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RESEARCH ARTICLE

# Transgenic *Arabidopsis thaliana* expressing a wheat oxalate oxidase exhibits hydrogen peroxide related defense response

**WEI Fang[1, 2*], HU Jie[1*], YANG Yan[1, 2], HAO Zhi-da[1], WU Rui-hua[1], TIAN Bao-ming[1, 2, 3], CAO Gang-qiang[1, 2], ZANG Xin[1, 3]**

[1] *School of Life Sciences, Zhengzhou University, Zhengzhou 450001, P.R.China*
[2] *Zhengzhou Key Laboratory of Plant Molecular Breeding, Zhengzhou 450001, P.R.China*
[3] *Henan Key Laboratory of Bioactive Macromolecules, Zhengzhou 450001, P.R.China*

**Abstract**

Oxalic acid (OA) is considered as an important pathogenetic factor of some destructive diseases caused by some fungal pathogens such as *Sclerotinia sclerotiorum*. Oxalate degradation is important for plant health, and plants that contain oxalate oxidase (OXO) enzymes could breakdown oxalate into $CO_2$ and $H_2O_2$, which subsequently evokes defense responses. However, some species, such as *Arabidopsis thaliana*, have no oxalate oxidase activity identified to date. The present study aims to develop transgenic *Arabidopsis* expressing a wheat oxalate oxidase, to test for the response to OA exposure and fungal infection by *S. sclerotiorum*. The results showed that the transgenic *Arabidopsis* lines that expressed the wheat OXO exhibited enhanced resistance to OA exposure and *S. sclerotiorum* infection in the tolerance assays. In the same manner, it could convert OA to $CO_2$ and $H_2O_2$ to a higher extent than the wild-type. Intensive osmotic adjustments were also detected in the transgenic *Arabidopsis* lines. The higher level of produced $H_2O_2$ subsequently induced an elevated activity of antioxidant enzymes including superoxide dismutase (SOD) and peroxidase (POD) in the transgenic *Arabidopsis* plants. The present study indicated that the expression of a gene encoding wheat OXO could induce intensive osmotic adjustments and hydrogen peroxide related defense response, and subsequently increased tolerance to *S. sclerotiorum* in transgenic *A. thaliana*.

**Keywords:** oxalate oxidase, oxalic acid, *Sclerotinia sclerotiorum*, hydrogen peroxide, *Arabidopsis thaliana*

## 1. Introduction

Oxalic acid (OA), one small dicarboxylic acid, is thought to be present in most plant tissues. In many plant species, oxalate is known to have been produced through ascorbate breakdown (Franceschi and Nakata 2005), and considered as a key pathogenetic factor of some destructive diseases caused by some fungal pathogens (Dutton and Evans 1996; Yadav *et al.* 2012). These diseases include *Sclerotinia sclerotiorum*, which has an extremely broad range of host plants (Zheng *et al.* 2007; Huang *et al.* 2013). The disease

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WEI Fang, Tel/Fax: +86-371-67739513, E-mail: fangwei@zzu.edu.cn; Correspondence TIAN Bao-ming, Tel/Fax: +86-371-67739513, E-mail: tianbm@zzu.edu.cn
[*] These authors contributed equally to this study.


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severity caused by *Sclerotinia sclerotiorum* is directly correlated with OA levels in the infected plant tissues (Williams *et al.* 2011). Oxalate could lower extracellular pH, allowing for optimum activity of plant cell wall-degrading enzymes produced by the fungus (Guimaraes and Stotz 2004). Oxalate was considered directly toxic to host plants because of its acidity (Alkan *et al.* 2013). Thus, the secreted OA by *S. sclerotiorum* might enhance virulence in some ways (Kim *et al.* 2008).

Basically, oxalate oxidase (OXO) catalyses oxidation of OA into carbon dioxide ($CO_2$) and hydrogen peroxide ($H_2O_2$). Degradation of toxic OA and generating $H_2O_2$ by OXO serves as dual roles in the defense responses (Sabater and Martin 2013). $H_2O_2$ is directly toxic to micro-organisms (Shetty *et al.* 2007), and also contributes to structural reinforcement of plants cell walls (Hammond-Kosack and Parker 2003). For example, the gene encoding wheat OXO has already been applied to many species to enhance plant resistance to OA and fungal pathogens, such as tobacco (Berna and Bernier 1997), American chestnut (Zhang *et al.* 2013), potato (Ahmad *et al.* 2008), oilseed rape (Dong *et al.* 2008), hybrid poplar (Liang *et al.* 2001), and so on.

Overexpression of OXO not only contributes to resistance to OA-producing pathogens in plants, but also triggers other defense responses. OXO could trigger synthesis of salicylic acid that co-ordinates defense responses, such as expressions of *PR5-1*, *defensin*, and *SCO* (Hu *et al.* 2003). OA is an important pathogenicity determinant of *S. sclerotiorum*, and could induce a programmed cell death (PCD) response in the diseased plant tissue. Oxalate could also induce increased reactive oxygen species (ROS) levels in the plant, which correlate closely to PCD (Kim *et al.* 2008). Thus the transgenic OXO plants could enhance their resistance by breakdown of OA that triggers PCD *via* ROS production (Rietz *et al.* 2012; Zhang *et al.* 2013).

Additionally, the emerging evidence suggests a more complicated interaction among ROS, OXO and antioxidant capacity under biotic stress, in which the enhanced level of ROS usually causes oxidative damage, but compromised by antioxidative defense system comprising of nonenzymatic and enzymatic components (Gururani *et al.* 2012; Daub *et al.* 2013). For instance, the expression level of OXO in the transformed tobacco was up-regulated by both abiotic and biotic stresses (Berna and Bernier 1999; Orozco-Cardenas *et al.* 2001).

*Arabidopsis* has been proposed as a host model to study interactions with *S. sclerotiorum* (Dickman and Mitra 1992; Dai *et al.* 2006), and no OXO activity has identified in *Arabidopsis thaliana* to date (Foster *et al.* 2012). Therefore, the strategy presented here is to express a gene encoding wheat oxalate oxidase that could degrade OA into $CO_2$ and $H_2O_2$, to investigate the response to OA exposure and fungus infection by *S. sclerotiorum* in the transgenic *Arabidopsis*.

## 2. Results

### 2.1. Development of transgenic *Arabidopsis thaliana* expressing wheat OXO

The pCAMBIA3304-OXO plasmid containing the gene encoding wheat oxalate oxidase under the control of a duplicated CaMV 35S promoter was employed for *Agrobacterium*-mediated transformation of *A. thaliana* inflorescence by using a modified floral-dip method (Martinez-Trujillo *et al.* 2004). The main inflorescences inoculated with *Agrobacterium tumefaciens* were then analyzed by histochemical GUS staining, and the results indicated that the *Arabidopsis* ovules could be efficiently transformed with the used *Agrobacterium* strain (Fig. 1-A). The collected $T_1$ seeds were grown on the MS media containing 12 mg $L^{-1}$ phosphinothricin to select phosphinothricin-resistant plants, which were then transferred to soil for development and histochemical GUS assay. The identified $T_1$ plants were independently retained in the growth chamber for yielding

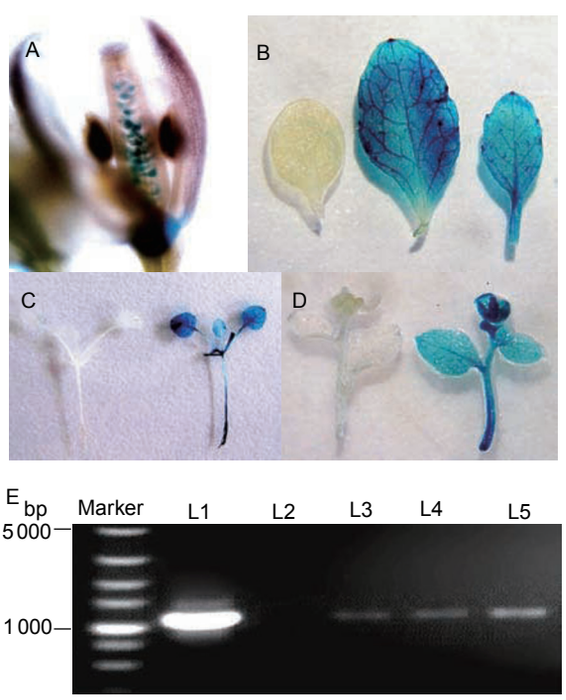



**Fig. 1** Transformant identification by GUS staining and genomic PCR analysis. The wheat oxalate oxidase (OXO) and GUS were driven with CaMV 35S promoter. A, the transformed *Arabidopsis* ovule. B, the transformed *Arabidopsis* leaves ($T_2$ generation). C, the transformed *Arabidopsis* seedling plant ($T_2$ generation). D, the transformed *Arabidopsis* flower and stem ($T_2$ generation). E, about 1.1 kb of amplified DNA product. L1, the plasmid pCAMBIA3304-OXO; L2, the wild-type *Arabidopsis*; L3–5, the $T_2$ *Arabidopsis* plants.

seeds ($T_2$ generation). $T_2$ plants was then confirmed by histochemical GUS assay (Fig. 1-B, C and D) and PCR analysis (Fig. 1-E).

### 2.2. Transgenic plants showed increased OXO expression and elevated $H_2O_2$ production

To determine whether the transgene OXO was functionally expressed in the transformed plants, the RT-PCR was used to analyze the expression level of OXO in the leaves of transgenic *Arabidopsis* inoculated with *S. sclerotiorum*. The results showed that a significantly increased level of OXO expression was detected in the transgenic plants, as well as in the inoculated transgenic plants, in contrast with the wild-type plants (Fig. 2). Moreover, expression of transgenic OXO was confirmed by histochemical detection of $H_2O_2$ production by diaminobenzidine (DAB) staining in the transgenic *Arabidopsis* after infection with *S. sclerotiorum.* The results showed that a stronger red-brown coloration was detected in the transgenic plants in comparison with the wild-type plants (Fig. 3).

### 2.3. Transgenic OXO plants exhibited enhanced resistance to OA and S. *sclerotiorum*

Resistance of the OXO transgenic plants was evaluated after OA exposure and *S. sclerotiorum* infection by the detached leaf assay. The brown lesion and yellowing area surrounding of the detached leaves were observed after OA treatment (10, 40 and 60 mmol $L^{-1}$) (Fig. 4-A), and the data indicated that the OXO transgenic *Arabidopsis* displayed the enhanced resistance for OA treatment, but the wild-type plants were comparatively more sensitive to OA treatment (Fig. 4-B). The resistance of transgenic *Arabidopsis* to

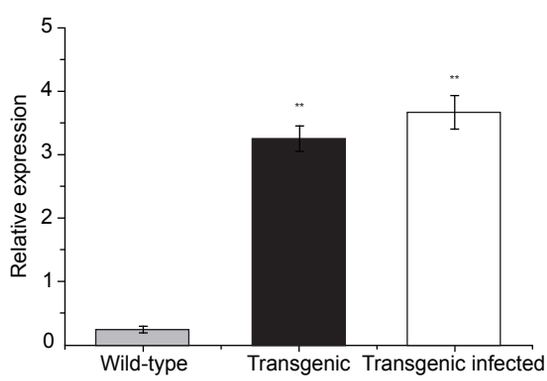
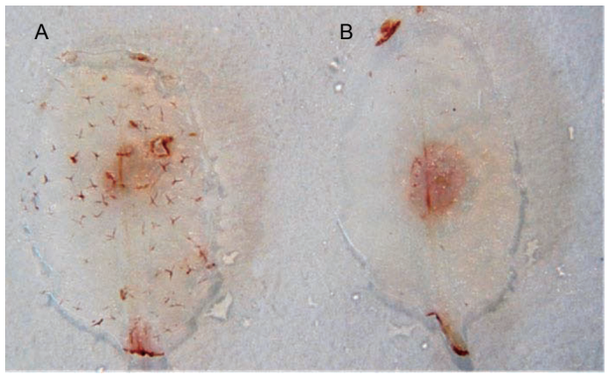



**Fig. 2** The quantitative real-time PCR analysis of OXO expression in transgenic plants. The normalized fold expression was calculated with the $2^{-\Delta\Delta CT}$ method, the wild-type without infection was used as a control. **, significant level at $P<0.05$. The same as below.



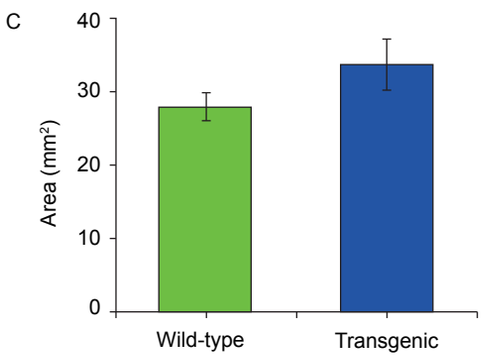




**Fig. 3** $H_2O_2$ production in leaves after infection with *Sclerotinia sclerotiorum*. A, the wild-type. B, the transgenic *Arabidopsis*. C, the infection area in the wild-type and transgenic plants.

*S. sclerotiorum* was also tested, and the results showed that the necrotic area become visible on the leaves of the wild-type plants after fungal treatment for 48 h (Fig. 5-A), but less area of necrosis appeared in the OXO-transformed leaves (Fig. 5-B).

### 2.4. Activity of antioxidant enzymes in the OXO transgenic *Arabidopsis*

The activity of endogenous antioxidant enzymes including SOD, POD and CAT was analyzed in the transgenic *Arabidopsis* plants after infection with *S. sclerotiorum*, and the results showed that a significantly enhanced activity of SOD and POD was identified in transgenic plants inoculated with *S. sclerotiorum* in comparison with that in the uninfected wild-type plants, but a reduced CAT activity was determined in the fungal-infected transgenic and wild-type plants in contrast with that in the uninfected plants (Fig. 6-A).

### 2.5. Osmotic adjustment in the OXO transgenic *Arabidopsis*

To analyze osmotic adjustments in the transgenic plants

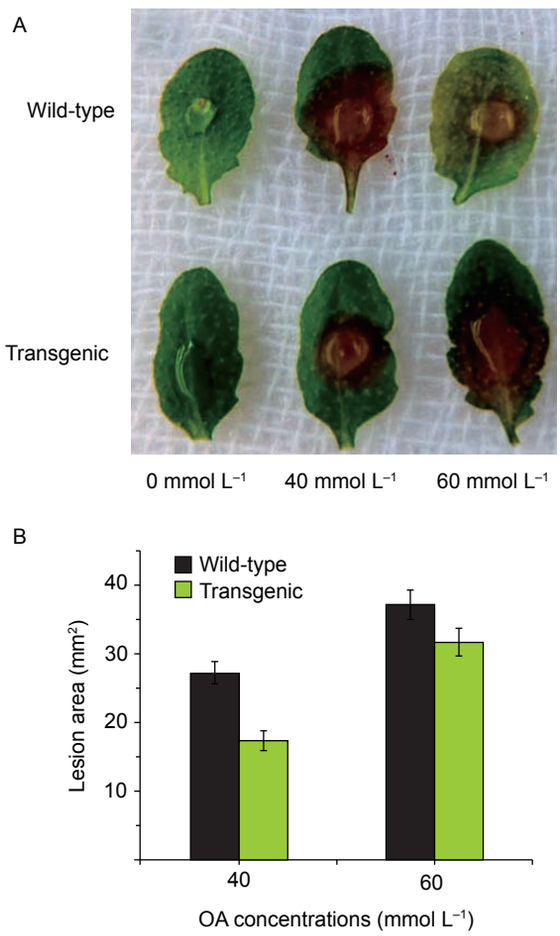



**Fig. 4** Oxalic acid (OA) resistance in the detached leaves. A, the leaves of wild-type and transgenic plants treated with OA for 24 h. B, the quantified brown lesion area in wild-type and transgenic leaves.

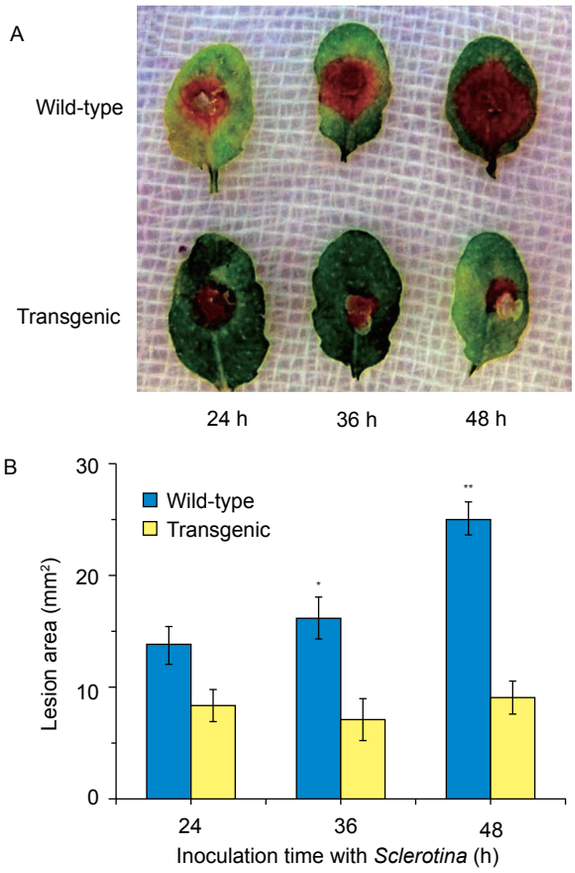



**Fig. 5** The fungal resistance in the detached leaves. A, images of wild-type and transgenic leaves for fungal treatments. B, measurements of fungal spread in wild-type and transgenic plant leaves. *, significant level at $P<0.01$. The same as below.

infected with *S. sclerotiorum*, soluble substance such as carbohydrates and proline was quantified in the transgenic plants after *S. sclerotiorum* treatment for 24 h. The data showed that both proline and sugar content significantly increased in the transgenic *Arabidopsis*, in comparison with those in the uninfected wild plants (Fig. 6-B).

## 3. Discussion

Expression of transgenic OXO could lead to production of the defense-induced molecule, $H_2O_2$ (Lane *et al.* 1993; Woo *et al.* 2000; Tamas *et al.* 2010; Molla *et al.* 2013; Zhang *et al.* 2013). However, it is not clear if $H_2O_2$ generated by OXO induces defense responses and plays a role in plant disease resistance. *Arabidopsis* wild-type was lack of wheat germin-like OXO activity as it was only found in the major crop cereals (Lane 2000), and thus provided us with a suitable system for function analysis of transgene with wheat OXO. In the present study, the OXO transgenic *Arabidopsis* was produced by using *A. tumefaciens*-mediated floral-dip method, and the transgenic plants functionally expressing this OXO gene showed increased accumulation of $H_2O_2$ compared with wild-type. The $H_2O_2$ accumulation was considerred closely associated with increased OXO activity in the transgenic sunflower plants (Hu *et al.* 2003). Moreover, the increasing evidence showed that plant pathogenesis was associated with $H_2O_2$ (Beneloujaephajri *et al.* 2013; Daub *et al.* 2013; Zhang *et al.* 2013), and more likely OXO could increase tolerance to pathogen through generating $H_2O_2$. Furthermore, the OXO transgenic *Arabidopsis* exhibited the enhanced resistance against OA exposure and *S. sclerotiorum* infection. The leaf inoculation assay showed that the expression of transgenic OXO generating $H_2O_2$ significantly inhibited *S. sclerotiorum* infection and reduced the lesion area in the leaves of transgenic plants. The OXO transgenic *Arabidopsis* lines exhibited the enhanced

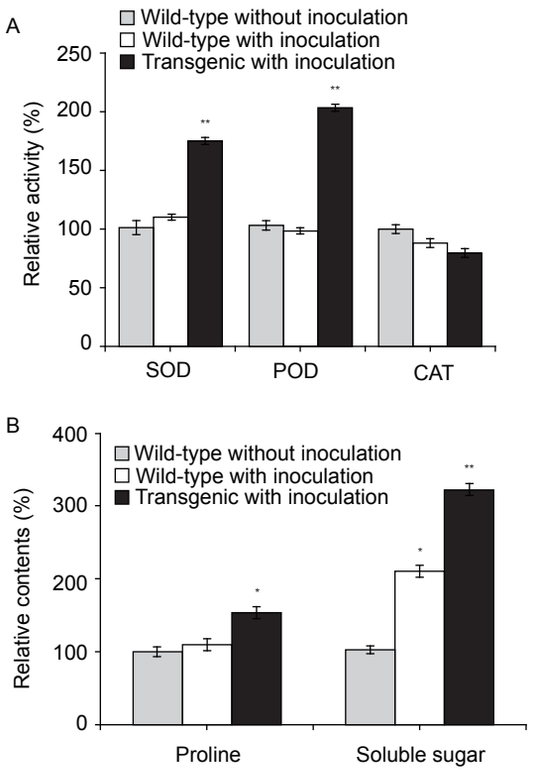



**Fig. 6** The measurements of antioxidant enzymes and soluble substance in transgenic plants after inoculation with *S. sclerotiorum*. A, activity of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) detected in the plants. B, relative amount of proline and sugar contents in the assayed plants.

resistance to *S. sclerotiorum* and that the resistance was apparently correlated to the expression levels of transgenic OXO. Furthermore, OA metabolism may also allow a more rapid or powerful modification of plant defenses by counteracting the OA-mediated suppression of the oxidative burst (Williams *et al.* 2011), hence the degradation of OA produced by *S. sclerotiorum* may reduce the damage in the plant tissues thereby slowing down the advance of this necrotrophic fungus.

In addition, the enhanced activity of antioxidant enzymes including SOD and POD was detected, and the osmotic adjustments were identified in transgenic plants inoculated with *S. sclerotiorum*. These results indicated that improvement of oxidative stress tolerance was not only associated with the increased activity of antioxidant enzymes but also related to the metabolic changes in the transgenic plants. As SOD catalyzing the dismutation of two superoxide radicals into $O_2^-$ and $H_2O_2$, it was regarded as a key component of the oxidative stress defense mechanism (Kuźniak 2002). For instance, overexpression of SOD was identified under cold and freezing stresses in maize (Kingston-Smith and Foyer 2000, and alfalfa (Van Camp *et al.* 1996). Combing with transgenic OXO catalyzing the oxidation of OA to $CO_2$ and $H_2O_2$, a burst of $H_2O_2$ was generated for defense responses in pathogen infection (Dong *et al.* 2008).

Meantime, the increased amount of proline and soluble sugars indicated that metabolite activities also probably contribute to oxidative stress in the transformed *Arabidopsis* plants. The higher accumulation of osmolytes involved in the oxidative stresses was induced by ROS production (Chen and Dickman *et al.* 2005). Soluble sugars were regarded as an important osmolyte, and the involvement of sugars in the antioxidant defense against paraquat-induced oxidative stress was identified in potato (Sinkevich *et al.* 2010), and higher accumulation of soluble sugars was also found to be related with the improved tolerance of maize plants under salt stress (Feng *et al.* 2002). Proline was also considered as a nonenzymatic antioxidant and potential inhibitor of programmed cell death (Chen and Dickman *et al.* 2005; Liang *et al.* 2013), thus the increased proline content may potentially reinforce cell wall to enhance resistance of transgenic plants against OA-induced lesion and fungal infection.

## 4. Conclusion

Here we demonstrated that transgenic *Arabidopsis* expressing OXO could efficiently improve resistance to OA exposure and *S. sclerotiorum* infection, which probably involved in hydrogen peroxide related defense responses, when considering a lack of endogenous OXO producing OA pathway in *A. thaliana*, and the efficiency of transgenic *Arabidopsis* expressing OXO to enhance the *Sclerotinia* resistance was likely associated with the improved OA detoxification and elevated $H_2O_2$ production.

## 5. Materials and methods

### 5.1. Plant materials and transformation vector

The *Agrobacterium* strain LBA4404 was provided by the Oilcrops Research Institute of Chinese Academy of Agricultural Sciences (Wuhan, China), and the strain carries the plasmid pCAMBIA3304 that harbors a 1.8-kb fragment of a wheat OXO gene and a GUS report gene driven by a duplicated CaMV 35S promoter (Dong *et al.* 2008), as well as a gene encoding phosphinothricin acetyltransferase as the selectable marker to confer phosphinothricin resistance in the transformed plants (Lutz *et al.* 2001). The transgenic lines were generated by introducing the wheat (*Triticum aestivum*) OXO gene into *Arabidopsis thaliana* wild-type (Columbia ecotype Col-0) by using *Agrobacterium tumefaciens*-mediated floral-dip method (Martinez-Trujillo *et al.*

2004). Transgenic plants were selected on MS (Murashige and Skoog 1962) media containing 12 mg $L^{-1}$ phosphinothricin and transferred to soil. The seedlings were grown in a growth chamber at 22°C in a 16/8 h light/dark photoperiod cycle.

### 5.2. PCR analysis and histochemical GUS assay

Genomic DNA was extracted from young leaves of the phosphinothricin-resistant plants and their offspring by the modified hexadecyltrimethylammonium bromide method (Paterson *et al.* 1993; Lin *et al.* 2003). PCR analysis was used to maintain the presence of the transgene until homozygous transgenic lines were obtained. The PCR primers used were 5′-CATAGCAAGCATGGGGTACTCCAAAAC-3′ and 5′-CCCAAGCTTGAATTCCCGATCTAGTAACATAG-3′, which generated a 1.1-kb product specific to wheat OXO. Meantime, the identified resistant plants were used for histochemical GUS analysis. In brief, the leaves and young seedlings were collected and incubated at 37°C in the dark for 24 h in the staining solution containing a mixture of 50 mmol $L^{-1}$ sodium phosphate (pH 7.0), 0.1% Triton X-100, 0.5 mmol $L^{-1}$ $K_3/K_4$FeCN, 10 mmol $L^{-1}$ EDTA, 20% methanol (v/v) and 0.5 mg $mL^{-1}$ 5-bromo-4-chloro-3-indolyl glucuronic acid. The staining solution was then replaced with an equal volume of 70% (v/v) ethanol to remove the chlorophyll.

### 5.3. *S. sclerotiorum* infection assay

For infection assay, *S. sclerotiorum* mycelium was isolated from the diseased oilseed rape plants and cultured on Petri dishes with potato dextrose agar medium at 25°C. Agar discs containing mycelial plugs were then excised from the edges of growing colonies and placed in the center of the detached rosette leaves. The inoculated leaves were then placed in the wet containers and covered with plastic wraps in the dark at 22°C. The lesion area was recorded after fungal incubation for 48 h.

### 5.4. OA tolerance assay

The transgenic lines ($T_2$ generation) were assessed for their resistance to OA exposure using the detached leaf assay, and the wild-type *Arabidopsis* were used as controls. Briefly, 5 μL droplet of OA solution with different concentrations of 10, 40, and 60 mmol $L^{-1}$ was applied on the detached leaves, respectively (Chen *et al.* 2013). The detached leaves were then placed in wet Petri dishes with a plastic cover to maintain high humidity at 22°C in the growth chamber. The symptoms, including the size of brown lesion and yellowing area surrounding the lesion were measured in length and width after OA treatment.

### 5.5. Analysis of transgenic OXO expression

The expression of OXO in the transgenic *Arabidopsis* lines was quantified by real-time PCR. In brief, the total RNA was extracted from leaves of the transgenic *Arabidopsis* plants incubated with *S. sclerotiorum* for 24 h, using EasyPure RNA Kit (Transgen Biotech., Beijing) according to manufacturer's instruction with minor modifications, and the template cDNA was then synthesized using First-Strand cDNA Synthesis SuperMix (Transgene Biotech.). The real-time PCR was quantitatively performed to measure the expression of transgenic OXO with specific primer pairs (OXO-F: 5′-GAGATCGGCATCGTGATGAA-3′; OXO-R: 5′-CTGGCTGTTGAAG GAGACTAC-3′), with an internal reference gene Actin (ACT-F: 5′-TGGTGTCATGGT TGGGATGG-3′; ACT-R: 5′-CGTGAGAAGAACAGGGTGCT-3′).

### 5.6. Determination of $H_2O_2$ production

*In situ* hydrogen peroxide ($H_2O_2$) was detected by 3,3-diaminobenzidine (DAB) staining as previously described (Chen *et al.* 2009). $H_2O_2$ reacts with DAB to form a reddish-brown stain. Briefly, the inoculated leaves of transgenic plants were placed in 1 mg $mL^{-1}$ of DAB solution (pH 3.8), and incubated in the dark at room temperature for 12 h, and the samples were then boiled for 5 min in the solution containing a mixture of glycerin, acetic acid and ethanol (1:1:1) and then rinsed twice with 50% ethanol solution.

### 5.7. Activity assay of antioxidant enzymes

The ROS detoxification enzymes including superoxide dismutase (SOD) and peroxidase (POD) that dismutate superoxide to $H_2O_2$, and catalase (CAT), which converts $H_2O_2$ to water, were examined to analyze antioxidant activity in the transgenic *Arabidopsis* plants. The crude enzyme extracts were firstly prepared by grinding 1.0 g fresh leaves in 10 mL extraction buffer which contains 50 mmol $L^{-1}$ PBS (pH 7.0), 3 mmol $L^{-1}$ DL-Dithiothreitol (DTT), 1 mmol $L^{-1}$ EDTA-Na and 5% polyvinyl pyrrolidone (PVP), and the supernatant was then obtained by centrifugation at 15000×g, for 20 min at 4°C. The activity of enzymes including SOD, POD and CAT was determined according to the protocol as previously described (Chen *et al.* 2009). In detail, to determine SOD activity, the assay medium was used containing 50 mmol $L^{-1}$ phosphate buffer (pH 7.8), 13 mmol $L^{-1}$ methionine, 75 μmol $L^{-1}$ nitroblue tetrazolium (NBT), 2 μmol $L^{-1}$ riboflavin, 10 μmol $L^{-1}$ $Na_2EDTA$. One unit of SOD activity was defined as the amount of enzyme demanded to inhibit reduction of

NBT by 50% per mg protein. The activity of peroxidase was assayed by an increase in absorbance at 470 nm for 1 min. The assay medium consisted of 50 mmol $L^{-1}$, 2% $H_2O_2$ 1.0 mL, 50 mmol $L^{-1}$ phospahate buffer 2.9 mL. After the addition of crude enzyme extract, the mixture was incubated at 37°C for 15 min, then cooled in ice bath, and added 20% (w/v) trichloroacetic acid (TCA) 2.0 mL to terminate the reaction, then centrifigated at 5 000×g for 15 min and measured the absorbance of the supernatant at 470 nm. One unit of POD activity was defined as an increase of 0.01 in absorbance at 470 nm per min per mg protein. CAT activity was assayed by ultraviolet absorption, the reaction mixture consisted of Tris-HCl (pH 7.0) 1.0 mL, 1.7 mL distilled water, 0.1 mL crude enzyme extract, and 0.2 mL 200 mmol $L^{-1}$ $H_2O_2$. One unit of CAT activity was defined as a decrease of 0.1 in absorbance at 240 nm per min per mg protein.

### 5.8. Analysis of metabolites

Carbohydrates and free proline were extracted from leaves of transgenic *Arabidopsis* plants after incubation with *S. sclerotiorum* for 24 h, and estimated by the method as previously described (Latef and He 2011). In brief, for determination of sugar contents, 1 mL of carbohydrate extracts were boiled for 7 min after an addition of 9 mL of anthrone sulfuric acid, and the absorbance of the solution was then measured at 620 nm using a UV/VIS spectrophotometer (UV-2450, Japan). Meantime, to quantitate proline contents, 1 g of fresh leaves were homogenized and boiled in 100 mL of 3% sulfosalicylic acid for 10 min followed with filtration. 5 mL of the filtrate was added with 4.0 mL glacial acetic acid and 5 mL acid-ninhydrin, and then heated at 100°C for 1 h. Finally, the absorbance of the liquid mixture was measured after benzene purification at 520 nm using a UV/VIS spectrophotometer (UV-2450, Japan).

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