Roles of hydrogen gas in plants: a review

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Abstract. Hydrogen gas (H2) was first identified as a unique molecular messenger in animals. Since H2 was reported as a novel antioxidant, it has been proven effective in treating many diseases. However, the studies concerning H2 in plants are just beginning to emerge. Here, two paths of H2 production in plants have been reported, namely, hydrogenase and nitrogenase. H2 has positive effects on seed germination, seedling growth, adventitious rooting, root elongation, harvest freshness, stomatal closure and anthocyanin synthesis. H2 also can enhance plant symbiotic stress resistance commonly through the enhancement of antioxidant defence system. Moreover, H2 shows cross talk with nitric oxide, carbon monoxide and other signalling molecules (for example, abscisic acid, ethylene and jasmonate acid). H2 can regulate the expression of responsive genes under abiotic stress and during adventitious roots formation and anthocyanin biosynthesis. Future work will need to focus on the molecular mechanism of H2 and its crosstalk with other signalling molecules in plants. With its promising application in agriculture, hydrogen agriculture will be welcomed in the near future.

Additional keywords: gene expression, reactive oxygen species.

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Introduction

Hydrogen is the most abundant element in the universe, occupying ~75% of the mass of the universe. It is also the most abundant element of human body composition. Hydrogen gas (H2), a colourless, odourless and tasteless gas is the structurally simplest gas in nature. In animals, H2 has come to the forefront of therapeutic medical gas research. Ohsawa et al. (2007) first reported that H2 is an antioxidant that can selectively reduce cytotoxic free radicals. This surprising discovery immediately attracted the attention of numerous researchers all over the world. Since then, many studies in a variety of biomedical fields using clinical and experimental models for many diseases have proven that H2 can act as a feasible therapeutic strategy in different disease models, including diabetes mellitus type (Kajiyama et al. 2008), organ transplantation (Cardinal et al. 2010), radiation therapy (Qian et al. 2010) and metabolic syndrome (Kamimura et al. 2011). Therefore, it is postulated that the hydrogen-containing agents would be universally applied in clinical practice.

Recently, attention in the role of H2 has shifted from animals to plants. H2 production in plants has been extensively studied (Stephenson and Stickland 1931; Gaffron and Rubin 1942). Accumulated evidence suggests that H2 is continually synthesised through various sources including enzyme and non-enzyme pathways in organisms (Das et al. 2008). Some researchers studied H2 for the purpose of developing H2-based fuel cells (Melis 2012). During the last decade, H2 has been one of the molecules that has received much attention from plant researchers. Similar to the beneficial roles in animals, H2 has been emerged as an important bio-regulator in plants under abiotic stresses and during diverse physiological processes (Xu et al. 2013; Cui et al. 2014; Zhang et al. 2015). Under these new development conditions, we noted that the physiological effects of H2 were often accompanied by changes in reactive oxygen species (ROS) (Zeng et al. 2013), suggesting that the function of H2 is partially dependent on ROS regulation.

The botanical effects of H2 in plants have been discussed elsewhere previously (Zeng et al. 2014). Considering the rapid increase in interest in studies of H2 in plants, this paper provides a critical review about the most recent works in the literature regarding some mechanisms about the basic physiological processes of H2 and its interaction with some gas molecules. In addition, we summarise briefly H2-mediated stress responses and related gene expression.

Production of H2 in plants

In organisms, the emission of H2 was first observed in bacteria by Stephenson and Stickland (1931). Then, Gaffron and Rubin (1942) reported the metabolism of H2 in algae. Until now, hydrogenase and nitrogenase have been reported to be involved the H2 production in organisms (Das et al. 2008; Fig. 1). As shown in Fig. 1a, electrons are formed and transmitted in photosynthetic systems. Then, by the activity of hydrogenase or nitrogenase, Fd(red) can be re-oxidised and form H2. Besides, compounds such as starch and glycogen accumulating during carbon dioxide (CO2) fixation are degraded...
root elongation and the H2 production in alfalfa leaves during indicated a positive interrelationship between the inhibition of induced by both hormone and abiotic stress. H2 production in of concentration of endogenous H 2 (25\text{mM}) in alfalfa seedlings in a dose-dependent manner. The highest detected after 24 h of treatment with 100\text{mM}. H2 production in rice seedlings was induced by abscisic acid (Kim and Kim 2011). These authors also found that exogenous hydrogenase can release H2 during the nitrogen fixation process (Fig. 1a). In addition, rhizobia can release H2 in the nitrogen fixation process (Fig. 1b).

As mentioned above, H2 production in bacteria and algae has been well studied. However, only a few studies have reported H2 production and hydrogenase in higher plants. Boichenko (1947) studied the H2 evolution in isolated chloroplasts and postulated the existence of hydrogenase in plants. Sanadze (1961) first demonstrated that H2 was released and absorbed in plant leaves. After that, Renwick et al. (1964) found that winter rye seeds could release H2. The authors also reported that exogenous H2 could promote the germination rate of winter rye seeds. In addition, rhizobia from symbiosis with leguminous plants could release H2 in the nitrogen fixation process (Dong et al. 2003; Irvine et al. 2004), but the existence of rhizobia in plants has not yet been elucidated. Recently, Xie et al. (2012) found that NaCl treatment promoted endogenous H2 release in Arabidopsis seedlings, reaching the highest level of more than 100 \text{μmol g}^{-1} \text{FW} after 6 h of treatment. Jin et al. (2013) reported that paraquat-induced oxidative stress (PQ) promoted endogenous H2 release in alfalfa seedlings in a dose-dependent manner. The highest concentration of endogenous H2 (25 \text{μmol g}^{-1} \text{FW}) was detected after 24 h of treatment with 100 \text{μM} PQ. They also indicated a positive interrelationship between the inhibition of root elongation and the H2 production in alfalfa leaves during exposure to PQ. The revolution of H2 in rice seedlings was 0.318 and 0.305 \text{mM} g FW 1 h^{-1} under light and in the dark respectively (Zeng et al. 2013). These authors also found that H2 production in rice seedlings was induced by abscisic acid (ABA), ethylene (ET), jasmonate acid (JA), salt and drought stresses. The production of H2 was consistent with hydrogenase activity and the expression of putative hydrogenase genes in rice seedlings. Some putative genes encoding hydrogenase have been found in rice (Zeng et al. 2013). Therefore, H2 production was induced by both hormone and abiotic stress. H2 production in plants is dependent not only on hydrogenase, but also dependent on nitrogenase. Although the activities of hydrogenase and nitrogenase have been revealed, the related coding genes and their synthetic pathways are still unknown. Thus, it is of great interest to know the H2 biosynthesis pathways in higher plants. H2 as a signalling molecule can move freely from one plant cell to another cell. It is also conceivable that H2 might serve as an informational signal to other parts of the plants, or to plants in the vicinity. Thus, how do plants sense the H2 signalling? So far, the answer to this question is still unknown.

**H2 regulates plant growth and development**

Hydrogen-rich water (HRW) was regarded as a safe and easily available way to mimic the physiological functions of endogenous H2 in plants and animals. In animals, our understanding about the biological roles of H2 has developed rapidly. It was reported that H2 could protect animal cells by increasing the activity of antioxidant enzymes such as catalase (CAT) and superoxide dismutase (SOD) (Xie et al. 2010). More importantly, a growing number of studies have found that H2 is an antioxidant that can selectively reduce cytotoxic free radicals (Hayashida et al. 2008; Matsumoto et al. 2009). Recently, the roles of H2 in plants have been studied during several plant growth and development stages including seeds germination, seedling growth, root development, stomatal closure and anthocyanin synthesis.

**Seeds germination**

Seed germination, which is a highly specialised phase in plant life, is essential for seedling establishment. It is a critical step in a plant’s life cycle, and is regulated by a wide range of endogenous and environment factors (Kong et al. 2015). A role for H2 in plants was first reported in winter rye seeds, where H2 was found to induce seeds germination (Renwick et al. 1964). Since then, Xu et al. (2013) has demonstrated that H2 exerted an advantageous
Table 1. Overview of H2-mediated physiological processes in plants

<table>
<thead>
<tr>
<th>Plant growth and development</th>
<th>Plant species</th>
<th>Tissues</th>
<th>H2-induced effect</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Seeds germination</td>
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<td>Seeds</td>
<td>Increased seed germination</td>
<td>Xu et al. (2013)</td>
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<tr>
<td>Rice</td>
<td>Seeds</td>
<td></td>
<td>Increased seed germination in NaCl</td>
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<tr>
<td>Seedlings growth</td>
<td>Chinese cabbage</td>
<td>Seedlings</td>
<td>Alleviated Cd-induced growth inhibition</td>
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</tr>
<tr>
<td>Maize</td>
<td>Seedlings</td>
<td></td>
<td>Improved net photosynthetic rate</td>
<td>Zhang et al. (2015)</td>
</tr>
<tr>
<td>Alfalfa</td>
<td>Seedlings</td>
<td></td>
<td>Increased alfalfa seedling growth</td>
<td>Dai et al. (2017)</td>
</tr>
<tr>
<td>Cucumber</td>
<td>Explants</td>
<td></td>
<td>Improved AR development, increased NO content, upregulated NR genes, improved AR development, increased NO content</td>
<td>Zhu et al. (2016a)</td>
</tr>
<tr>
<td>Adventitious rooting (AR)</td>
<td>Cucumber</td>
<td>Explants</td>
<td>Upregulated cell cycle-related genes, promoted AR formation</td>
<td>Zhu et al. (2016a)</td>
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<tr>
<td>Rice</td>
<td>Root</td>
<td></td>
<td>50% HRW had the most significant effect</td>
<td>Lin et al. (2014)</td>
</tr>
<tr>
<td>Alfalfa</td>
<td>Root</td>
<td></td>
<td>50% HRW significantly recovered in Cd</td>
<td>Chen et al. (2014)</td>
</tr>
<tr>
<td>Root elongation</td>
<td>Rice</td>
<td>Root</td>
<td>50% HRW significantly increased root length</td>
<td>Xu et al. (2013)</td>
</tr>
<tr>
<td>Alfalfa</td>
<td>Root</td>
<td></td>
<td>10% HRW significantly recovered in Cd</td>
<td>Cui et al. (2014)</td>
</tr>
<tr>
<td>Postharvest freshness</td>
<td>Kiwifruit</td>
<td>Fruit</td>
<td>80% HRW had the most significant effect</td>
<td>Hu et al. (2014)</td>
</tr>
<tr>
<td>Cut lily and rose</td>
<td>Flower</td>
<td></td>
<td>Regulated the antioxidant defence, regulated the senescence process</td>
<td>Ren et al. (2017)</td>
</tr>
<tr>
<td>Anthocyanin synthesis</td>
<td>Radish</td>
<td>Hypocotyls</td>
<td>Increased anthocyanin and total phenols</td>
<td>Su et al. (2014)</td>
</tr>
<tr>
<td>Alfalfa</td>
<td>Seeding</td>
<td></td>
<td>Increased (iso)flavonoids metabolism</td>
<td>Xie et al. (2015)</td>
</tr>
<tr>
<td>Arabidopsis</td>
<td>Leaves</td>
<td></td>
<td>Increased intracellular H2 production, reduced stomatal aperture, enhanced drought tolerance</td>
<td>Jin et al. (2016b)</td>
</tr>
<tr>
<td>Stomatal closure</td>
<td>Alfalfa</td>
<td>Leaves</td>
<td>Enhanced plant sensitivity to ABA, modified leaf apoplastic pH, reduced stomatal aperture</td>
<td>Xie et al. (2014)</td>
</tr>
</tbody>
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The authors also suggested that H2 dependently accelerated the physiological process of seed germination in O. sativa under salt stress (Table 1). The effects of H2 on seed germination suggest that the level of soil hydrogen is one factor determining seed germination. In future agriculture, HRW might be widely used to promote seed germination and increase yields of crops.

**Seedlings growth**

It has been observed that HRW treatments increased the growth of seedlings of Chinese cabbage (Wu et al., 2015), maize (Zhang et al., 2015) and alfalfa (Dai et al., 2017; Table 1). HRW treatment significantly increased the FW of Chinese cabbage seedlings (Wu et al., 2015). Zhang et al. (2015) reported that 50% HRW increased the maize seedlings growth through the improvement of net photosynthetic rate. Compared with the chemical-free control samples, HRW resulted in the increase of alfalfa seedlings growth in aboveground and belowground parts (Dai et al., 2017). HRW treatment also increased non-protein thiols (NPTs) in seedlings. These authors also further confirmed that HRW upregulated 21 proteins and downregulated nine proteins during that process (Dai et al., 2017). Thus, a potential role of H2 in increasing seedlings growth is achieved by regulating various proteins.

**Adventitious rooting**

It was well known that adventitious roots are post-embryonic roots originating from the stem, leaf petiole, and non-pericycle tissue of old roots. H2 produced a positive effect on adventitious root development in cucumber (Lin et al., 2014; Zhu et al., 2016a; Table 1). Lin et al. (2014) found that H2-triggered adventitious root formation might be dependent on the auxin response in cucumber. Results from our laboratory also suggested that 50% HRW treatment significantly promoted adventitious root development in cucumber (Zhu et al., 2016a). We have also reported that exogenous nitric oxide (NO) generated from nitric oxide synthase (NOS) and nitrate reductase (NR) activity might be required for H2-induced adventitious root formation (Zhu et al., 2016b). Therefore, H2 can trigger adventitious root initiation and development, and the process is most likely to be accompanied with a combinational signalling process. However, whether there are some other novel singleing molecule(s) involved in adventitious root formation remains to be investigated. In addition, further work will likely show that H2 and NO are involved in many more physiological responses in plants.

**Root elongation**

Root systems have been identified as playing important roles in plant nutrient and water acquisition. H2 has exhibited positive effects on regulating root elongation in plants. Xu et al. (2013) found that 50% HRW significantly increased the root length in cucumber. Treatment with 50% HRW achieved maximum alleviated aluminium (Al)-induced inhibition of root elongation in alfalfa via a reduction in Al accumulation (Chen et al., 2014). In addition, 10% HRW significantly recovered the inhibition of root elongation in Cd-treated alfalfa by increasing the total or isozymatic activities of representative antioxidant enzymes and their corresponding transcripts (Cui et al., 2013; Table 1). Therefore, in the future, HRW may be used to irrigate crops and as a substitute for chemical rooting agents, thus reducing environmental pollution.
Postharvest freshness

Due to the harmlessness of H₂, it plays important roles in postharvest freshness of agricultural products. Hu et al. (2014) demonstrated that H₂ delayed the ripening process in kiwifruit by alleviating pectin solubilisation and reducing the activities of lipid peroxidation and maintaining the free radical scavenging activity (Table 1). Ren et al. (2017) suggested that H₂ might regulate the senescence process by keeping high levels of FW and leaf relative water content of cut lily and rose flowers. Therefore, reduction of oxidative damage was considered to be one of the main mechanisms by which HRW treatment delayed senescence of plant. Therefore, application of H₂ to perishable postharvest freshness of agricultural products. Hu et al. (2014) demonstrated that H₂ delayed the ripening process in kiwifruit by alleviating pectin solubilisation and reducing the activities of lipid peroxidation and maintaining the free radical scavenging activity (Table 1). Ren et al. (2017) suggested that H₂ might regulate the senescence process by keeping high levels of FW and leaf relative water content of cut lily and rose flowers. Therefore, reduction of oxidative damage was considered to be one of the main mechanisms by which HRW treatment delayed senescence of plant. Therefore, application of H₂ to perishable postharvest freshness of agricultural products. Hu et al. (2014) demonstrated that H₂ delayed the ripening process in kiwifruit by alleviating pectin solubilisation and reducing the activities of lipid peroxidation and maintaining the free radical scavenging activity (Table 1). Ren et al. (2017) suggested that H₂ might regulate the senescence process by keeping high levels of FW and leaf relative water content of cut lily and rose flowers. Therefore, reduction of oxidative damage was considered to be one of the main mechanisms by which HRW treatment delayed senescence of plant. Therefore, application of H₂ to perishable

Anthocyanin synthesis

Recently, it was reported that H₂ enhanced UV-A-induced anthocyanin accumulation in the hypocotyls of sprouts by re-establishing ROS homeostasis and upregulating the anthocyanin biosynthesis-related genes (Su et al. 2014; Table 1). Further, there are six main anthocyanidins in plants, namely, cyanidin (Cy), delphinidin (Dp), pelargonidin (Pg), peonidin (Pn), petunidin (Pt), and malvidin (Mv). Su et al. (2014) further evidenced that Cy was predominant in radish sprouts. Anthocyanin is one of important (iso)flavonoids in plants. Xie et al. (2015) indicated that H₂ conferred tolerance to UVB-induced oxidative damage partially by the manipulation of (iso)flavonoids metabolism and antioxidative defense in alfalfa. Anthocyanins contribute to the quality of fruits and vegetables and are beneficial to human health. Anthocyanins are recognised as compounds with potential health benefits because of their valuable nutritional antioxidative activities. Beyond that, anthocyanin production is also the typical plant response to environmental stress. Therefore, in the future, H₂ may be widely used in plants. Apart from anthocyanin, the effects of H₂ on other nutrients in plants are worth studying, such as vitamins, amino acid and inorganic salt.

Stomatal closure

Stomatal movement is critical in controlling plant water status, and can be triggered by numerous environment or hormonal factors. Recently, Xie et al. (2014) found that H₂ could regulate stomatal movement via an ABA signalling cascade in which rbhf-complex-dependent ROS and nitric reductase-associated NO production, as well as subsequent guard cell outward-rectifying K⁺ channel (GORK) activation, was causally involved. Jin et al. (2016a) found that H₂-regulated stomatal closure under drought stress in alfalfa was dependent on ABA. These authors also reported that H₂ rapidly increased hydrogen peroxide (H₂O₂), and modified apoplastic pH of leaves in alfalfa via an ABA-based mechanism. To summarise, HRW-induced drought tolerance was, at least partially, conferred via an H₂O₂-dependent pathway, further confirming the speculation that H₂ positively regulated the ABA-dependent drought response.

From the above, we suggest that H₂ might confer antioxidant protection to plants by eliminating the cytotoxic free radicals and increasing activities of representative antioxidant enzymes. H₂ may also function as a signalling molecule through interaction with plant hormones, ROS and other small molecules, thus affecting numerous physiological processes. Therefore, H₂ has broad application prospects in plants.

H₂ enhances plant resistance to environmental stimuli

Environmental stimuli are a major constraint to plant growth, survival and yield, which also result in oxidative stress and overproduction of ROS by disruption of cellular redox homeostasis. There are accumulating data showing the participation of H₂ in different types of environmental stresses. In the following sections, the way in which H₂ is associated with major environmental stresses is discussed in more detail.

Osmotic stress

Xie et al. (2012) reported that H₂ could modulate related antioxidant defence enzymes in Arabidopsis under salinity stress. For example, H₂ increased ZAT12 gene expression to reverse the salt-triggered inhibition of total ascorbate peroxidase (APX) activity. Additionally, H₂ could maintain ion homeostasis in Arabidopsis under salt stress by regulating the antiporters and H⁺ pump responsible for Na⁺ exclusion and compartmentation (Table 2). H₂ also enhanced corresponding transcripts of antioxidant enzymes, including SOD, CAT and APX, and alleviated oxidative damage in rice under salt stress, consequently alleviating the inhibition of seed germination and seedling growth caused by salinity stress (Xu et al. 2013). It is likely that H₂ does not act alone, but rather, interacts with other signalling molecules such as ABA and H₂O₂ to effect stomatal closure. Xie et al. (2014) indicated that H₂ could enhance tolerance to drought stress in Arabidopsis by triggering ABA signalling. In addition, these authors also identified that H₂-mediated ROS-dependent NO production operated downstream of ABA, promoting stomatal closure partially through the GORK. According to Jin et al. (2016a), H₂ was induced by drought stress and ABA treatment, which was catalysed by hydrogenase enzyme. Jin et al. (2016a) also confirmed that H₂ positively regulated the ABA-promoted H₂O₂ production and stomatal closure, which may reduce water loss in response to drought stress. In further work, Jin et al. (2016b) showed that H₂ could decrease lipid peroxidation and rapidly elevate H₂O₂ levels in alfalfa under osmotic stresses (Table 2). Besides, H₂O₂ could play a significant role in the HO-1-dependent induction of osmotic stress tolerance triggered by H₂. During the last 5 years, a certain number of studies have been conducted to investigate how H₂ may be involved in the response against osmotic stress. We note that the mechanisms associated with such responses in plants remain elusive.

Heavy metals stress

Heavy metals such as Al, mercury (Hg) and Cd result in serious environmental pollution in many places across the world, and are a threat to human health and plant development. Cui et al. (2013) found that HRW treatment significantly inhibited the appearance of Cd toxicity symptoms in alfalfa seedlings, and decreased the contents of TBARS under Cd stress. They also suggested that H₂-attenuated Cd toxicity functioned through an increase in activities of representative antioxidant enzymes or their corresponding transcripts and enhancing the ratio of reduced/oxidised (homo) glutathione ((h)GSH) (Table 2). Cui et al. (2014) proposed that H₂
inhibited Hg toxicity in alfalfa seedlings by alleviating growth stunting, reducing Hg accumulation, avoiding oxidative stress and re-establishing redox homeostasis, especially GSH/GSSG and AsA pools. H2 could alleviate Al-induced inhibition of root growth in alfalfa and Al accumulation in root apexes (Chen et al. 2014). Additionally, HRW treatment, similar to the NO scavenger, could decrease endogenous NO production during that process (Table 2). HRW treatment also significantly alleviated the Cd toxicity symptoms in Chinese cabbage (Wu et al. 2015). During that process, H2 could alleviate oxidative damage, as indicated by the decrease of TBARS and ROS production. Application of H2 could alleviate Cd-induced growth inhibition of alfalfa seedlings (Dai et al. 2017). This was accompanied by decreasing oxidative damage, enhancing sulfur compound metabolic process, and maintaining nutrient element homeostasis. Thus, the authors revealed the main target genes and metabolic processes associated with Cd resistance conferred by H2.

As mentioned above, H2 alleviates heavy metals toxicity mainly by decreasing oxidative damage and increasing the activities of representative antioxidant enzymes. Despite the identified importance of HRW, the mechanisms of action of H2 under heavy metals stress are not yet clear. Given that heavy metals stresses impose considerable constraints on crop production, there is a real need for continued research in this area in order to reveal the underlying molecular mechanisms of H2 under heavy metal stresses.

**High light stress**

Application of HRW significantly improved maize seedling growth under high light stress, suggesting that H2 had an ameliorating effect against high light-induced stress (Zhang et al. 2015). Increased activities of antioxidative enzymes were also reported in HRW treatment under high light stress. The authors confirmed that H2 could alleviate oxidative damage under high light stress by decreasing the level of O2•− and H2O2 as well as the content of TBARS (Table 2). Whether these enhanced enzyme activities followed from increased gene expression or from post-translational modification of the respective proteins has not yet been investigated.

**Temperature stress**

Xu et al. (2017) showed that H2 alleviated the decrease of chlorophyll content, photosynthetic activity, lipid peroxidation and the overproduction of ROS (Table 2). Additionally, H2 might contribute to the enhancement of cold tolerance by the re-establishment of redox homeostasis via miR398 and miR319. Recently, a large number of genes coding for small RNAs or miRNAs response to cold stress have been identified (Zhou et al. 2008). The low temperatures resulted in the formation of secondary structures in RNA, thus affecting gene expression. Therefore, H2 might alleviate cold stress through regulating the expression of RNA. H2 alleviated the detrimental effects of high-temperature stress on the growth of cucumber seedlings, as indicated by improving the photosynthetic capacity, increasing the antioxidant response, and promoting the accumulation of heat shock protein 70 (HSP70) and osmolytes (Chen et al. 2017b). Along with these studies, we suggest that H2 may enhance temperature tolerance in plants via improvement in the photosynthetic capacity, increasing the antioxidant response, and promoting the accumulation of HSP70 and osmolytes that are required for redox homeostasis.

**Paraquat-induce oxidative stress**

Under paraquat-induced oxidative stress conditions, H2 can alleviate the inhibition of root growth by reducing lipid
peroxidation and decreasing H₂O₂ and superoxide anion radical levels in alfalfa seedlings (Jin et al. 2013; Table 2). This result revealed that H₂ or the heme oxygenase-1 (HO-1) inducer hemin treatment enhanced the tolerance to oxidative stress induced by paraquat in alfalfa seedlings. In addition, H₂ pre-treatment could dramatically increase HO-1 activity. Therefore, the author of this work suggested that H₂ might function as an important gaseous molecule that alleviates oxidative stress via HO-1 signalling (Table 2).

**Ultraviolet radiation**

Some recent studies have demonstrated that ultraviolet radiation (UV) increased the amount of ROS, oxygen-derived free radicals and lipid peroxidation, thus leading to cellular damage and subsequent growth inhibition (Su et al. 2014; Xie et al. 2015; Table 2). Further, the involvement of H₂ in UV radiation-resistance has also been reported. For example, in radish sprouts, HRW treatment significantly blocked the UV-A-induced increase of H₂O₂ and O₂•− accumulation, and enhanced the UV-A-induced increase of enzyme activities (Su et al. 2014). Additionally, the HRW treatment with UV-A induced a rise in the content of anthocyanin. In another study, the application of H₂ in alfalfa seedlings subjected to UV-B radiation reduced the UV-B effects characterised by upregulating the transcription levels of (iso)flavonoids biosynthetic-related genes and strengthened the related transcripts of representative antioxidant enzymes (Xie et al. 2015). Combined with the above results, we suggest that H₂ will have practical applications for enhancing the nutritional quality of plants.

Taken together, abiotic stresses that adversely affect plant growth and development have become a global problem. Almost all abiotic stresses inhibit plant growth and development and enzyme activities, resulting in oxidative stress in terms of an increased level of ROS in plant cells. The application of exogenous H₂ modulates the response against abiotic stresses by reducing lipid peroxidation, strengthening related transcripts of antioxidant enzymes and regulating related genes. It is likely that H₂ may act as an effective signal molecule in stress response. In future studies it will also be important to seek further information about the underlying molecular mechanisms of H₂, and to identify and characterise its direct targets and their functions under abiotic stresses.

**Cross-talk between H₂ and other signalling molecules**

Previous studies have shown that H₂ can crosstalk with other molecules to regulate various physiological processes, showing that H₂ signal transduction doesn’t usually operate as a linear pathway. Here, we provide a brief overview of the interaction between H₂ and NO, CO or other signalling molecules.

**Cross-talk between H₂ and NO**

Research on the mechanisms for H₂ signalling in plant are fragmented, although rapid progress in being made this field. H₂ signal transduction pathway ways are closely linked to NO. As reported, H₂ was able to mimic, to some extent, the effect of NO in dose-dependent induction of stomatal closure (Xie et al. 2014) and adventitious root development (Zhu et al. 2016a, 2016b; Fig. 2). Xie et al. (2014) discovered that exposure of *Arabidopsis* to HRW resulted in an increase in NO content. They also demonstrated that NO production contributed to H₂-promoted stomatal closure in *Arabidopsis*. Thus, H₂-induced NO synthesis was required for stomatal closure in *Arabidopsis*. Recently, Zhu et al. (2016a) indicated that H₂ mediated cell cycle activation via the NO pathway during adventitious root formation in cucumber. Then, they reported that HRW also increased NOS and NR activity and NO content in both a dose- and time-dependent fashion (Zhu et al. 2016b). In addition, an increase in H₂-mediated NO production catalysed by NOS-like protein and NR might be required and be part of the molecular events involved in H₂ action (Zhu et al. 2016b). These results suggested that NO might be one of the main pathways involved in H₂-induced adventitious root development. Furthermore, Chen et al. (2014) demonstrated that functional interaction of H₂ and NO could alleviate Al toxicity symptoms and enhanced AI stress in alfalfa. So, there are some clear interactions between H₂ and NO, but the exact nature of this interaction appears to depend on the system studied (different physiological processes). Further, H₂ and NO systems include complex process in plants, so further studies are needed to determine the crosstalk between H₂ and NO in different physiological processes.

**Cross-talk between H₂ and CO**

It is well known that H₂ and CO act as gas signalling modulators, and are involved in plant development and stress response. Studies have shown the involvement of HO-1/CO in H₂-induced osmotic stress tolerance in alfalfa (Jin et al. 2016b). In addition, H₂ mediates target genes related to auxin signalling and adventitious root development through CO pathways during adventitious rooting (Lin et al. 2014).
reported that CO could be involved in H2-induced adventitious rooting under drought stress, and alleviate oxidative damage during that process (Fig. 2). As mentioned above, both H2 and CO might be involved in stress responses and adventitious root development in plants. However, it is unclear whether these two molecules work in concert in other developmental processes. It also unknown whether the H2-activated target genes related to auxin signalling lie upstream of and/or regulate CO production.

Cross-talk between H2 and other signalling molecules
Stress can trigger the production of H2 in plants. Zeng et al. (2013) indicated that H2 production was induced by ABA, ET and JA. In contrast, H2 might modulate the output of hormone signalling pathways in response to a stress challenge. Both H2O2 and H2 can activate the apoplastic pH and change stomatal aperture in alfalfa seedlings, suggesting a novel regulating mechanism of H2 in plant drought response (Jin et al. 2016a). Interactions between ABA and H2 during stomatal closure in Arabidopsis were highlighted by Xie et al. (2014). ABA induced H2 biosynthesis in Arabidopsis seedlings under osmotic stress. Also, NO and ROS were found to participate in the change of stomatal aperture in Arabidopsis seedlings by stimulating H2 biosynthesis (Xie et al. 2014; Fig. 2). For a signalling molecule to be effective it needs to be produced quickly and efficiently on demand, and to be removed rapidly and effectively when no longer required. However, it is still unclear whether H2 interacts with other signals molecules or is removed rapidly when no longer required.

Clearly, H2 can, and does interact with many other signals, but a complete ‘H2 signalling map’ will take some time to complete.

Modulation of gene expression by H2
As mentioned above, a large number of physiological and developmental responses to H2 have been verified in plants. Thus, some genes must inevitably be changed following exposure to H2. Liu et al. (2016) reported that H2 upregulated the expression of hydrogenase genes (OsHypB and OsFhdB), synthesis genes (OsAOS2, OsLOX, OsEDS1, OsPAD4 and OsACS1) and receptor genes of salicylic acid (SA) (OsNRPR4), JA (OsCOI1), and ET (OsERS1, OsERS2 and OsETR2) signalling pathways in wild rice (Table 3). Additionally, the effect of H2 on the expression level of these genes was more obvious in the seedling stage than in the reproductive stage.

Abiotic stress response
By using Arabidopsis thaliana capx1 and sos1 mutants, Xie et al. (2012), demonstrated that cAPX1 and SOS1 might be the downstream target genes of H2 signalling under salt stress. Zeng et al. (2013) also quantified the expression of genes for phytohormone receptors in rice seedlings by reverse-transcription quantitative PCR (RT-qPCR) and suggested that H2 could regulate the expression of these genes. They found that transcription of the three putative rice hydrogenase genes (OsHydA1, OsFhdB and OsHypB) was dramatically upregulated by a 2 week exposure to HRW. They also analysed the transcription level of genes encoding antioxidant enzymes (OsFeSOD, OsMnSOD, OsCu/ZnSOD, OsCAT-A, OsCAT-B, OsAPX and OsGPX) and found that H2 could trigger the expression of these genes under drought and salt stresses. HRW pre-treatment could alleviate Cd stress in alfalfa seedlings, which might be related to the regulation of ECS, GS, hGS and GR1/2 transcripts (Cui et al. 2014; Table 3). Wu et al. (2015) showed that H2 induced the expression of IRT1, Nramp1, HMA3, HMA2 and HMA4 genes responsible for heavy metal homeostasis in Chinese cabbage. H2 induced the expression of antioxidant enzymes and miRNAs in rice seedlings (Xu et al. 2017). Moreover, miR398 transcripts were downregulated by H2, and the expression levels of its targets Cu/Zn-SOD1 (CSD1) and Cu/Zn-SOD2 (CSD2) were increased. By contrast, miR319 transcripts were differentially increased, showing a relatively

### Table 3. Overview of the regulate genes of H2

<table>
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<tr>
<th>Plant species</th>
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<th>H2-mediated genes</th>
<th>Physiological and developmental processes</th>
<th>Reference</th>
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<tr>
<td>Arabidopsis</td>
<td>Seedlings</td>
<td>SOS1, cAPX1, OsERS1, OsERS2, OsETR1, OsETR2, OsETR4, OsAOS2–1, OsAOS2–2, OsAOS2–3, OsNRPR4, OsACS1, OsSLR1, OsPR1, OsHydA1, OsFhdB, OsHypB, OsFeSOD, OsMnSOD, OsCu/ZnSOD, OsCAT-A, OsCAT-B, OsAPX, OsGPX</td>
<td>Salt stress</td>
<td>Xie et al. (2012)</td>
</tr>
<tr>
<td>Rice</td>
<td>Shoot/Root</td>
<td></td>
<td>Drought stress and Salt stress</td>
<td>Zeng et al. (2013)</td>
</tr>
<tr>
<td>Alfalfa</td>
<td>Seedlings</td>
<td>OsAOS2, OsLOX, OsEDS1, OsPAD4, OsACS1, OsNRPR4, OsCOI1, OsERS1, OsERS2, OsETR2</td>
<td>Cd stress</td>
<td>Cui et al. (2014)</td>
</tr>
<tr>
<td>Chinese cabbage</td>
<td>Roots</td>
<td>ECS, GS, hGS, GR1/2</td>
<td>Cd stress</td>
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<tr>
<td>Rice</td>
<td>Seedlings</td>
<td>CSD1, CSD2, PF1, PF2</td>
<td>Cold stress</td>
<td>Xu et al. (2017)</td>
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<tr>
<td>Cucumber</td>
<td>Seedlings</td>
<td>HSP70</td>
<td>High-temperature stress</td>
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</tr>
<tr>
<td>Wild rice</td>
<td>Seedlings</td>
<td>OsHypB, OsFhdB, OsAOS2, OsLOX, OsEDS1, OsPAD4, OsACS1, OsNRPR4, OsCOI1, OsERS1, OsERS2, OsETR2</td>
<td>Adventitious rooting</td>
<td>Liu et al. (2016)</td>
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<tr>
<td>Cucumber</td>
<td>Hypocotyls</td>
<td>CsDNAJ1, CsDCDK1/5, CsCDC6, CsAUX2B, CsAUX2D</td>
<td></td>
<td>Lin et al. (2014)</td>
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<tr>
<td>Cucumber</td>
<td>Hypocotyls</td>
<td>CycA, CycB, CDKA, CDKB</td>
<td>Adventitious rooting</td>
<td>Zhu et al. (2016a)</td>
</tr>
<tr>
<td>Radish</td>
<td>Hypocotyls</td>
<td>PAL, CHS, CHI, F3H, DFR, ANS</td>
<td>Anthocyanin biosynthesis</td>
<td>Sa et al. (2014)</td>
</tr>
<tr>
<td>Alfalfa</td>
<td>Seedlings</td>
<td>CHS, FLS, 6OMT, Mn-SOD, POD1B, POD2 CAT</td>
<td>Anthocyanin biosynthesis</td>
<td>Xie et al. (2015)</td>
</tr>
</tbody>
</table>
negative correlation with its target genes PROLIFERATING CELL FACTORS (PCF5) and PROLIFERATING CELL FACTORS (PCF8) (Xu et al. 2017; Table 3). Recently, Chen et al. (2017b) found that H2 alleviated the detrimental effects of high-temperature stress on the growth of cucumber seedlings by upregulating HSP70 expression. Therefore, it is likely that H2 participates in biotic stress resistance through regulating gene expression that involved in H2 production and antioxidant enzymes. However, the exact mechanisms by which this process occurs are not yet known.

Adventitious roots formation

Direct exposure to H2 via treatment with HRW induced CsDNAJ-1, CsCDPK1/5, CsCDC6, CsAUX22B-like and CsAUX22D-like gene expression, suggesting that H2 mediated adventitious roots formation by inducing these genes expression (Lin et al. 2014; Table 3). The authors concluded that CsDNAJ-1, CsCDPK1/5, CsCDC6, CsAUX22B-like and CsAUX22D-like were target genes to HRW-induced adventitious roots formation (Lin et al. 2014). In our laboratory, Zhu et al. (2016a) indicated that the target genes related to adventitious rooting were also upregulated by HRW in cucumber explants. In addition, we also found that HRW upregulated cell cycle-related genes, CycA, CycB, CDK4 and CDKB expression during adventitious rooting (Table 3). Therefore, H2 mediated the representative target genes related to auxin signalling and cell cycle during adventitious root formation. Further verification through employment of different growth and development stages is needed to more fully understand the effects of H2 on plants.

Anthocyanin biosynthesis

Recently, Su et al. (2014) found that H2 could regulate anthocyanin biosynthesis-related genes. Direct exposure to UV-A induced PAL, CHS, CHI, F3H, DFR and ANS gene expression in radish, suggesting that UV-A induced anthocyanin biosynthesis-related genes expression (Table 3). Exposure to UV-A plus HRW also induced the genes expression. These results imply that H2 strengthened UV-A induction of PAL, CHS, CHI, F3H, DFR and ANS gene expression (Su et al. 2014). Additionally, UV-B exposure caused increases in the transcription levels of all detected (iso) flavonoids biosynthetic-related genes, including PAL, CHS, CHI, FLS, IPS and 6/OMT (Table 3). Furthermore, theses increasing tendencies were substantially strengthened by HRW. They further established that H2 regulated anthocyanin biosynthesis-related target genes under UV-B radiation (Xie et al. 2015). These results provide a more solid foundation for improving the nutritional value of crops by H2.

As described above, H2 may mediate the transcription of some specific genes, including hydrogenase-related genes, antioxidant enzymes-related genes, miRNAs-related genes, cell cycle-related genes and anthocyanin biosynthesis-related genes. Although these specific genes may function in H2 metabolism, their exact receptors and downstream molecules remain unclear.

Conclusions and perspectives

Recently, there have been increasing studies regarding the synthesis and physiological roles of H2 in plants. Although the accurate H2 biosynthetic pathway has not been fully determined, it has been established that there are two pathways to produce H2 in plant cells, namely, hydrogenase and nitrogenase. H2 might confer antioxidant protection to plants in various developmental processes. Further, H2 functions as a signalling molecule and interacts with other signalling molecules (NO, CO and plant hormones) to affect growth and development processes through regulation of many genes in plants.

Future studies regarding H2 biosynthesis should focus on the molecular details of each pathway in higher plants. The intricate mechanisms associated with its responses to abiotic stimuli are still a subject of great interest. Further research is required to investigate the role of H2 in modulating plant growth and development. Deeper insights into the interplay of various phytohormones with H2 at a signalling level will help provide a road-map for addressing the problem more holistically. Another question that remains is “what is the receptor(s) of H2 in plants?” Also, the insights into various components associated with the perception of H2 signals and their transduction need further investigation. More research work will improve knowledge concerning possible application of hydrogen water to crop plants with the aim of enhancing agriculture in areas of yield and quality. Solving these problems will mean that application of hydrogen agriculture will move one step closer to reality, and in the future H2 may be widely applied to improve the stress resistance of crops and to achieve the purpose of disaster prevention and environment protection.

Conflicts of interest

The authors declare no conflicts of interest.

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