

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

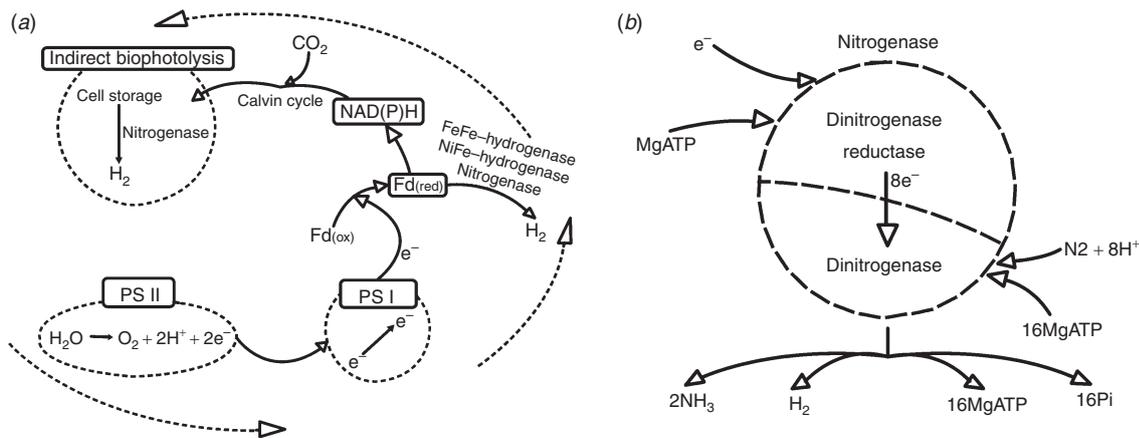


Fig. 1. Two H_2 production pathways in organisms: nitrogenase is viewed as the main productive route of H_2 in organisms (Tamagnini *et al.* 2002; Kim and Kim 2011). In Fig. 1a, rhizobia can release H_2 in the nitrogen fixation process. Hydrogenase is viewed as the main productive route of H_2 in organisms (Das *et al.* 2008; Kim and Kim 2011). In Fig. 1b, photons derived from light energy split water into oxygen (O_2) and electrons. The electrons are then activated and reduce ferredoxin (Fd). By the activity of hydrogenases, Fd (red) can be reoxidised, forming H_2 . Additionally, compounds accumulated during carbon dioxide (CO_2) fixation are degraded through the Calvin cycle and release H_2 .

through the Calvin cycle. Then, H_2 is synthesised through the activity of nitrogenase (Kim and Kim 2011; Fig. 1a). In addition, rhizobia can release H_2 during the nitrogen fixation process (Fig. 1b).

As mentioned above, H_2 production in bacteria and algae has been well studied. However, only a few studies have reported H_2 production and hydrogenase in higher plants. Boichenko (1947) studied the H_2 evolution in isolated chloroplasts and postulated the existence of hydrogenase in plants. Sanadze (1961) first demonstrated that H_2 was released and absorbed in plant leaves. After that, Renwick *et al.* (1964) found that winter rye seeds could release H_2 . The authors also reported that exogenous H_2 could promote the germination rate of winter rye seeds. In addition, rhizobia from symbiosis with leguminous plants could release H_2 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 H_2 release in *Arabidopsis* seedlings, reaching the highest level of more than $100 \mu\text{mol g}^{-1}$ FW after 6 h of treatment. Jin *et al.* (2013) reported that paraquat-induced oxidative stress (PQ) promoted endogenous H_2 release in alfalfa seedlings in a dose-dependent manner. The highest concentration of endogenous H_2 ($25 \mu\text{mol g}^{-1}$ FW) was detected after 24 h of treatment with $100 \mu\text{M}$ PQ. They also indicated a positive interrelationship between the inhibition of root elongation and the H_2 production in alfalfa leaves during exposure to PQ. The evolution of H_2 in rice seedlings was 0.318 and $0.305 \mu\text{mol g FW}^{-1} \text{h}^{-1}$ under light and in the dark respectively (Zeng *et al.* 2013). These authors also found that H_2 production in rice seedlings was induced by abscisic acid (ABA), ethylene (ET), jasmonate acid (JA), salt and drought stresses. The production of H_2 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, H_2 production was induced by both hormone and abiotic stress. H_2 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 H_2 biosynthesis pathways in higher plants.

H_2 as a signalling molecule can move freely from one plant cell to another cell. It is also conceivable that H_2 might serve as an informational signal to other parts of the plants, or to plants in the vicinity. Thus, how do plants sense the H_2 signalling? So far, the answer to this question is still unknown.

H_2 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 H_2 in plants and animals. In animals, our understanding about the biological roles of H_2 has developed rapidly. It was reported that H_2 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 H_2 is an antioxidant that can selectively reduce cytotoxic free radicals (Hayashida *et al.* 2008; Matsumoto *et al.* 2009). Recently, the roles of H_2 in plants have been studied during several plant growth and development stages including seeds germination, seedlings 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 H_2 in plants was first reported in winter rye seeds, where H_2 was found to induce seeds germination (Renwick *et al.* 1964). Since then, Xu *et al.* (2013) has demonstrated that H_2 exerted an advantageous

Table 1. Overview of H₂-mediated physiological processes in plants

Plant growth and development	Plant species	Tissues	H ₂ -induced effect	Reference
Seeds germination	Winter rye	Seeds	Increased seed germination	Xu <i>et al.</i> (2013)
	Rice	Seeds	Increased seed germination in NaCl	Renwick <i>et al.</i> (1964)
Seedlings growth	Chinese cabbage	Seedlings	Alleviated Cd-induced growth inhibition	Wu <i>et al.</i> (2015)
	Maize	Seedlings	Improved net photosynthetic rate	Zhang <i>et al.</i> (2015)
	Alfalfa	Seedlings	Increased alfalfa seedling growth	Dai <i>et al.</i> (2017)
	Cucumber	Explants	Improved AR development, increased NO content, upregulated NR genes, improved AR development, increased NO content	Zhu <i>et al.</i> (2016b)
	Adventitious rooting (AR)	Cucumber	Explants	Upregulated cell cycle-related genes, promoted AR formation
Root elongation	Cucumber	Explants	50% HRW had the most significant effect	Lin <i>et al.</i> (2014)
	Alfalfa	Root	50% HRW significantly recovered in Cd	Chen <i>et al.</i> (2014)
	Rice	Root	50% HRW significantly increased root length	Xu <i>et al.</i> (2013)
Postharvest freshness	Alfalfa	Root	10% HRW significantly recovered in Cd	Cui <i>et al.</i> (2014)
	Kiwifruit	Fruit	80% HRW had the most significant effect	Hu <i>et al.</i> (2014)
Anthocyanin synthesis	Cut lily and rose	Flower	Regulated the antioxidant defence, regulated the senescence process	Ren <i>et al.</i> (2017)
	Radish	Hypocotyls	Increased anthocyanin and total phenols	Su <i>et al.</i> (2014)
	Alfalfa	Seedling	Increased (iso)flavonoids metabolism	Xie <i>et al.</i> (2015)
Stomatal closure	<i>Arabidopsis</i>	Leaves	Increased intracellular H ₂ production, reduced stomatal aperture, enhanced drought tolerance	Jin <i>et al.</i> (2016b)
	Alfalfa	Leaves	Enhanced plant sensitivity to ABA, modified leaf apoplastic pH, reduced stomatal aperture	Xie <i>et al.</i> (2014)

effect on promoting seed germination in *Oryza sativa* L. under salt stress (Table 1). The authors also suggested that H₂ dependently accelerated the physiological process of seed germination in *O. sativa* under salt stress via activating amylase activity and increasing the formation of energy resources. Therefore, these 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,

2016b; 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 signaling 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 reduce lipid peroxidation and maintain 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 be one of the main mechanisms by which HRW treatment delayed senescence of plant. Therefore, application of H₂ to perishable cut flowers, vegetables and fruits may delay senescence and extend their postharvest life.

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 antioxidant defence 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 antioxidant 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 H₂ could regulate stomatal movement via an ABA signalling cascade in which *rbohF*-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 though 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 maintenance 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

Table 2. Overview of the responses of H₂ in abiotic stress

Plant species	Tissues	Abiotic stress	H ₂ -mediated effect	H ₂ -mediated antioxidant enzyme	Reference
<i>Arabidopsis</i>	Seedling	Salt	Ion homeostasis	APX	Xie <i>et al.</i> (2012)
Rice	Seedling	Salt	Oxidative damage	CAT, SOD, APX	Xu <i>et al.</i> (2013)
<i>Arabidopsis</i>	Leaves	Drought	Stomatal closure	–	Xie <i>et al.</i> (2014)
Alfalfa	Seedling	Drought	Stomatal closure	–	Jin <i>et al.</i> (2016a)
Alfalfa	Seedling	Osmotic	lipid peroxidation	–	Jin <i>et al.</i> (2016b)
Alfalfa	Seedling	Cadmium	Cd toxicity symptoms TBARS	SOD, POD	Cui <i>et al.</i> (2013)
Alfalfa	Seedling	Mercury	Hg accumulation, oxidative damage, redox homeostasis	APX, POD	Cui <i>et al.</i> (2014)
Alfalfa	Seedling	Aluminium	Al accumulation, NO production	–	Chen <i>et al.</i> (2014)
Chinese cabbage	Seedling	Cadmium	Cd toxicity symptoms, oxidative damage	SOD, POD	Wu <i>et al.</i> (2015)
Alfalfa	Seedling	Cadmium	Oxidative damage, sulfur compound metabolic, nutrient element homeostasis	–	Dai <i>et al.</i> (2017)
Maize	Seedling	High light	Oxidative damage	SOD, CAT, APX	Zhang <i>et al.</i> (2015)
Rice	Seedling	Cold	Chlorophyll contents, lipid peroxidation, redox homeostasis	SOD, CAT, POD, APX	Xu <i>et al.</i> (2017)
Cucumber	Seedling	Heat	Photosynthetic capacity, HSP70 and osmolyte accumulation	SOD, CAT, POD, APX	Chen <i>et al.</i> (2017b)
Alfalfa	Seedling	PQ	Lipid peroxidation, H ₂ O ₂ and superoxide anion radical	SOD, POD, CAT	Jin <i>et al.</i> (2013)
Radish	Seedling	UVA	H ₂ O ₂ and O ₂ ^{•-} accumulation, anthocyanin	SOD, APX	Su <i>et al.</i> (2014)
Alfalfa	Seedling	UVB	Oxidative damage(iso)flavonoids biosynthetic	SOD, POD, CAT	Xie <i>et al.</i> (2015)

inhibited Hg toxicity in alfalfa seedlings by alleviating growth stunting, reducing Hg accumulation, avoiding oxidative stress and re-establishing redox homeostasis, especially GSH/hGSH and AsA pools. H₂ could alleviate Al-induced inhibition of root growth in alfalfa and Al accumulation in root apices (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 H_2O_2 and superoxide anion radical levels in alfalfa seedlings (Jin *et al.* 2013; Table 2). This result revealed that H_2 or the heme oxygenase-1 (HO-1) inducer hemin treatment enhanced the tolerance to oxidative stress induced by paraquat in alfalfa seedlings. In addition, H_2 pre-treatment could dramatically increase HO-1 activity. Therefore, the author of this work suggested that H_2 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 H_2 in UV radiation-resistance has also been reported. For example, in radish sprouts, HRW treatment significantly blocked the UV-A-induced increase of H_2O_2 and $O_2^{\bullet-}$ 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 H_2 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 H_2 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 H_2 modulates the response against abiotic stresses by reducing lipid peroxidation, strengthening related transcripts of antioxidant enzymes and regulating related genes. It is likely that H_2 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 H_2 , and to identify and characterise its direct targets and their functions under abiotic stresses.

Cross-talk between H_2 and other signalling molecules

Previous studies have shown that H_2 can crosstalk with other molecules to regulate various physiological processes, showing that H_2 signal transduction doesn't usually operate as a linear pathway. Here, we provide a brief overview of the interaction between H_2 and NO, CO or other signalling molecules.

Cross-talk between H_2 and NO

Research on the mechanisms for H_2 signalling in plant are fragmented, although rapid progress in being made this field. H_2 signal transduction pathways are closely linked to NO. As reported, H_2 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*

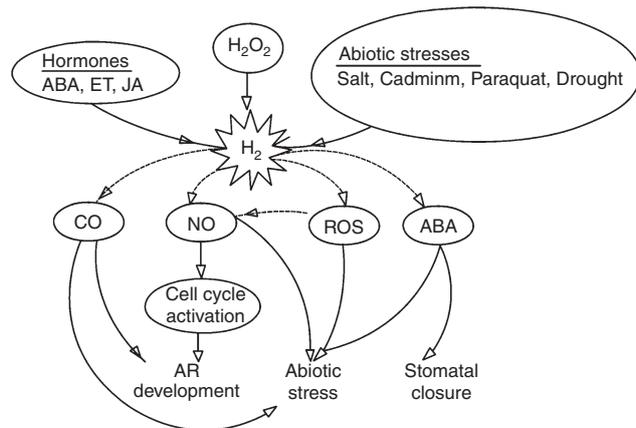


Fig. 2. Schematic model of the interaction among H_2 , NO, CO and other signalling molecules in different plant physiological and abiotic stress processes. H_2 production in plants is induced by H_2O_2 , specific abiotic stresses (salt stress, drought stress, paraquat stress and Cd stress) and hormones (ABA, ET and JA). H_2 , NO, CO, reactive oxygen species (ROS) and other signalling molecules may receive various stimuli through signalling sensors. They might interact via cross-regulation and transduce signalling to regulate plant adventitious roots (AR) development, stomatal closure and abiotic stress responses. Dashed arrows indicate that there is no direct evidence showing the interaction between the signals. Dashed arrows indicate that there is no direct evidence showing the interaction between the molecules.

to HRW resulted in an increase in NO content. They also demonstrated that NO production contributed to H_2 -promoted stomatal closure in *Arabidopsis*. Thus, H_2 -induced NO synthesis was required for stomatal closure in *Arabidopsis*. Recently, Zhu *et al.* (2016a) indicated that H_2 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 H_2 -mediated NO production catalysed by NOS-like protein and NR might be required and be part of the molecular events involved in H_2 action (Zhu *et al.* 2016b). These results suggested that NO might be one of the main pathways involved in H_2 -induced adventitious root development. Furthermore, Chen *et al.* (2014) demonstrated that functional interaction of H_2 and NO could alleviate Al toxicity symptoms and enhanced Al stress in alfalfa. So, there are some clear interactions between H_2 and NO, but the exact nature of this interaction appears to depend on the system studied (different physiological processes). Further, H_2 and NO systems include complex process in plants, so further studies are needed to determine the crosstalk between H_2 and NO in different physiological processes.

Cross-talk between H_2 and CO

It is well known that H_2 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 H_2 -induced osmotic stress tolerance in alfalfa (Jin *et al.* 2016b). In addition, H_2 mediates target genes related to auxin signalling and adventitious root development through CO pathways during adventitious rooting (Lin *et al.* 2014). Chen *et al.* (2017a)

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 *OsACSI*) and receptor genes of salicylic acid (SA) (*OsNPR4*), JA (*OsCOII*), 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 *GRI/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

Plant species	Tissues	H ₂ -mediated genes	Physiological and developmental processes	Reference
<i>Arabidopsis</i>	Seedlings	<i>SOS1 cAPX1</i>	Salt stress	Xie <i>et al.</i> (2012)
Rice	Shoot/Root	<i>OsERS1</i> , <i>OsERS2</i> , <i>OsETR2</i> , <i>OsETR3</i> , <i>OsETR4</i> , <i>OsAFB2-1</i> , <i>OsAFB2-2</i> , <i>OsTIR1</i> , <i>OsPYL</i> , <i>OsGID1</i> , <i>OsHk6</i> , <i>OsNPR4</i> , <i>OsACSI</i> , <i>OsSLR1</i> , <i>OsPRI</i> , <i>OsHydA1</i> , <i>OsFhdB</i> , <i>OsHypB</i> , <i>OsFeSOD</i> , <i>OsMnSOD</i> , <i>OsCu/ZnSOD</i> , <i>OsCAT-A</i> , <i>OsCAT-B</i> , <i>OsAPX</i> , <i>OsGPX</i>	Drought stress and Salt stress	Zeng <i>et al.</i> (2013)
Alfalfa	Seedlings	<i>ECS</i> , <i>GS</i> , <i>hGS</i> , <i>GRI/2</i>	Cd stress	Cui <i>et al.</i> (2014)
Chinese cabbage	Roots	<i>IRT1</i> , <i>Nramp1</i> , <i>HMA3</i> , <i>HMA2</i> , <i>HMA4</i> ,	Cd stress	Wu <i>et al.</i> (2015)
Rice	Seedlings	<i>CSD1</i> , <i>CSD2</i> , <i>PCF5</i> , <i>PCF8</i>	Cold stress	Xu <i>et al.</i> (2017)
Cucumber	Seedlings	<i>HSP70</i>	High-temperature stress	Chen <i>et al.</i> (2017b)
Wild rice	Seedlings	<i>OsHypB</i> , <i>OsFhdB</i> , <i>OsAOS2</i> , <i>OsLOX</i> , <i>OsEDS1</i> , <i>OsPAD4</i> , <i>OsACSI</i> , <i>OsNPR4</i> , <i>OsCOII</i> , <i>OsERS1</i> , <i>OsERS2</i> , <i>OsETR2</i>		Liu <i>et al.</i> (2016)
Cucumber	Hypocotyls	<i>CsDNAJ-1</i> , <i>CsCDPK1/5</i> , <i>CsCDC6</i> , <i>CsAUX22B</i> , <i>CsAUX22D</i>	Adventitious rooting	Lin <i>et al.</i> (2014)
Cucumber	Hypocotyls	<i>CycA</i> , <i>CycB</i> , <i>CDKA</i> , <i>CDKB</i>	Adventitious rooting	Zhu <i>et al.</i> (2016a)
Radish	Hypocotyls	<i>PAL</i> , <i>CHS</i> , <i>CHI</i> , <i>F3H</i> , <i>DFR</i> , <i>ANS</i>	Anthocyanin biosynthesis	Su <i>et al.</i> (2014)
Alfalfa	Seedlings	<i>CHS</i> , <i>FLS</i> , <i>6IOMT</i> , <i>Mn-SOD</i> , <i>POD1B</i> , <i>POD 2 CAT</i>	Anthocyanin biosynthesis	Xie <i>et al.</i> (2015)

negative correlation with its target genes *PROLIFERATING CELL FACTOR5 (PCF5)* and *PROLIFERATING CELL FACTOR8 (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*, *CDKA* 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*, *IFS* and *6IOMT* (Table 3). Furthermore, these 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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