVG treatment, suggesting that $H_2$ may increase the
381  capacity of disulfide-bonded proteins by enhancing PDI expression. Meanwhile, ETH inhibitor
382  AVG reverses the inducing effects of HRW on PDI, indicating that ETH may play important
383  roles in $H_2$-induced CAPX and PDI expression during rooting.
384

## Conclusion

386  Our results show that both $H_2$ and ETH play crucial roles in promoting adventitious root
387  development. ETH might work as a downstream signaling molecule during $H_2$-induced rooting.
388  Further proteomic studies show that photosynthesis-related proteins (Rubisco and SBPase),
389  amino and metabolism-related protein (TDH), stress response-related protein (CAPX), and
390  folding, modification and degradation-related protein (PDI) may play positive roles in the ETH-
391  $H_2$-induced adventitious rooting process. Oxygen-evolving enhancer protein (OEE1) may play
392  inhibiting roles during that process. The mechanisms underlying adventitious root development
393  are very complex and more research will need to be carried out to fully understand adventitious
394  rooting signaling.
395

## Acknowledgments

397  We thank Dr. Yongchao Zhu from Zhejiang University for providing guidance on experimental
398  methods. We also thank Dr. Chen Bai from Gansu Academy of Agricultural Sciences for
399  supplying a gas chromatographic analyzer.

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586

List of the genes whose transcription profile was evaluated by RT-PCR

2 **Table 1** List of the genes whose transcription profile was evaluated by RT-PCR

| Gene name | NCBI accession number | primer | 5′-3′ primer sequence |
|---|---|---|---|
| CsACS3 | AB006805.1 | F | 5'-CCTTGCAGAGGCTGGCGATG-3' |
| | | R | 5'-GGTGACTTGGAAGCCGTTGGAG-3' |
| CsACO1 | AB006806.1 | F | 5'-AGGTAGGTGGCCTGCAACTCC-3' |
| | | R | 5'-CTCCGAGGTTGACGACAATGGC-3' |
| Rubisco | NP_198657.1 | F | 5'-GAGATTGAGGAGGCTAAGAAGGAA-3' |
| | | R | 5'-GGGCTTGTAGGCGATGAAAC -3' |
| SBPase | NP_001267658.1 | F | 5'-GAGTTCGTTATTTGGGGAGTCATT -3' |
| | | R | 5'-TTATCAGGGGTTGCTTTGGTG -3' |
| TDH | XM_004143321.2 | F | 5'-CCATTCAACTTTCCAACAGAACC -3' |
| | | R | 5'- CGAGCCATCAACAACAGCA-3' |
| CAPX | NM_001280706 | F | 5'-TTGGCTGGTGTTGTTGCTGT -3' |
| | | R | 5'-GGCTCGGGTTTGTCCTCTCT -3' |
| PDI | XM_004149750.2 | F | 5'- TGAGTTTTACGCCCCTTGGT-3' |
| | | R | 5'-TCTCTGTTTGACTCCTCGTTGG -3' |
| OEE1 | XM-004141898.2 | F | 5'-TTTGAAGTTGGTGCTGATGGTT-3' |
| | | R | 5'- GGTGAAGAGGAACGGGACA-3 |
| Actin | DQ115883.1 | F | 5'-TGGACTCTGGTGATGGTGTTA-3' |
| | | R | 5'-CAATGAGGGATGGCTGGAAAA-3' |

Identification and analysis of proteins differentially expressed in response to ETH inhibitor on $H_2$-induced adventitious root development in cucumber explants

1    **.Table 2** Identification and analysis of proteins differentially expressed in response to ETH

2    inhibitor on $H_2$-induced adventitious root development in cucumber explants

| Spot No.[a] | Identified protein [b] | Species | Score [c] | NCBI accession Number | MW [d] | Pi [e] | Peptides Count [f] | Up/Down [g] |
|---|---|---|---|---|---|---|---|---|
| K9/1628 | Glutamine synthetase | *Cucumis sativus* | 406 | KGN57792.1 | 39251.8 | 5.82 | 13 | ↓ |
| K10/2129 | ribulose bisphosphate carboxylase/oxygenase precursor peptide | *Cucumis sativus* | 78 | AAA33131.1 | 21049.4 | 7.55 | 12 | ↑ |
| K11/1937 | Unknow protein | *Cucumis sativus* | 234 | KGN44928.1 | 22556.6 | 4.45 | 13 | ↓ |
| K12/1908 | Cytosolic ascorbate peroxidase | *Cucumis sativus* | 595 | KGN65655.1 | 27377.8 | 5.43 | 17 | ↑ |
| K13/1561 | Sedoheptulose-1,7-bisphosphatase | *Cucumis sativus* | 534 | KGN50632.1 | 42075.4 | 5.96 | 22 | ↑ |
| K14/1799 | Eukaryotic translation elongation factor 1 delta isoform X1 | *Cucumis sativus* | 417 | XP_011652060.1 | 24670.3 | 4.45 | 15 | ↓ |
| K15/1817 | Oxygen-evolving enhancer protein 1 | *Cucumis sativus* | 905 | KGN48464.1 | 34938.8 | 6.24 | 24 | ↓ |
| K16/2139 | PREDICTED:11S globulin seed storage protein 2-like | *Cucumis sativus* | 458 | XP_011652795.1 | 54382 | 5.71 | 11 | ↓ |
| K17/2062 | Cytochrome b6-f complex iron-sulfur subunit | *Cucumis sativus* | 112 | KGN43571.1 | 24255.2 | 8.45 | 9 | ↓ |
| K18/1169 | Chloroplast transketolase | *Cucumis sativus* | 712 | KGN56609.1 | 80569.6 | 6 | 27 | ↓ |
| K19/2156 | Ribulose bisphosphate carboxylase small chain | *Cucumis sativus* | 68 | KGN52085.1 | 20685.2 | 8.24 | 11 | ↓ |
| K20/1284 | ATPase alpha subunit (chloroplast) | *Cucumis sativus* | 385 | AAZ94637.1 | 55348 | 5.13 | 24 | ↑ |
| K21/2064 | ATPase alpha subunit (chloroplast) | *Cucumis sativus* | 246 | AAZ94637.1 | 55348 | 5.13 | 20 | ↓ |
| K22/361 | 26S protease regulatory subunit 6A homolog | *Cucumis sativus* | 230 | XP_004135596.1 | 38614.9 | 5.16 | 21 | ↓ |
| K23/748 | Oxygen-evolving enhancer protein 1 | *Cucumis sativus* | 752 | KGN48464.1 | 34938.8 | 6.24 | 20 | ↓ |
| K24/533 | Mg-protoporphyrin IX chelatase | *Cucumis sativus* | 236 | KGN49283.1 | 45728 | 5.72 | 20 | ↓ |
| L1/203 | Protein disulfide-isomerase | *Cucumis sativus* | 83 | KGN47715.1 | 57045.6 | 4.88 | 12 | ↑ |
| L2/230 | Threonine dehydratase | *Cucumis sativus* | 620 | KGN48214.1 | 67198.9 | 6.22 | 25 | ↑ |
| L3/1196 | PSII reaction center subunit V (chloroplast) | *Cucumis sativus* | 103 | AAZ94668.1 | 9380.7 | 4.83 | 4 | ↓ |
| L4/858 | Cytosolic ascorbate peroxidase | *Cucumis sativus* | 716 | KGN65655.1 | 27377.8 | 5.43 | 15 | ↑ |
| L6/656 | Proteasome subunit alpha type | *Cucumis sativus* | 466 | KGN62618.1 | 30861.4 | 4.98 | 9 | ↓ |
| L7/1009 | Unknow | *Cucumis sativus* | 412 | KGN57117.1 | 18646.5 | 4.56 | 9 | ↓ |
| L8/158 | Malic enzyme | *Cucumis sativus* | 496 | KGN66170.1 | 65093.8 | 5.72 | 23 | ↑ |
| L9/975 | Chlorophyll a-b binding protein, | *Cucumis sativus* | 270 | KGN51208.1 | 26517.6 | 5.84 | 7 | ↑ |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | chloroplastic | | | | | | | |
| L10/1116 | 60S acidic ribosomal protein P1 | *Cucumis sativus* | 355 | KGN56349.1 | 11414.7 | 4.34 | 3 | ↑ |
| L11/747 | Cysteine protease | *Cucumis sativus* | 114 | KGN62647.1 | 40614.9 | 5.2 | 8 | ↓ |
| L12/200 | Protein disulfide-isomerase | *Cucumis sativus* | 90 | KGN47715.1 | 57045.6 | 4.88 | 18 | ↓ |
| L13/527 | Mg-protoporphyrin IX chelatase | *Cucumis sativus* | 200 | KGN49283.1 | 45728 | 5.72 | 19 | ↓ |
| L14/1132 | Ribulose bisphosphate carboxylase small chain | *Cucumis sativus* | 100 | KGN52085.1 | 20685.2 | 8.24 | 11 | ↑ |
| L15/502 | Unknow | *Cucumis sativus* | 312 | KGN57401.1 | 31417.5 | 4.82 | 5 | ↓ |
| L16/626 | Sedoheptulose-1,7-bisphosphatase | *Cucumis sativus* | 546 | KGN50632.1 | 42075.4 | 5.96 | 18 | ↑ |
| L17/896 | PREDICTED: 29 kDa ribonucleoprotein, chloroplastic | *Cucumis sativus* | 298 | XP_004137828.1 | 30479.5 | 5.84 | 7 | ↓ |
| L18/103 | Chloroplast HSP70 | *Cucumis sativus* | 412 | KGN61439.1 | 75350 | 5.18 | 25 | ↓ |
| L19/447 | 30S ribosomal protein S1, chloroplastic | *Cucumis sativus* | 496 | XP_004147619.1 | 45304.5 | 5.34 | 18 | ↓ |
| L20/1129 | PREDICTED: 60S acidic ribosomal protein P2-4 | *Cucumis sativus* | 93 | XP_004150488.1 | 11419.9 | 4.53 | 8 | ↓ |
| L21/982 | PREDICTED: 11S globulin subunit beta | *Cucumis sativus* | 479 | XP_011651441.1 | 57575.8 | 7.21 | 15 | ↓ |
| L22/1140 | ribulose-1,5-bisphosphate carboxylase small subunit | *Cucumis sativus* | 113 | CCF55356.1 | 20713.2 | 8.24 | 15 | ↑ |
| L23/732 | PREDICTED: 14-3-3-like protein D | *Cucumis sativus* | 120 | XP_004136830.1 | 29379.5 | 4.76 | 10 | ↓ |
| M15/139 | PREDICTED: ruBisCO large subunit-binding protein subunit alpha | *Cucumis sativus* | 401 | XP_004145754.1 | 61399.9 | 5.06 | 26 | ↓ |
| M17/239 | Oxygen-evolving enhancer protein 1 | *Cucumis sativus* | 216 | KGN48464.1 | 34938.8 | 6.24 | 19 | |
| M18/1018 | PREDICTED: 60S acidic ribosomal protein P1 | *Cucumis sativus* | 170 | KGN56349.1 | 11414.7 | 4.34 | 2 | ↓ |
| M19/1030 | Cytosolic ascorbate peroxidase | *Cucumis sativus* | 407 | KGN65655.1 | 27377.8 | 5.43 | 17 | ↓ |
| M20/340 | ribulose-1,5-bisphosphate carboxylase small subunit | *Cucumis sativus* | 137 | CCF55356.1 | 20713.2. | 8.24 | 14 | ↓ |
| M21/162 | Threonine dehydratase | *Cucumis sativus* | 206 | KGN48214.1 | 67198.9 | 6.22 | 22 | ↓ |
| M23/462 | Sedoheptulose-1,7-bisphosphatase | *Cucumis sativus* | 250 | KGN50632.1 | 42075.4 | 5.96 | 18 | ↓ |
| M24/354 | Beta-form rubisco activase | *Cucumis sativus* | 241 | KGN50568.1 | 48292.6 | 8.19 | 19 | ↓ |
| N1/351 | Unknow | *Cucumis sativus* | 238 | KGN59544.1 | 36680 | 4.7 | 23 | ↓ |
| N2/117 | Protein disulfide-isomerase | *Cucumis sativus* | 62 | KGN47715.1 | 57045.6 | 4.88 | 16 | ↓ |

3  Spots K9/1628 to K21/2064 were the proteins in ETH treatment; K22/361 to L23/732 were the proteins in ETH treatment and the
4  control; M15/139 to N2/117 were the proteins in HRW+AVG treatment.

5    [a] Spot numbers are arbitrary as assigned by the software of PD-Quest 8.0.

6    [b] The name and functional categories of the proteins using MALDI TOF-TOF MS.

7    [c] The Mascot score obtained after searching against the NCBI nr database.

8    [d, e] Theoretical molecular mass (MV) and isoelectric point (PI) of the identified protein.

9    [f] Number of peptides sequenced.

10   [g] " ↑ " represents up-regulated expression, " ↓ " represents down-regulated expression.

Analysis of differential protein expression during $H_2$, ETH and AVG-induced adventitious root development

2 **Table 3** Analysis of differential protein expression during $H_2$, ETH and AVG-induced adventitious

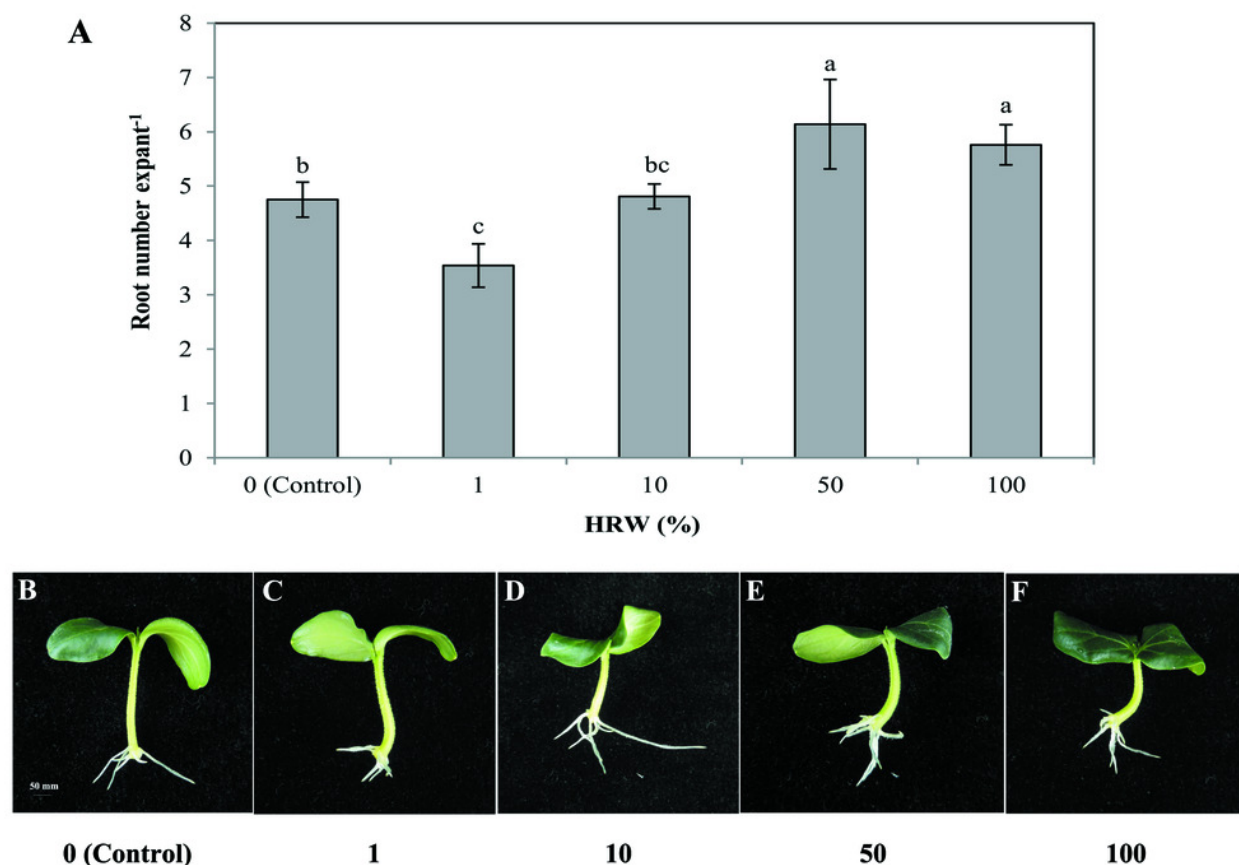
3 root development

| Protein name | Functional category | Control | $H_2$ | ETH | $H_2$+AVG |
|---|---|---|---|---|---|
| ribulose-1,5-bisphosphate carboxylase small subunit | Photosynthesis | ⎯⎯ | ↑ | ↑ | ↓ |
| Sedoheptulose-1,7-bisphosphatase | photosynthesis | ⎯⎯ | ↑ | ↑ | ↓ |
| Threonine dehydratase | amino and metabolism | ⎯⎯ | ↑ | ⎯⎯ | ↓ |
| Cytosolic ascorbate peroxidase | stress response | ⎯⎯ | ↑ | ↑ | ↓ |
| Protein disulfide-isomerase | Protein folding, modification and degradation | ⎯⎯ | ↑ | ⎯⎯ | ↓ |
| Oxygen-evolving enhancer protein 1 | photosynthesis | ⎯⎯ | ↓ | ↓ | ↑ |

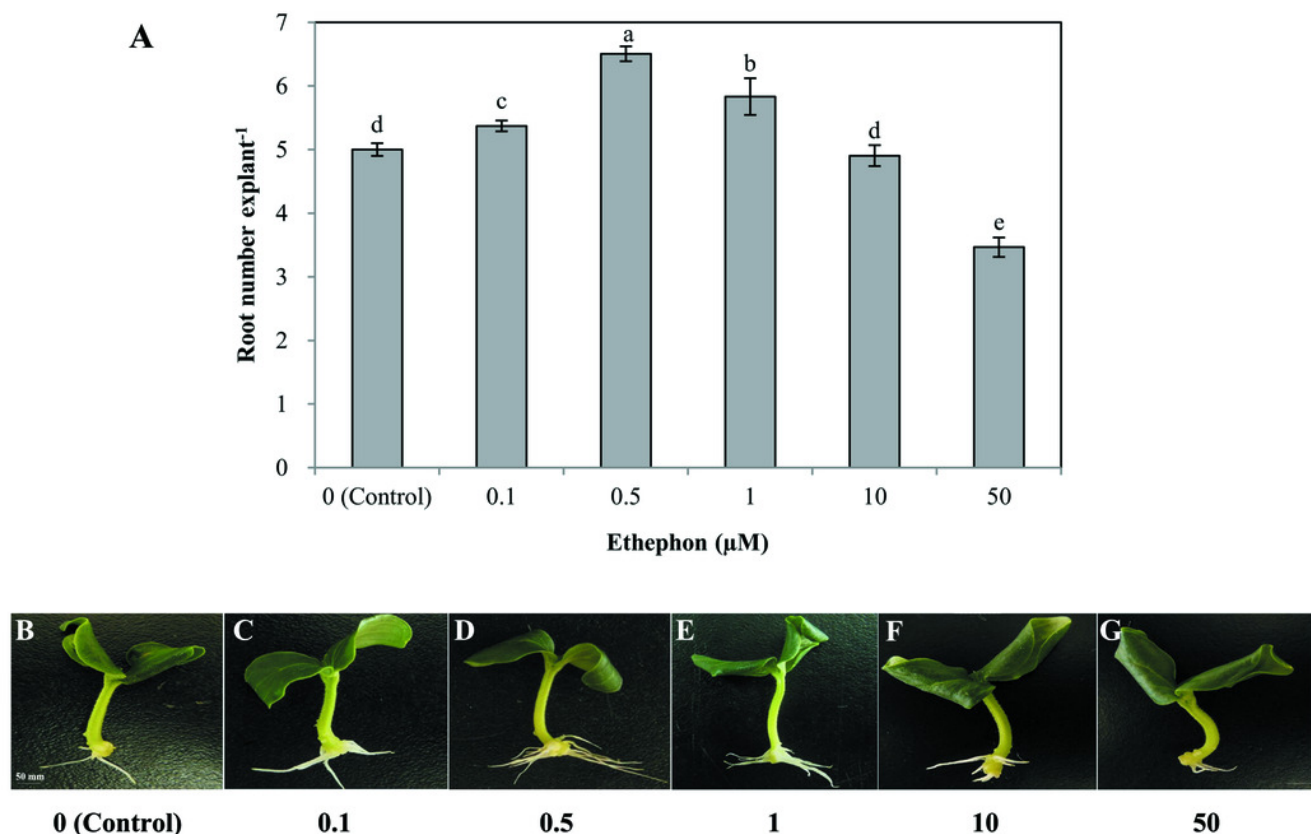
4 " ↑ " represents up-regulated expression, " ↓ " represents down-regulated expression.

# Figure 1

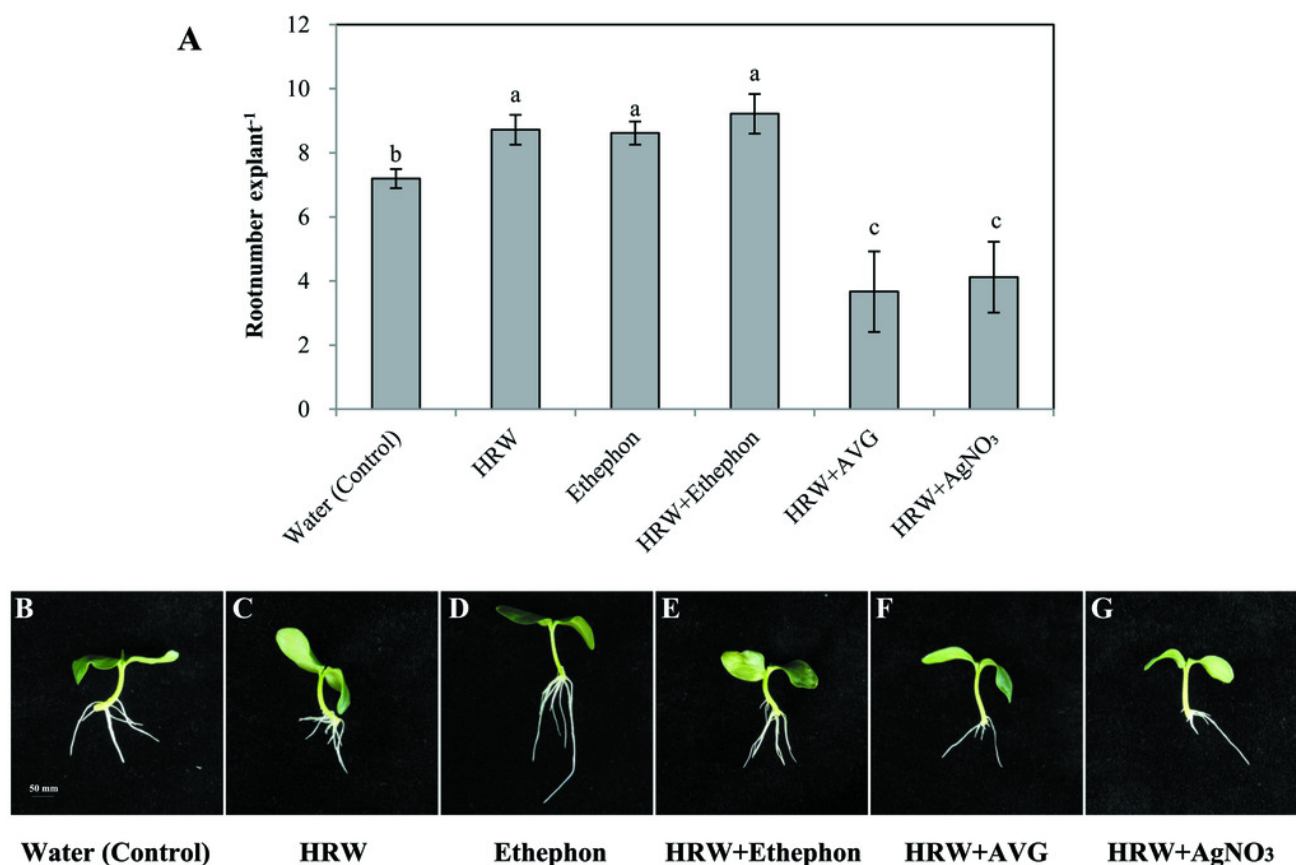
Effects of different concentrations of HRW on adventitious root development.

The primary root system was removed from hypocotyls of 7-day-old germinated cucumber. Explants were incubated with distilled water or different concentrations of HRW and indicated for 7 days. The number (**A**) of adventitious roots per explant were expressed as mean ± SE (n = 20 explants from each of four independent experiments). Bars with different lowercase letters were significantly different (Duncan's multiple range test, *P* < 0.05. Photographs (**B-F**) show hypocotyl explants after 7 days of the treatments indicated.

# Figure 2

Effects of different concentrations of ethephon on adventitious root development.

The primary root system was removed from hypocotyls of 7-day-old germinated cucumber. Explants were incubated with distilled water or different concentrations of ethephon and indicated for 7 days. The number (**A**) of adventitious roots per explant were expressed as mean ± SE (n = 20 explants from each of four independent experiments). Bars with different lowercase letters were significantly different (Duncan's multiple range test, $P < 0.05$. Photographs (**B-G**) show hypocotyl explants after 7 days of the treatments indicated.

# Figure 3

Effects ETH inhibitor on $H_2$-induced adventitious root development.

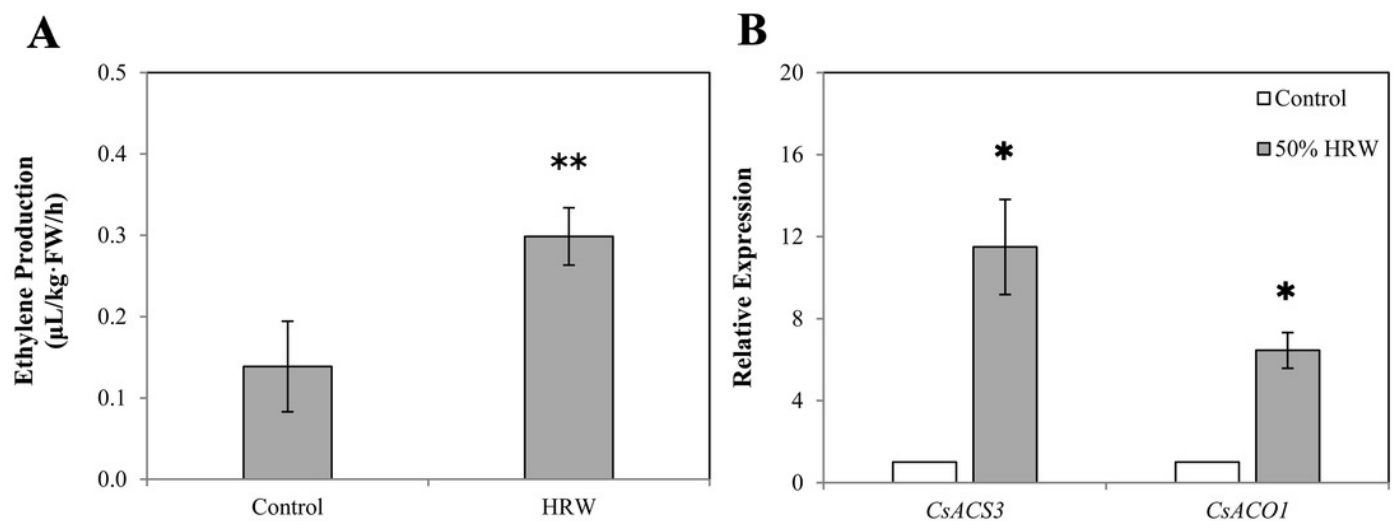
The primary root system was removed from hypocotyls of 7-day-old germinated cucumber. Explants of cucumber were incubated with 0.5 µM ethephon, 50% HRW, 50% HRW + 0.5 µM ethephon, 50% HRW + 1 µM AVG, 50% HRW+ 0.1 µM AgNO$_3$/NaNO$_3$ and indicated for 7 days. The number (**A**) of adventitious roots per explant were expressed as mean ± SE (n = 20 explants from each of four independent experiments). Bars with different lowercase letters were significantly different (Duncan's multiple range test, $P < 0.05$. Photographs (**B-G**) show hypocotyl explants after 7 days of the treatments indicated.

# Figure 4

The effect of HRW on the ETH production (A) and on the expression level (B) of two ETH-related genes in cucumber explant.

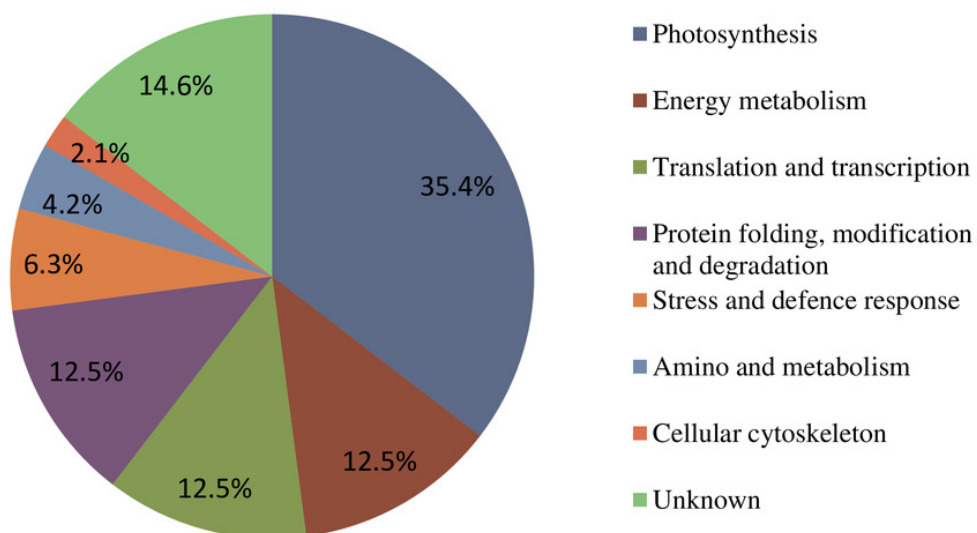
The values (means ± SE) are the average of three independent experiments. Bars with an asterisk presents significant ($P < 0.05$), and two asterisks presents very significant ($P < 0.01$).

# Figure 5

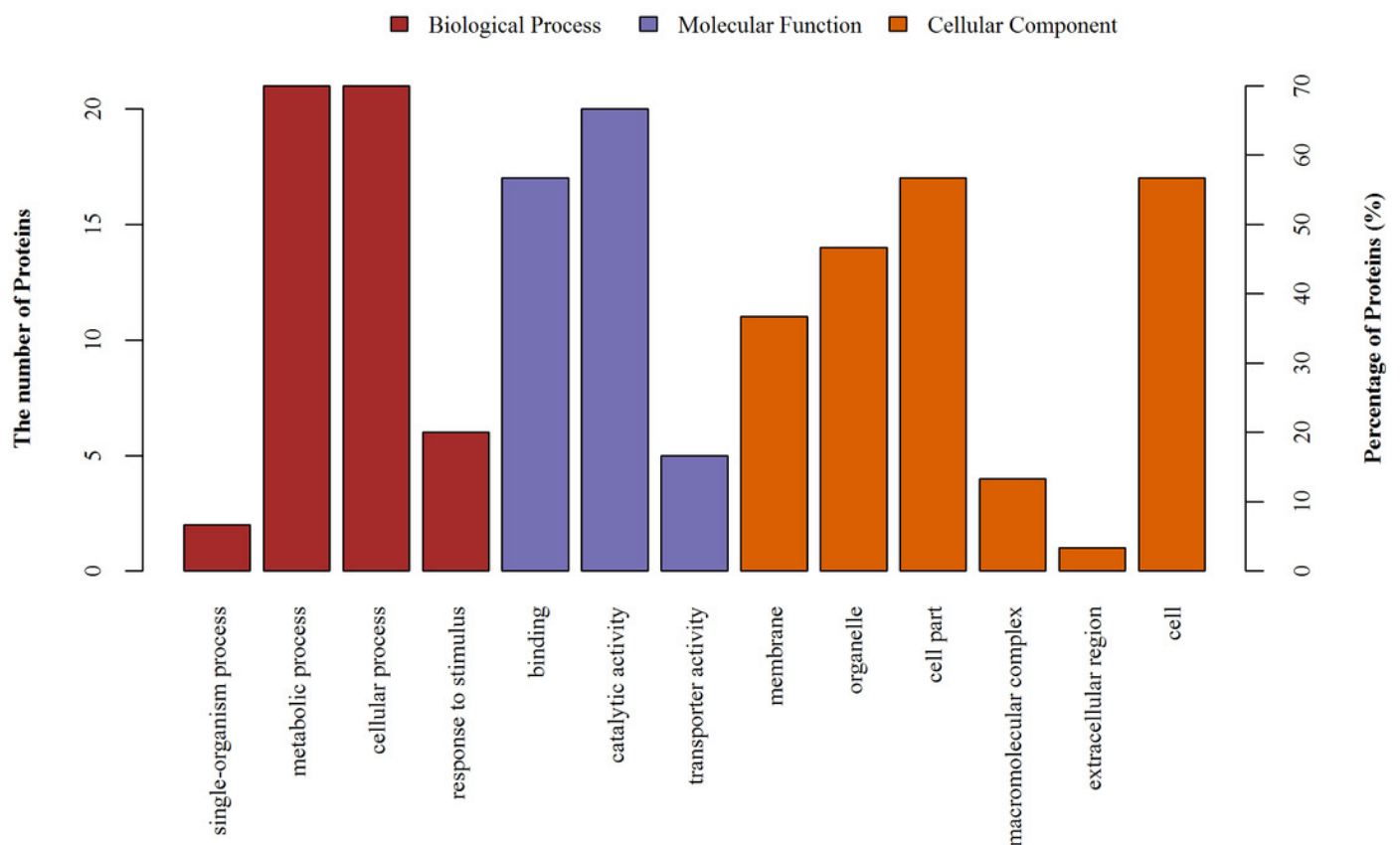
Separation of the total proteins extracted from the explants of cucumber on 2-DE gels over the pH range 4-7.

Each map depicts one representative gel (of three replicates). A total of 48 protein spots showing difference are numbered on the gels. Arrows indicate the protein spots that were positively identified. **A**, Control. **B**, HRW. **C**, ethephon. **D**, HRW + AVG.

# Figure 6

The functional classification and distribution of all 41 identified proteins.

Unknown proteins include those whose functions have not been described.

# Figure 7

The gene ontology (GO) analysis of the identified proteins.

Information of number and percentage of involved proteins in a term are shown in left and right y-axis.

# Figure 8

The effect of ETH and HRW on the expression levels of *rbcS* (A), *SBPase* (B), *TDH* (C), *OEE1* (D), *CAPX* (E) and *PDI* (F).

The values (means ± SE) are the average of three independent experiments. Bars with different lowercase letters were significantly different by Duncan's multiple range test (P < 0.05).