BcGR1.1 Enhanced Tolerance to Copper Stress in Arabidopsis

Subjects: Horticulture

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Copper is a mineral element, which is necessary for the normal growth and development of plants, but high levels of copper will seriously damage plants. Studies have shown that *AtGR1* improves the tolerance of *Arabidopsis* to aluminum and cadmium stress. Researchers identified four genes (named *BcGR1.1*, *BcGR1.2*, *BcGR2.1* and *BcGR2.2*, respectively) encoding glutathione reductase (GR) in non-heading Chinese cabbage (*Brassica campestris (syn. Brassica rapa) ssp. chinensis*), which could be divided into two types based on the subcellular localization. Among them, *BcGR1.1*, which belonged to the cytoplasmic localization type, was significantly upregulated under copper stress. Compared to WT (the wild type), *Arabidopsis thaliana* heterologously overexpressed *BcGR1.1* had longer roots, higher fresh weight, higher GSH levels and GSH/GSSG (oxidized form of GSH) ratio, and accumulated more superoxide dismutase and peroxidase under copper stress. However, in the AsA-GSH cycle under copper stress, the contents of AsA and AsA/DHA were significantly downregulated, and the contents of DHA and T-AsA (total AsA) were upregulated, in the *BcGR1.1*-overexpressing *Arabidopsis*. Therefore, *BcGR1.1* could improve the scavenging ability of reactive oxygen species (ROS) by increasing the activity of GR, antioxidant enzymes and the utilization of AsA, and then enhance the copper stress tolerance of plants.

non-heading Chinese cabbage BcGR1.1 GR copper stress ROS

1. Introduction

In addition to the common abiotic stresses in agricultural production, such as drought, flooding and extreme temperatures [1][2], heavy-metal pressure has become another stress threatening crop production [3]. This is mainly due to the unrestricted industrialization and urbanization that has occurred in the last few decades. Heavy-metal pollution has become the focus of global attention due to its wide range, strong toxicity, long-term effects and irreversibility [4][5]. The heavy metals with the greatest influence on plant metabolism are Pb, Cd, Cu and Zn. As an essential heavy-metal element [6], copper plays a key role in many biological processes of plants, including photosynthesis and respiratory electron transfer, cell wall remodeling, superoxide scavenging, lignification and ethylene sensing [7][8][9][10][11][12][13]. If the copper content in plants is insufficient, the growth and development of plant reproductive organs are affected [14]. However, when the concentration of copper exceeds the requirements for plant growth, the structure and function of the plant cell membrane will be damaged, the permeability of plant cell membranes will be affected, and the antioxidant enzyme system and chloroplast structure of plants will be damaged, resulting in inhibition of the growth and development of plants [15][16][17][18][19][20][21].

Under copper stress conditions, the growth of plant primary roots is inhibited [22], which, in turn, affects the growth and development of aerial parts. Under the conditions of copper stress, there will be an excessive accumulation of ROS in plants, which is toxic to plants [23]. In the process of plant evolution, some mechanisms for coping with copper stress have been developed, including adjusting the dynamic balance of copper [24], activating the antioxidant defense response [25], and so on. The defense system includes enzymes that remove ROS, such as superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) as well as low-molecular-weight antioxidants, such as reduced ascorbic acid (AsA) and reduced glutathione (GSH) [26].

As an important component of the antioxidant system [27], GSH can play an important role in the physiological activity of organisms. It actively participates in the formation of disulfide, sulfide, thiolipid and other sulfides. It also participates in the elimination of excess ROS [28], the reduction in peroxides, the transmission of redox-sensitive signals [29], and the elimination of superoxide free radicals in the process of cell metabolism. It can coordinate with heterologous toxic substances, regulate plant growth and development, and resist various stresses (e.g., temperature, heavy metals, osmotic stress, and pathogen infection) [30][31][32][33][34][35][36][37][38]. GSH can chelate with copper in plants to excrete copper from the plant, thereby significantly reducing the copper content in plant tissues [39]. In addition, GSH is the electron donor of oxidized ascorbic acid (DHA), and its redox state is associated with the AsA-GSH cycle. As the main antioxidant system in plants, the AsA-GSH cycle can inhibit the production of ROS. This plays an important role in plant anti-aging and stress adaptation [40].

In general, glutathione mainly exists in two completely different forms: the reduced form (GSH) and the oxidized form (GSSG). Glutathione in plants is mostly reduced [41]. Therefore, glutathione is commonly referred to as the reduced form (GSH). In the face of stress, the activity of GR in an organism is improved, and the content of GSH is increased by the catalytic reduction in GSSG to increase plants' ability to resist environmental stress [42][43][44][45]. Therefore, high GR activity is necessary for plants to maintain high levels of GSH, especially under stress conditions.

In recent years, arable land area is decreasing. Thus, how to safely use the heavy-metal-contaminated arable land has become a major problem. However, since plants are immovable, they lack the ability to avoid the polluted environment. Therefore, the only chance for them to survive under adverse conditions is the mobilization of defense mechanisms and the evolution of a more tolerant genotype [46]. In *Arabidopsis thaliana*, overexpression of *AtGR1* results in high levels of GSH and GSH/GSSG ratios that help suppress ROS and lipid-peroxide-derived reactive carbonyl species (RCS) damage to plants, and enhance the dual detoxification function of plants, thereby enhancing the aluminum tolerance of *Arabidopsis thaliana* [47]. However, unlike aluminum stress, copper, as one of the components of heavy-metal pollution, is itself an essential trace element for plants. Therefore, it is particularly important to improve the plants' ability to cope with copper stress. The copper content in the soil should be not too much or too little. Therefore, it is particularly important to study the coordinated evolution of copper in plants and soils.

2. Phylogeny and Conserved Domains Analysis of GRs

Four *GR* genes in non-heading Chinese cabbage were identified, and named *BcGR1.1*, *BcGR1.2*, *BcGR2.1* and *BcGR2.2*. The results of phylogenetic tree analysis and the differential distribution of conserved motifs indicate that the GRs of non-heading Chinese cabbage are similar to other varieties, with two subcellular localization types: cytoplasm (**Figure 1a**, green box) and chloroplast (**Figure 1a**, pink box). Phylogenetic tree analysis showed that there is a close evolutionary relationship among non-heading Chinese cabbage, *Brassica rapa*, *Brassica napus* and *Raphanus sativus* (**Figure 1a**). Motif analysis showed that the motifs of BcGRs are overwhelmingly the same as those of other species (**Figure 1b**). For the cytoplasmic localized GR, there was only a difference in the first motif, motif 13, which was only identified in several other species, except RsGR, CrGR, and CsGR. Chloroplast-localized GR, according to the difference between the fourth and fifth conserved motifs, can be divided into four categories. EsGR, BoGR, BnaGR, RsGR, BcGR2.2, CsGR have exactly the same motifs and belong to the same class; compared with this class, BrGR lacks motif 18 and motif 15, and BcGR2.1 does not contain motif 18, The fourth motif of CrGR and AtGR is motif 10 instead of motif 18.

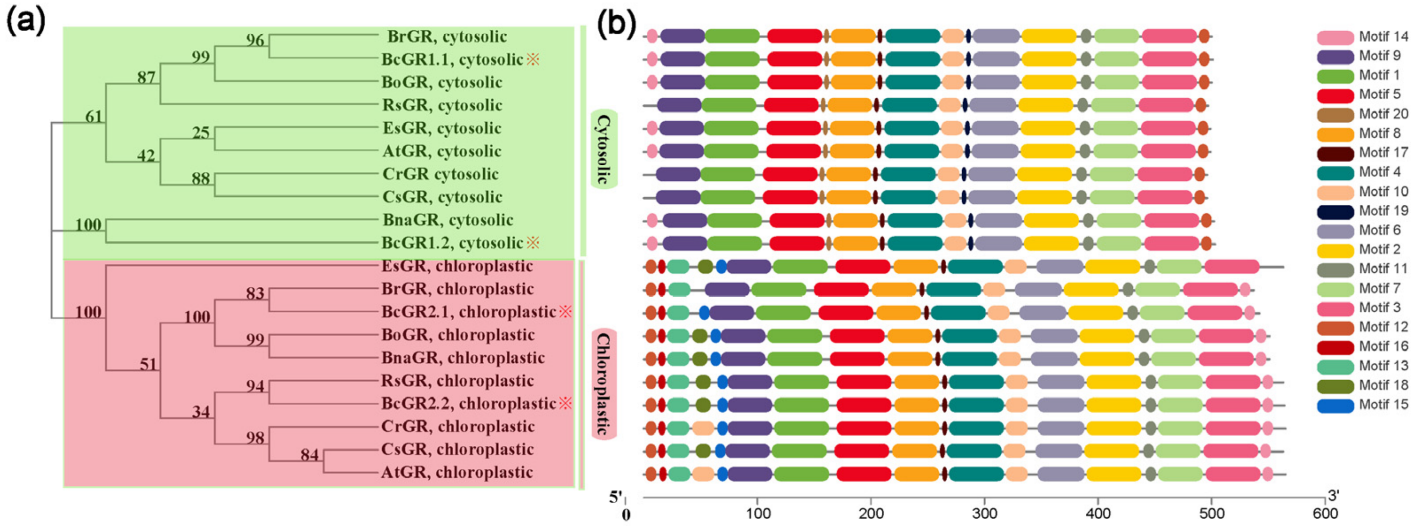
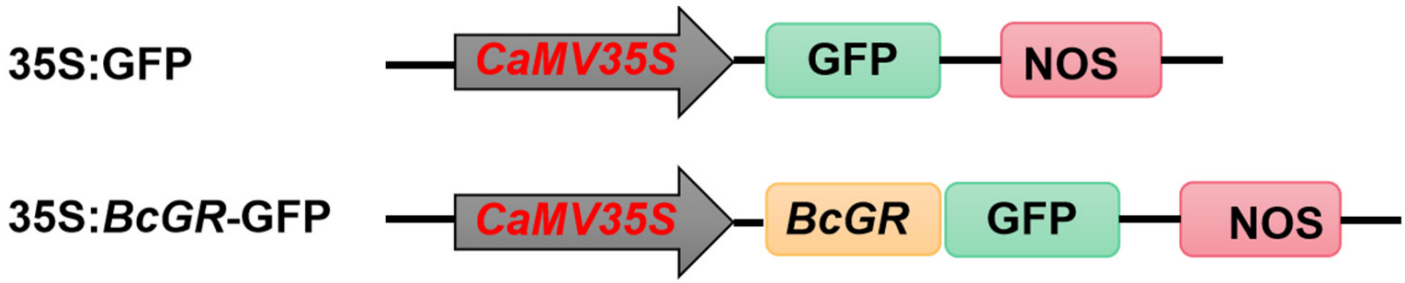


Figure 1 Phylogenetic relationships **(a)** and conserved motif distributions **(b)** of GRs. Each motif is represented by a coloured box numbered at the right.

3. Subcellular Localization of BcGRs

To further determine the specific location of BcGR proteins in cells, subcellular localization was performed. Researchers constructed a vector (**Figure 2a**), fused BcGRs with GFP, and detected the localization of BcGRs protein in cells by observing GFP. Results show that GFP protein was expressed in the whole cell, while BcGR1.1-GFP and BcGR1.2-GFP fusion proteins were mainly expressed in the cell membrane and cytoplasm, BcGR2.1-GFP and BcGR2.2-GFP fusion proteins were mainly expressed in the chloroplast (**Figure 2b**). This result indicated that the non-heading Chinese cabbage had two types of GR proteins: cytosolic localization and chloroplastic localization, which is consistent with the results of evolutionary analysis.



(a)

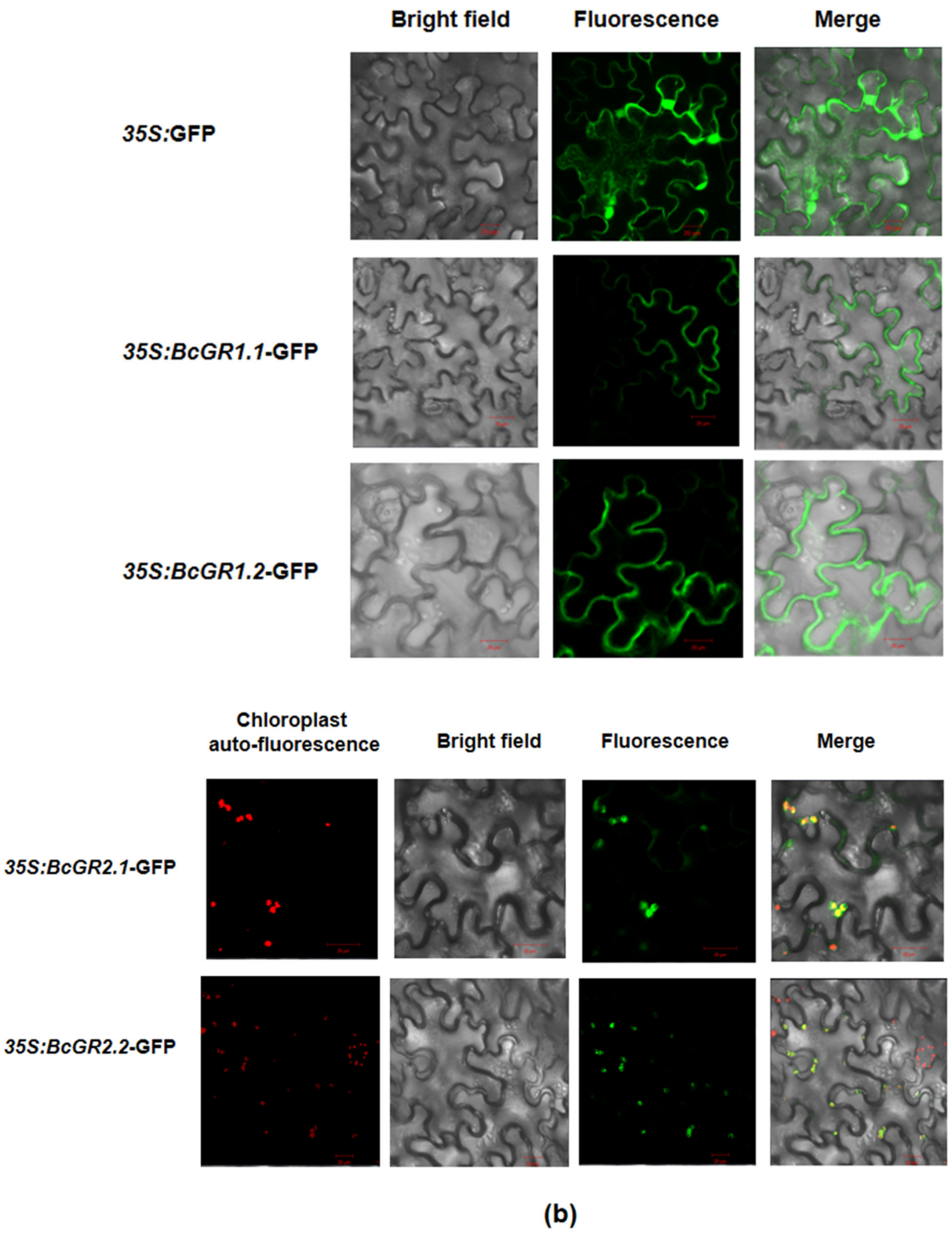


Figure 2 Subcellular localization of BcGRs in *Nicotiana benthamiana* plants. **(a)** Schematic diagram of the construction of subcellular localization vector. **(b)** *N. benthamiana* cellullar images of BcGRs. Researchers used 35S:GFP empty vector as a control in this experiment, and fused BcGRs protein with GFP to observe their subcellular localization. Chloroplast autofluorescence, red fluorescence of chloroplasts under excitation light at 488 nm and collection light at 650-750 nm. Bright field, bright field images of tobacco leaf cells. Fluorescence, green fluorescence of fusion protein of 35S: BcGR-

GFP or 35S: GFP. Merged, overlay of bright field, green fluorescence, or bright field, green fluorescence and red fluorescence images. Bars = 20 μm.

4. Expression patterns of *BcGR*s

Here, researchers used one-month-old, non-heading Chinese cabbage for copper treatment. The qRT-PCR showed that the four *GR* homologous genes in non-heading Chinese cabbage have different responses under copper stress (**Figure 3**). *BcGR1.1*, *BcGR1.2* and *BcGR2.1* were upregulated, especially *BcGR1.1*. The expression rapidly increased 6 h after copper stress, reaching a peak at 9 h and then slowly decreasing. However, the expression pattern of *BcGR2.2* was completely different from the other three *BcGR*s. *BcGR2.2* was downregulated, and showed the lowest expression at 9 h. The results showed that, among the four *GR* homologous genes, *BcGR1.1* may play a major role in copper stress.

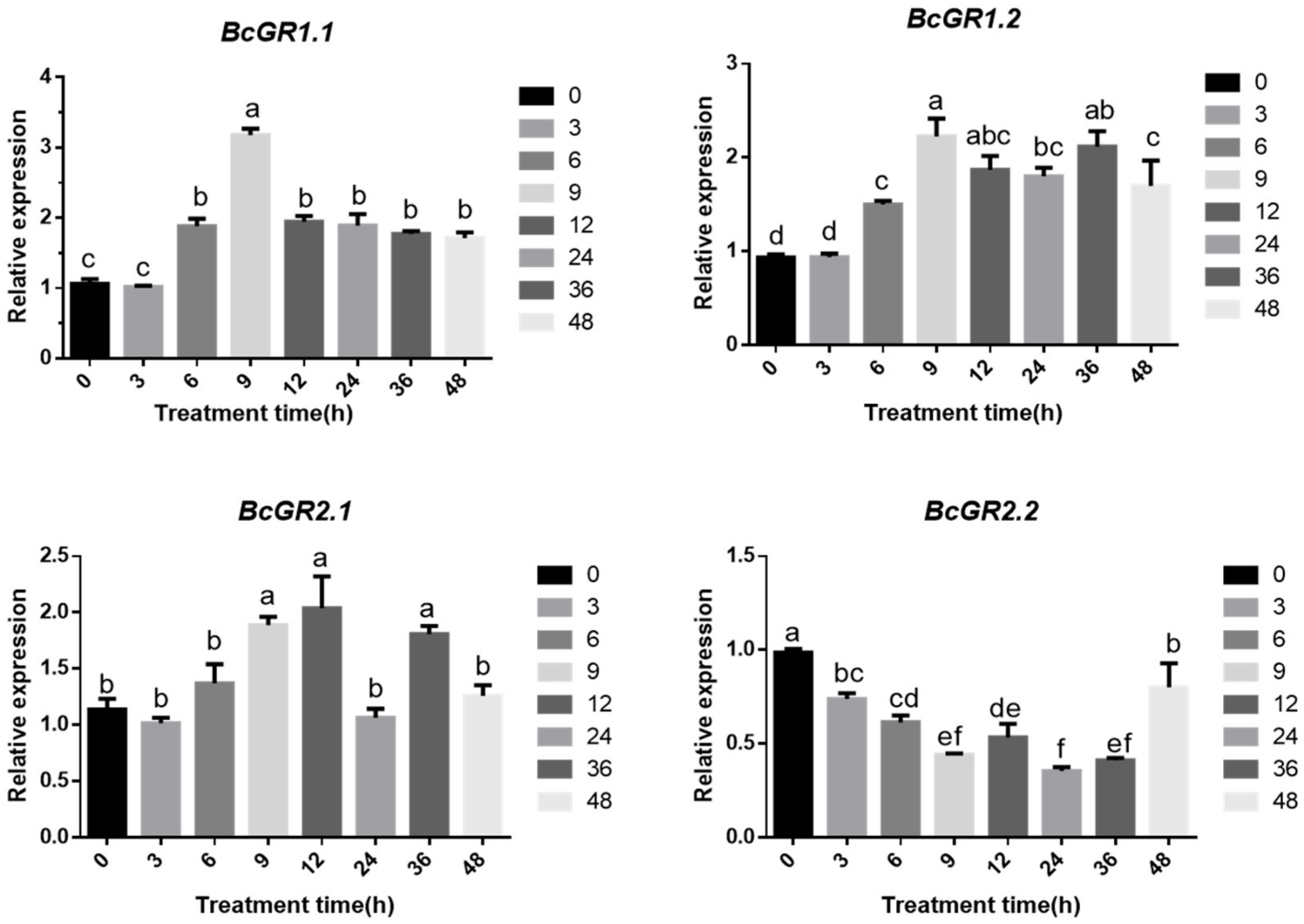


Figure 3 Expression patterns of *BcGR*s genes in the non-heading Chinese cabbage under copper stress. Analysis of *BcGR*s expression at different time points (0 h, 3 h, 6 h, 9 h, 12 h, 24 h, 36 h and 48 h) under copper stress. The expression levels of *BcGR*s represented the expression fold of each *BcGR* in seedlings treated with copper stress at different times, relative to seedlings without copper stress treatment, using the *BcGAPC* gene as an internal reference gene. The expression level of

*BcGR*s at each timepoint is the average of three replicates. Error bars indicate standard error of mean (SEM) of three independent experiments. According to the LSD test, the values within the same treatment followed by the same letter are not significantly different ($p < 0.05$).

5. Heterologous Overexpression of *BcGR1.1* in *Arabidopsis thaliana*

To further study the function of *BcGR1.1*, researchers transformed *Arabidopsis thaliana* by dipping flowers to obtain *BcGR1.1*-overexpressing plants. For each generation of transgenic plants, DNA level detection (**Figure 4b**), GUS detection (**Figure 4c**) and expression analysis (**Figure 4d**) were performed, respectively. After obtaining the transgenic lines, researchers tested the GR activity in the WT and transgenic lines. The results showed that the expression levels of *BcGR1.1*-0E3, *BcGR1.1*-0E7, and *BcGR1.1*-0E8 were increased to 274, 228, and 255 times of the WT, and the GR activity were significantly higher than that of the WT, which were 4.3, 3.1, and 3.3 times that of the WT, respectively (**Figure 4e**).

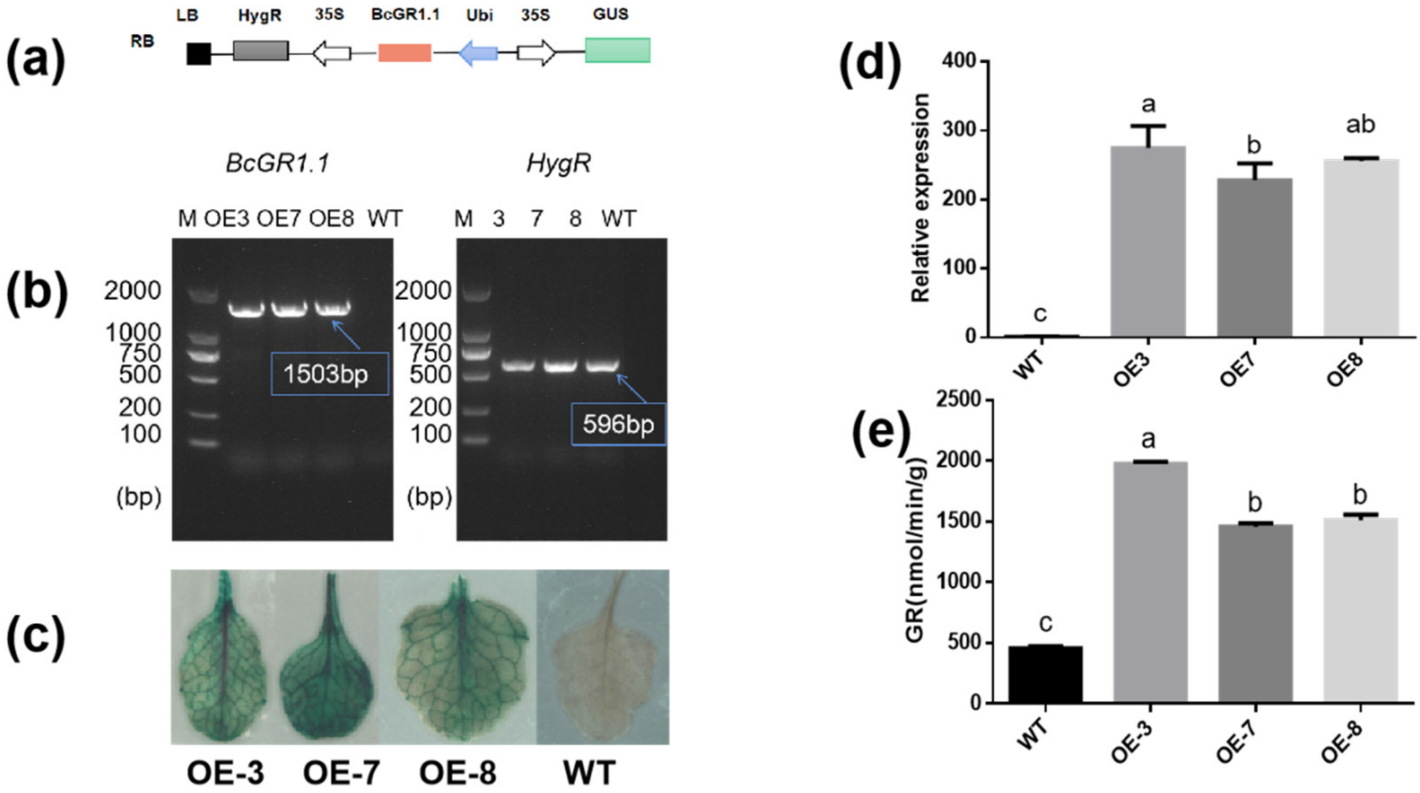


Figure 4 Creation and identification of *Arabidopsis thaliana* overexpressing *BcGR1* gene. (**a**) Construction of a vector expressing *BcGR1.1* under the control of Ubi promoter. (**b**) DNA level identification of positive seedlings. DNA from overexpression plants (OE3, OE7, OE8) and wild-type (WT) were used as PCR templates. Specific primers for *BcGR1.1* and hygromycin genes were used, and M represents DNA marker. (**c**) GUS detection of *BcGR1.1* in overexpression plants and WT. (**d**) Analysis of *BcGR1.1* expression level in overexpression plants and WT. (**e**) Detection of GR activity in overexpression plants and WT. Error bars indicate standard error of mean (SEM) of three

independent experiments. According to the LSD test, the values within the same treatment followed by the same letter are not significantly different ($p < 0.05$).

6. Overexpression of *BcGR1.1* Improved Copper Stress Tolerance in *Arabidopsis thaliana*

The expression of *BcGR1.1* was significantly upregulated under copper stress, and its heterologous overexpression in *Arabidopsis* obviously increased the GR activity. Therefore, researchers further studied the role of *BcGR1.1* in the response of plant to copper-stress . The GR, root length and fresh weight of WT and *BcGR1.1*-OE plants were measured under normal conditions (CK) and copper stress. Regardless of the presence of copper stress, the activity of GR in *BcGR1.1*-OE plants was always higher than that of WT. The GR activities of *BcGR1.1*-OE and WT were both increased under copper stress (**Figure 5d**). Under normal growth conditions, the roots of WT were longer than that of transgenic lines. Under copper treatment, root growth of WT and transgenic plant lines was significantly inhibited, and the root growth of WT plants was shorter than that of *BcGR1.1*-OE plants (the inhibition rates were 75% and 56%, respectively) (**Figure 5b**). There was no significant difference between the WT and *BcGR1.1*-OE plants under normal growth conditions, except OE3. However, the fresh weight of the transgenic lines was significantly higher than that of WT under copper treatment (**Figure 5c**). The results showed that the *BcGR1.1*-OE plants grew better under copper stress due to the longer roots and increased biomass.

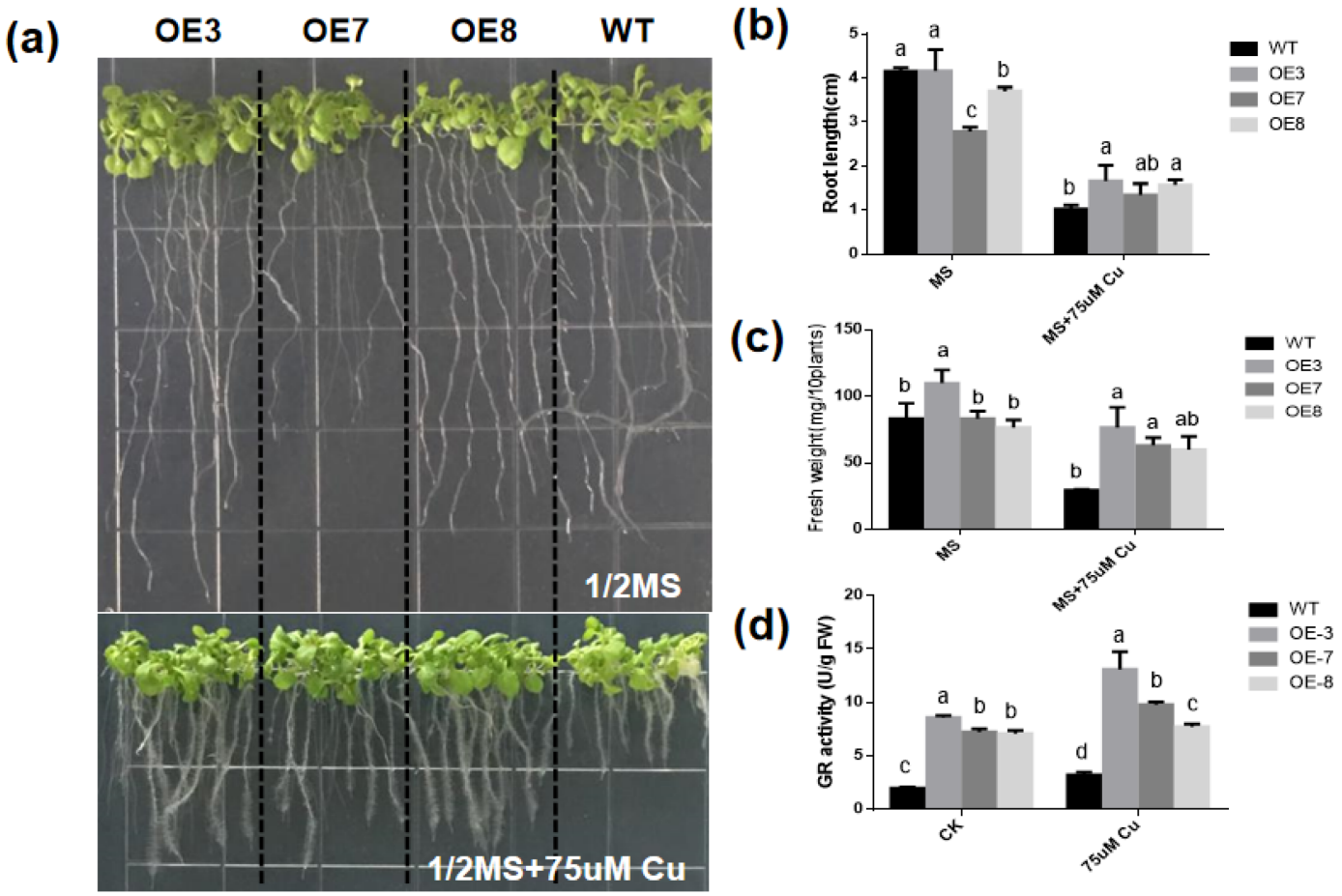


Figure 5 The growth state of *Arabidopsis thaliana* overexpressing *BcGR1.1* gene under copper stress. (**a**) Image of 21-day growth on half-strength Murashige and Skoog (1/2 MS) medium with or without 75 µM copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$). (**b**) Root growth. (**c**) Fresh weight. The fresh weight (FW) of wild-type and transgenic plants treated with or without 75 µM $CuSO_4 \cdot 5H_2O$ for 21 days. (**d**) Detection of GR activity in overexpression plants and WT. According to the LSD test, the values within the same treatment followed by the same letter were not significantly different ($p < 0.05$). Error bars indicate standard error of mean (SEM) of three independent experiments.

7. Effects of *BcGR1.1* Overexpression on the Status and Content of glutathione and AsA in *A. thaliana*

In the cycle of ASA-GSH (**Figure 6a**) [48], ASA and GSH are both important reducing substances in plants. Their oxidation/reduction status is closely related to the stress tolerance ability of plants. Therefore, researchers analyzed the changes in the status and content of these two antioxidant substances under copper stress.

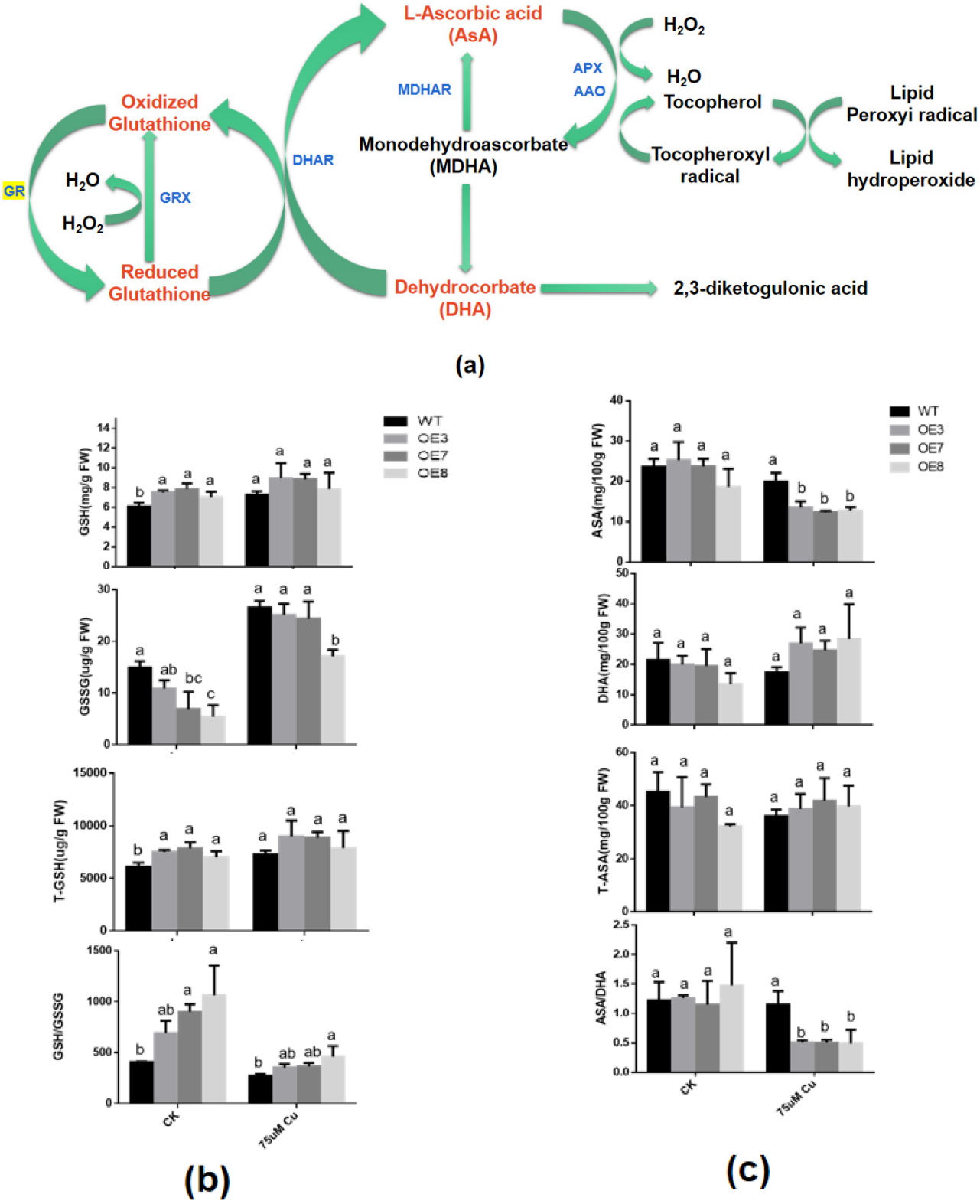


Figure 6 Analysis of antioxidant (AsA, GSH) content in WT and transgenic lines under copper stress. (**a**) The cycle of ASA-GSH. (1. ascorbate peroxidase (APX). 2. ascorbate oxidase (AAO). 3. monodehydroascorbate reductase (MDHAR). 4. dehydroascorbate reductase (DHAR). 5. glutathione peroxidase (GRX). 6. glutathione reductase (GR). (**b**) GSH, GSSG, T-GSH, and GSH/GSSG. (**c**) AsA, DHA, T-AsA and AsA/DHA. The plants were sampled and analyzed after exposure to normal or excess copper for 24 h. The data error is expressed as standard error of mean (SEM).

According to the LSD test, there was no significant difference in the values with the same letter after the same treatment ($p < 0.05$).

As far as glutathione is concerned, the GR enzyme activity in transgenic lines are significantly increased under normal growth conditions and under copper treatment (**Figure 5d**), which may result in a higher GSH content and lower GSSG and total glutathione (T-GSH) content. However, this difference is significant under normal growth conditions, but not under copper stress. This may be the result of the significant upregulation of *GR* in WT under copper stress. GSH/GSSG in both wild-type and transgenic plants decreased under copper stress. However, in transgenic lines, GSH/GSSG is still higher than WT (**Figure 6b**). These results indicated that higher levels of GSH may be beneficial for plants to cope with copper stress.

As for AsA, there is no significant difference in AsA, DHA, T-AsA and AsA/DHA content in WT and transgenic lines under normal conditions. Under copper stress, the AsA content in the transgenic line was significantly lower than that of the WT, while the content of DHA was the opposite. However, there was no significant difference between T-AsA. This resulted in a significant reduction in AsA/DHA in transgenic lines, to about 50% of the WT plants (**Figure 6c**). These results suggest that the heterologous overexpression of *BcGR1.1* in *Arabidopsis* may improve the efficiency of transforming AsA into DHA, thereby efficiently clearing ROS and improving plant tolerance to copper stress.

8. Antioxidant Enzyme Activities Are Altered in Transgenic *A. thaliana*

To determine whether the increase in copper-stress tolerance of transgenic plants is related to the change in antioxidant activity, the activities of SOD (**Figure 7a**), POD (**Figure 7b**), CAT (**Figure 7c**) and the content of H_2O_2 were measured in WT and transgenic plants with or without excessive copper treatment. With or without copper stress, the SOD and POD activities in *BcGR1.1*-OE plants were higher than those in WT. Under copper-stress treatment, SOD activity did not significantly change in WT and *BcGR1.1*-OE lines, but POD enzyme activity significantly increased. Under copper stress, the POD activities of OE3 and OE8 were 2.9 times and 2.3 times higher than that of WT, respectively. The CAT activity of *BcGR1.1*-OE line was lower than that of WT plants under normal conditions, but showed no difference under copper stress treatment. Upregulation of POD activity levels in transgenic lines under copper stress resulted in significantly lower H_2O_2 content in transgenic lines than in WT (**Figure 7d**). The results indicated that the heterologous overexpression of *BcGR1.1* in *Arabidopsis* can regulate the activity of certain antioxidant enzymes, reduce the ROS damage to plants, and improve the tolerance of plants to copper stress.

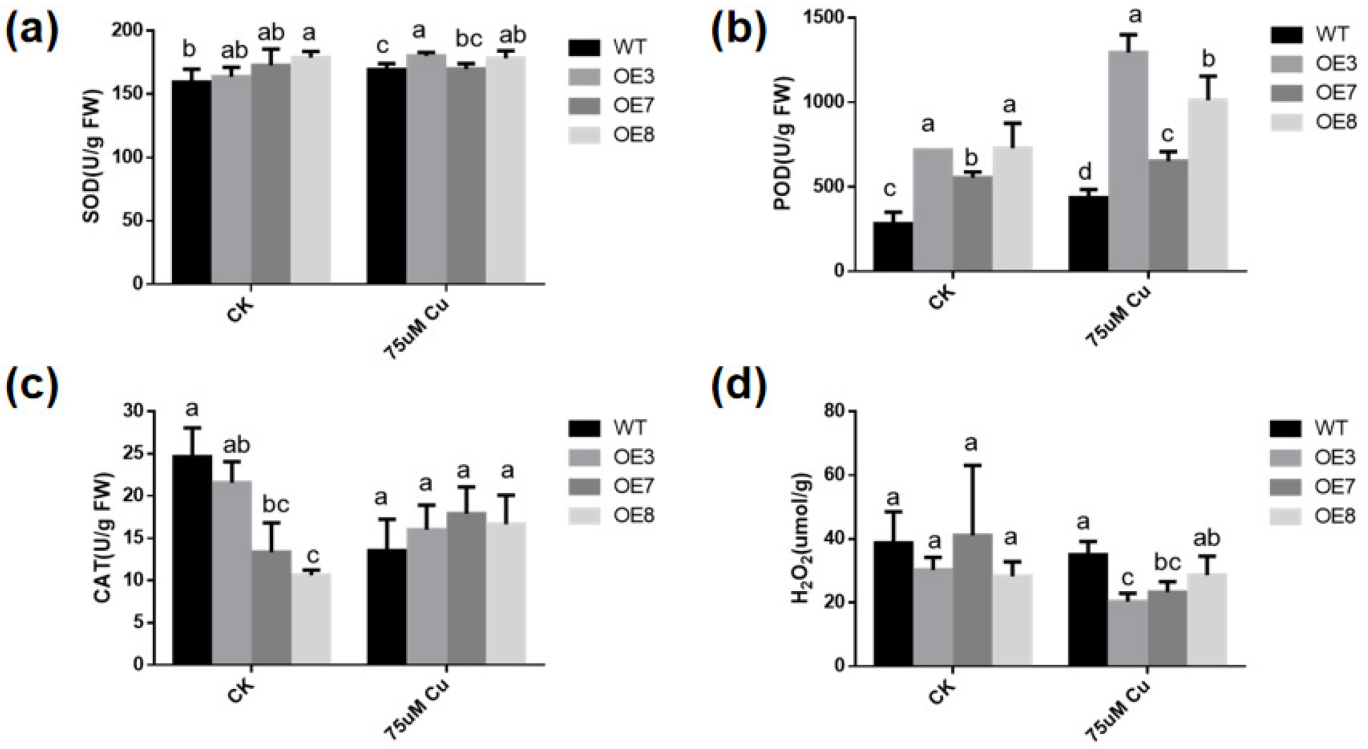


Figure 7 Determination of antioxidant enzyme activity. Analysis of the enzyme activities of SOD (**a**), POD (**b**), CAT (**c**) and the content of H_2O_2 (**d**) with or without excessive copper treatment. The plants were sampled and analyzed after exposure to normal or excess copper for 24 h. And the data error is expressed as standard error of mean (SEM). According to the LSD test, there was no significant difference in the values with the same letter after the same treatment ($p < 0.05$).

9. Virus-Induced *BcGR1.1* Silencing in Non-Heading Chinese Cabbage

Researchers used VIGS experiment to further study the function of *BcGR1.1* in non-heading Chinese cabbage. Two weeks after virus inoculation, PTY-*BcGR1.1* and PTY showed a mosaic leaf phenotype (**Figure 8a**). Plants with potential *BcGR1.1* function loss were sampled, and qRT-PCR was used to evaluate the silencing efficiency of *BcGR1.1*. As shown in **Figure 8b**, compared with PTY plants, *BcGR1.1* expression was significantly reduced by 70.4%, 81.4%, 64.9% and 82.6% in pTY-*BcGR1.1*#1, #2, #3, and #5 seedlings, respectively. There was no significant difference in GR activity between PTY-*BcGR1.1* and PTY plants (**Figure 8c**), which may be the result of functional complementarity between *BcGR* homologous genes.

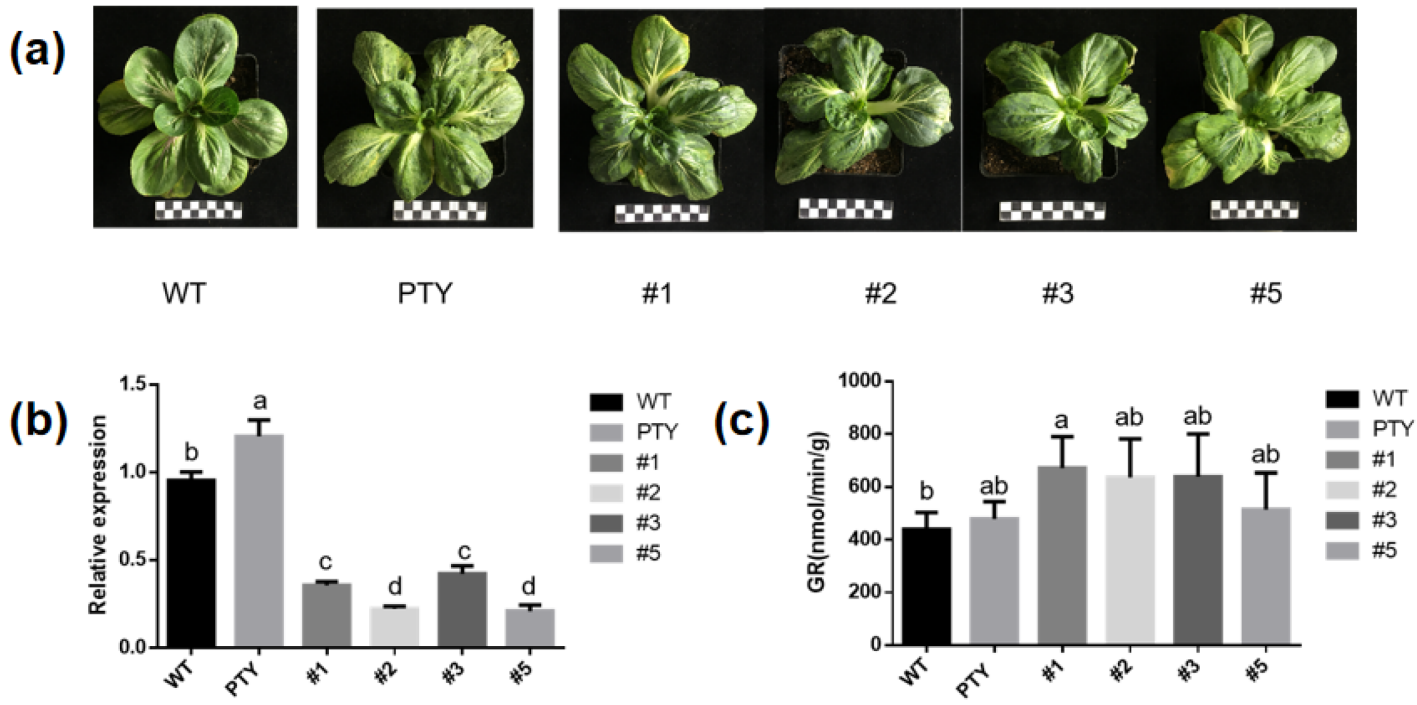


Figure 8 Virus-induced *BcGR1.1* silencing in non-heading Chinese cabbage. (**a**) Virus symptoms of the plants. (**b**) qPCR detection in *BcGR1.1* silenced plants. (**c**) Determination of GR activity of *BcGR1.1*-silenced plants. The data error is expressed as standard error of mean (SEM). According to the LSD test, the values within the same treatment followed by the same letter are not significantly different ($p < 0.05$).

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