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ORIGINAL ARTICLE

Expressing a gene encoding wheat oxalate oxidase enhances resistance to *Sclerotinia sclerotiorum* in oilseed rape (*Brassica napus*)

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Abstract *Sclerotinia sclerotiorum* causes a highly destructive disease in oilseed rape (*Brassica napus*). Oxalic acid (OA) secreted by the pathogen is a key pathogenicity factor. Oxalate oxidase (OXO) can oxidize OA into CO_2 and H_2O_2. In this study, we show that transgenic oilseed rape (sixth generation lines) constitutively expressing wheat (*Triticum aestivum*) OXO displays considerably increased OXO activity and enhanced resistance to *S. sclerotiorum* (with up to 90.2 and 88.4% disease reductions compared with the untransformed parent line and a resistant control, respectively). Upon application of exogenous OA, the pH values in transgenic plants were maintained at levels slightly lower than 5.58 measured prior to OA treatment, whereas the pH values in untransformed plants decreased rapidly and were markedly lower than 5.63 measured prior to OA treatment. Following pathogen inoculation, H_2O_2 levels were higher in transgenic plants than in untransformed plants. These results indicate that the enhanced resistance of the *OXO* transgenic oilseed rape to Sclerotinia is probably mediated by OA detoxification. We believe that enhancing the OA metabolism of oilseed rape in this way will be an effective strategy for improving resistance to *S. sclerotiorum*.

Keywords Hydrogen peroxide · Oilseed rape · Oxalic acid · Oxalate oxidase · Resistance · *Sclerotinia*

Abbreviations

DAB	3,3-Diaminobenzidine
OA	Oxalic acid
OXO	Oxalate oxidase
PA	Polyaniline
PB	Percentage branches with lesions
PS	Percentage stem circumference with lesions

Introduction

Sclerotinia sclerotiorum is pathogenic to more than 400 plant species (Boland and Hall 1994) and distributes worldwide. On oilseed rape (*Brassica napus*), it causes rots of

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leaves, stems and pods, resulting in a tremendous seed yield loss in China. No immune or highly resistant cultivars of oilseed rape have been reported to date, and few genetic sources of resistance to the pathogen are available to breeders (Zhou et al. 1994; Liu et al. 2005). Control of the disease depends heavily on application of fungicides to the crop, but this is expensive and can be ineffective due to the difficulties associated with applying sprays to thick canopies (Luo and Zhou 1994) and a lack of suitable forecasting methods to enable timely application of fungicides. Therefore, transgenic modification of the crop may provide a novel strategy for control of Sclerotinia disease on oilseed rape.

Oxalic acid (OA) secreted by *S. sclerotiorum* during infection is a key pathogenicity factor of the fungus (Marciano et al. 1983; Godoy et al. 1990; Liu et al. 1998; Cessna et al. 2000). The compound acidifies plant tissue surrounding the site of infection and causes tissue damage (Dutton and Evans 1996). The optimal pH value for many fungal cell wall-degrading enzymes lies in the acidic range and thus acidification by OA may enhance activities of these enzymes (Lumsden 1979; Marciano et al. 1983). Additionally, OA is a strong chelator of divalent cations and sequestration of calcium may weaken cell walls (Bateman and Beer 1965). Phenolics have important roles in plant defence (Métraux and Raskin 1993) and there are reports that OA can inhibit the activities of *0*-diphenol oxidase (Ferrar and Walker 1993) and polyphenol oxidase (Marciano et al. 1983; Liu et al. 1998) of host plants during fungal infection.

An attractive strategy for engineering resistance to *S. sclerotiorum* is to express an exogenous protein to degrade OA in oilseed rape. OA is generally catabolised by two pathways, i.e. decarboxylation and oxidation. Decarboxylation is mediated by oxalyl-CoA decarboxylase or oxalate decarboxylase, producing oxalyl-CoA or formic acid, respectively (Kesarwani et al. 2000). Oxidation can be accomplished by oxalate oxidase (OXO, a member of the germin family of proteins) (Chiriboga 1966; Lane et al. 1993). OXO breaks down OA into CO_2 and H_2O_2, the latter being suggested to play pivotal roles in plant defence responses (Lamb and Dixon 1997; Hu et al. 2003). H_2O_2 at micromolar concentrations in plant tissues is directly toxic to microbes, contributes to structural reinforcement of plant cell walls, triggers salicylic acid (SA) synthesis, and has a central role in the signaling cascades that coordinate various defence responses (Peng and Kuc 1992; Hammond-Kosack and Parker 2003). It has been proposed that, through the production of H_2O_2, OXO may cause cross-linking of plant cell wall proteins in papillae at the site of infection (Wei et al. 1998) and functions in the plant hypersensitive response (Lane 1994; Zhou et al. 1998a). Therefore, the *OXO* transgene may endow plants with multiple resistance mechanisms through detoxification of OA and H_2O_2-mediated activation of defence response genes.

These functions of OXO have attracted considerable attention due to the potential for engineering plant disease resistance. Transgenic soybean (*Glycine max*) and sunflower (*Helianthus annuus*) expressing wheat (*Triticum aestivum*) OXO showed increased resistance to *S. sclerotiorum* in both laboratory bioassays and field tests (Scelonge et al. 2000; Donaldson et al. 2001; Hu et al. 2003). It has been demonstrated in transformed sunflower that expression of wheat OXO can result in the induction of plant defence proteins (Hu et al. 2003). Transgenic oilseed rape and peanut (*Arachis hypogaea*) expressing barley (*Hordeum vulgare*) OXO enhanced the ability to break down exogenously supplied OA (Thompson et al. 1995; Livingstone et al. 2005). However, to our knowledge there has not been a specific report on whether heterogeneously expressed OXO in oilseed rape can enhance resistance to *S. sclerotiorum*.

Unlike sunflower, which possesses very low OXO activity (Hu et al. 2003), oilseed rape has an endogenous ability to metabolize OA. Studies using [^{14}C] OA indicated that oilseed rape plants can metabolize OA into organic acids and carbohydrates, and that resistant lines can endure higher concentrations of OA than susceptible ones (Liu et al. 1998). Whether the increase in the ability of transgenic oilseed rape to degrade OA can enhance resistance of the plant to *S. sclerotiorum* has not been determined.

To address the above question, here we report that the stably inherited transgenic oilseed rape (sixth generation lines) constitutively expressing wheat OXO exhibits significantly greater OXO activity, and higher resistance to OA and *S. sclerotiorum* in comparison with the untransformed parental cultivar 84039M and a resistant cultivar Zhongyou 821.

Materials and methods

Plant and fungal materials

A *B. napus* L. cultivar 84039M with moderate resistance to *S. sclerotiorum*, was used as the recipient of the transgene and as a control in disease resistance evaluation of transgenic lines. Another *B. napus* cultivar, Zhongyou 821, with the highest *S. sclerotiorum* resistance ratings during more than 10 years of testing (China National Rapeseed Variety Regional Trials), was used as a resistance control in the disease resistance evaluation. For plants grown in a plant growth room, the growth conditions were 20 ± 2°C under a 16/8 h photoperiod at a light intensity of 44 μmol m^{-2} s^{-1} and 60–90% relative humidity. For plants sown in a glasshouse, the conditions were 14–22°C and natural light. Fresh

sclerotia of the fungus *S. sclerotiorum*, collected from oilseed rape stems in the field in Wuhan, China, were germinated to produce mycelial inoculum on potato dextrose agar.

Vector construction and *Agrobacterium*-mediated transformation

A cDNA encoding wheat OXO with its native signal peptide sequence was derived from pRPA-BD-OX16 (kindly gifted by Rhone-Poulenc Agrochimie, Lyon, France) and cloned into the multiple cloning site (*Pst* I/*Xho* I) of pTΩ4A (Wang et al. 2003), thus generating p4A-OXO. A 1.8-kb *Eco*R I/*Hind* III fragment, comprising a CaMV 35S promoter, *OXO* and a *NOS* terminator, was excised from p4A-OXO and ligated with pBI121 (Jefferson et al. 1987), which had been cut with *Eco*R I/*Hind* III. The resulting OXO-expressing vector, designated pBOXO, was transformed into *Agrobacterium tumefaciens* strain LBA4404 and used for *Agrobacterium*-mediated transformation.

Agrobacterium-mediated transformation of *B. napus* cultivar 84039M was performed according to the protocol described previously (Guo and Wang 1999). Kanamycin-resistant plantlets that rooted well in selective medium were transferred to pots and grown in a glasshouse.

PCR-based screening and Southern-blot analysis

DNA was extracted from leaves of transformed plants and their offspring. PCR-based screening was used to maintain the presence of the transgene until homozygous transgenic lines were obtained. The PCR primers used were 5′-GTCC TGCAGCATGGGGTACTCCAAAAC-3′ and 5′-CCCA AGCTTGAATTCCCGATCTAGTAACATAG-3′, which generated a 1.1-kb product specific to wheat *OXO*. The integration of the target gene was also confirmed by Southern-blot analysis. Total DNA was extracted from T_6 generation transgenic plants using E.Z.N.A® plant DNA Mini kit (Omega Bio-tek Inc., Guangzhou, China), and five micrograms of total DNA were digested with *Hin*d III. As oilseed rape *OXO* is highly similar to the wheat *OXO* transgene, we utilized a ^{32}P-labeled probe specific to the *35S* promoter to avoid non-specific hybridization. The primers used for the 35S promoter probe were 5′-GCCATCATTGCGATAAA GGA-3′ (forward) and 5′-AAGGATAGTGGGATTGTGC GT-3′ (reverse), which amplified a 485-bp product. DNA electrophoresis, digestion, blotting and hybridization were done following the procedures described by Sambrook and Russell (2001). After hybridization, membrane was scanned using a Cyclone Storage Phosphor Scanner B431200 (Perkin Elmer, Waltham, MA, USA).

OXO activity and H_2O_2 content assays of the *OXO* transgenic plants

The activity of OXO in homozygous transgenic plants was measured according to the method described previously (Zhang et al. 1995). Untransformed 84039M was used as a control. Briefly, four grams of young leaves were excised from each plant grown in the growth room and homogenized in 5 ml of distilled water at 4°C. The crude enzyme was precipitated with ammonium sulphate at 70% saturation and dissolved in 1 ml of OXO reaction solution [in a 50 ml solution, 0.286 g succinic acid, 12.6 mg OA, 30 ml absolute ethanol, 4 mg 4-aminoantipyrine, 200 U horseradish peroxidase (Sigma, St. Louis, MO, USA), and 10 µl *N, N*-dimethylaniline, pH 3.3]. The absorbance of the different samples was measured at 550 nm using a spectrophotometer (DU650, Beckman, Fullerton, CA, USA).

H_2O_2 was visually detected in leaves of line OX10 (sixth generation) using 3,3-diaminobenzidine (DAB) as a substrate. Twelve hours after inoculation with *S. sclerotiorum*, leaves were excised and incubated with DAB-HCl (1 mg/ml, pH 3.8) in the growth room for 8 h. After the leaves were cleared in boiling ethanol (96%) for 10 min, H_2O_2 in the leaves was visualized as red brown precipitate and photographed using a microscope (Leica DMRE, Wetzlar, Germany).

Detection of leaf pH values in transgenic plants

The leaf pH value of line OX10 (sixth generation) was analysed using a pH micro-sensor equipped with a modified polyaniline (PA) electrode (Zou et al. 2007). Plants were grown in the glasshouse up to the four-true-leaf stage. The fabrication and optimization procedure of the pH microsensor was similar to the method described previously (Wan et al. 1997; Zhang et al. 2002). The surfaces of leaves were lightly rubbed with a cylindrical eraser so that they would retain drops of OA solution. One hundred microlitres of OA (10 mM) was applied onto the surface of leaves of transgenic and control plants. The Ag/AgCl reference electrode was inserted into the main vein 1 cm away from the petiole, and the PA working electrode into the lamina 1 cm away from the OA drop. The experiment was repeated three times (three different plants) and for each plant, three leaves were measured.

Resistance of transgenic plants to OA

The *OXO* transgenic lines (sixth generation) were assessed for their resistance to OA using detached leaf assays. Untransformed 84039M and Zhongyou 821 were used as controls. Plants were grown in the glasshouse up to the

three-true-leaf stage. Detached leaves were excised and arranged on wet paper towels placed in boxes. In each of four replicates, eight leaves, one for each concentration of OA, were used for each plant line. Two small areas (7 mm in diameter) on each side of the main vein of each leaf were lightly rubbed with a cylindrical eraser so that they would retain drops of OA. Twenty microlitres of OA (0, 5, 10, 20, 40, 100 or 200 mM) was applied onto the rubbed area. Thereafter, the boxes were covered with transparent polyethylene bags and placed in the growth room at 20 ± 2°C with a 16/8 h photoperiod at an intensity of 44 μmol m^{-2} s^{-1}. Every 12 h, symptoms, including the sizes of brown lesions and yellowing area surrounding the lesions, were measured in length and width.

Disease resistance evaluation

Sclerotinia resistance of the transgenic lines (sixth generation) was assessed using two methods: detached leaf inoculation tests and field disease nursery tests (Liu et al. 2005). Untransformed 84039M and Zhongyou 821 were used as controls. For the detached leaf inoculation tests, plants were grown in the glasshouse up to the four-true leaf stage. Mycelia of *S. sclerotiorum* were cultured on potato dextrose agar. Agar discs were excised from the edges of growing colonies and upended onto detached leaves. Each of four replicates included eight leaves from each transgenic line. All leaves were labelled and randomly arranged on wet gauze in containers that were collectively covered with transparent polyethylene bags. The leaves in the containers were incubated at 20 ± 2°C in a dark room. Twenty hours after inoculation and at intervals thereafter, lesion sizes were measured in length and width.

The field disease nursery tests were arranged as a randomized block design with two blocks and a plot size of 1.0 × 2.2 m (16–20 plants per plot). Plots were covered with nylon net to prevent pollen drift. *S. sclerotiorum* inoculum in the disease nursery was maintained by growing oilseed rape consecutively for four seasons and placing two sclerotia in each row before sowing. Crop management followed the standard agronomic practice, but without any pesticide application. Seven days before harvest, disease severity was assessed on a 0–4 scale [0, no lesions; 1, percentage stem circumference with lesions (PS) <25 or percentage branches with lesions (PB) <30; 2, $25 \leq PS < 50$ or $30 \leq PB < 60$; 3, $50 \leq PS < 75$ or $PB \geq 60$; 4, $PS \geq 75$ or almost all branches with lesions]. Disease index was calculated by $100\Sigma(I\ n_I)/(N\ k)$, where I is a disease severity score on the 0–4 scale, n_I is number of plants with each score, N is total number of plants assessed and k is the highest score (here it is 4) (Liu et al. 2005).

Statistical analysis

Variance analysis for all the data relating to OXO activity, pH value, brown lesion size, yellowing area, Sclerotinia lesion size and disease index was done using the SAS program (SAS Institute Inc.).

Results

Generation of the stably inherited *OXO* transgenic oilseed rape lines

The binary vector pBOXO (see "Materials and methods"; Fig. 1a) was constructed and used for transformation of 84039M. A total of 13 independent *OXO* transgenic plants were obtained. PCR-based screening and, from the third generation and onwards, Sclerotinia disease resistance selection were used to maintain the presence of the *OXO* transgene and associated resistance. As a consequence, five independent *OXO* transgenic lines, including OX1, OX6, OX9, OX10 and OX14, were selected and selfed to the stably inherited generations. The transgenic nature for each of them was confirmed by PCR and Southern-blot analysis (Fig. 1b, c). In the second generation, lines OX1, OX9, OX10 and OX14 segregated in a 3:1 ratio for the transgene as determined by PCR detection. For line OX6, the ratio was 29:15. From the fourth generation, these lines were no longer segregating for the transgene and thus were considered homozygous. Southern-blot analysis indicated these transgenic lines had a single copy of the transgene.

In terms of agronomic characters (within lines and between lines), all five homozygous transgenic lines were uniform and comparable to untransformed 84039M.

Transgenic plants exhibit significantly increased OXO activity and elevated H_2O_2 levels

To determine whether the *OXO* transgene was functionally expressed in transformed plants, OXO activity in leaf extracts was measured using a spectrophotometric assay and expressed as OD_{550}. The five homozygous transgenic lines OX1, OX6, OX9, OX10 and OX14, exhibited significantly ($P < 0.05$) greater OXO activities than did untransformed 84039M (Fig. 2a). Lines OX1 and OX14 had similar OD_{550} values (0.2433 and 0.2845, respectively) and were twice that of 84039M. The OD_{550} value of OX10 was 0.4512, three times greater than that of 84039M (0.1229). This result was confirmed by histochemical detection of H_2O_2 in leaves of OX10 (sixth generation) infected with *S. sclerotiorum*. Upon staining with DAB, compared with 84039M, the leaves (especially the veins) of the transgenic plants displayed stronger red-brown colouration in the

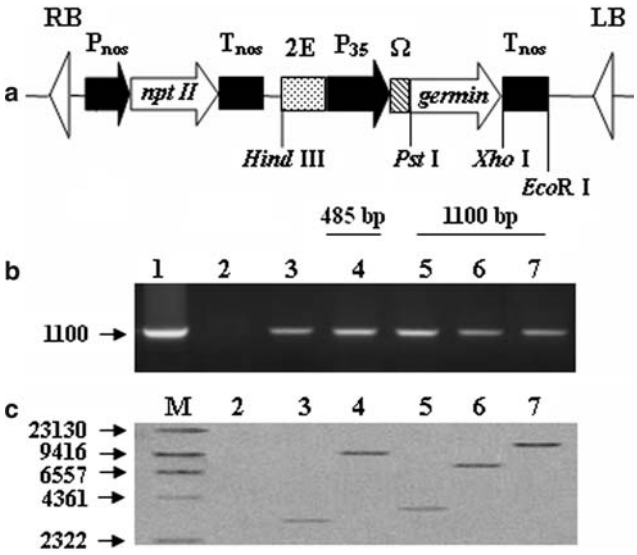

Fig. 1 Transformation of oilseed rape with a gene encoding wheat oxalate oxidase (OXO). **a** Schematic diagram of T-DNA of the binary vector pBOXO. *RB*, right border; P_{nos}, nopaline synthase promoter; *npt II*, neomycin phosphotransferase (II) coding region; T_{nos}, nopaline synthase terminator; *E*, enhancer; P_{35s}, cauliflower mosaic virus 35S promoter; *Ω*, the lead sequence of tobacco mosaic virus; *germin*, wheat *OXO* cDNA; *LB*, left border. The sites of a 485-bp probe used for Southern blot and a 1.1-kb fragment used for PCR template are indicated. **b** PCR analysis of the *OXO* transgenic plants using genomic DNA as the template. *Lane 1*, positive control (pBOXO); *lane 2*, untransformed 84039M (CK1); *lanes 3–7*, the transgenic plants tested: OX14, OX10, OX9, OX6 and OX1. **c** Southern-blot analysis of the *OXO* transgenic plants. *Lane 1*, DNA marker (unit, bp); *lane 2*, untransformed 84039M (CK1); lanes *3–7*, the transgenic plants tested: OX14, OX10, OX9, OX6 and OX1. Genomic DNA from all transgenic and CK1 plants was digested by *Hind* III

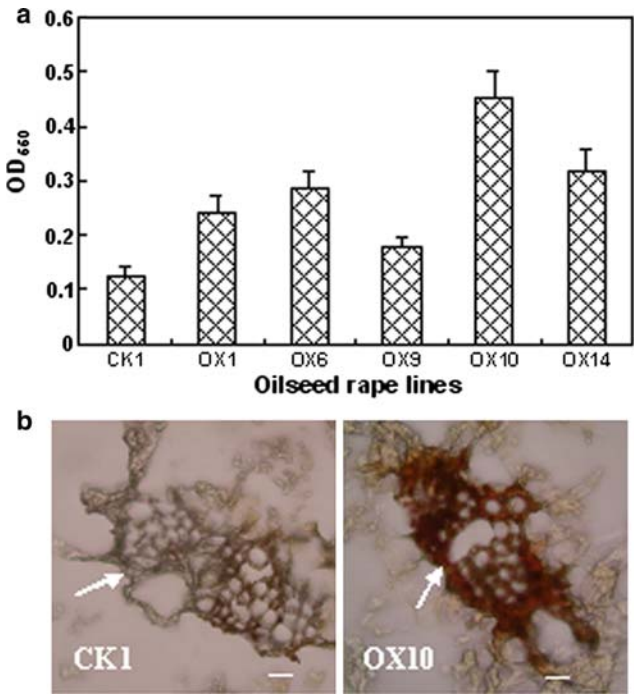

Fig. 2 Expression and functional analysis of the wheat *oxalate oxidase* (*OXO*) transgene in oilseed rape. **a** OXO activities in the transgenic plants. The activity was measured using a spectrophotometric assay and expressed as OD_{550}. CK1 is 84039M, an untransformed control; OX1, OX6, OX9, OX10 and OX14 were the transgenic plants tested. The data were obtained from three independent experiments and expressed as mean ± SD. **b** Histochemical detection of H_2O_2 levels in line OX10. Leaves were inoculated with *S. sclerotiorum* and stained with DAB, and observed from transverse sections. The *white arrow* indicates the red-brown colouration caused by H_2O_2 precipitate in the leaf veins. The *short scale bar* stands for 12.5 μm in length

transverse sections 12 h after inoculation with *S. sclerotiorum*, indicating higher levels of H_2O_2 accumulation in this transgenic line (Fig. 2b).

Transgenic OXO ameliorates acidification of leaf tissue by OA

It is important to investigate whether the *OXO* transgenic plants can degrade OA and thus prevent pH values from decreasing after exogenous OA application or Sclerotinia infection. Here, we used a modified, steady PA electrode (Zou et al. 2007) for in situ and real-time detection of leaf pH values. The experiments were carried out on leaves of the homozygous line OX10 (sixth generation) and untransformed 84039M. Following OA application to the leaf surface, the pH values in both transgenic and untransformed plants decreased immediately (Fig. 3). However, the rate of decrease was greater in the untransformed plants than in the transgenic plants. By 110 min after OA application, the pH values were significantly ($P < 0.05$) higher in the transgenic plants than in 84039M plants. By 392 min after OA application, the pH values in the transgenic plants remained at 4.9, whereas the pH values in the untransformed plants had dropped to 3.2 (Fig. 3). Therefore, the transgenic line displayed a considerable ability to protect the transgenic plants from OA-induced acidification.

The *OXO* transgenic oilseed rape shows enhanced resistance to OA

The *OXO* transgenic lines (sixth generation) were evaluated for their resistance to OA by detached leaf assays. Two types of symptoms caused by exogenous OA were observed: brown lesions and yellowing areas surrounding the brown lesions (Fig. 4). The symptom severity of all the lines tested increased with the concentrations of OA, but the non-transgenic controls were more sensitive to OA than the transgenic lines in terms of both brown lesion size and yellowing area (Figs. 4, 5). Eighty-four hours after OA treatment, brown lesion sizes of the transgenic lines and two controls showed slow increases with the concentrations of OA in the range of from 0 to 40 mM and rapid increases

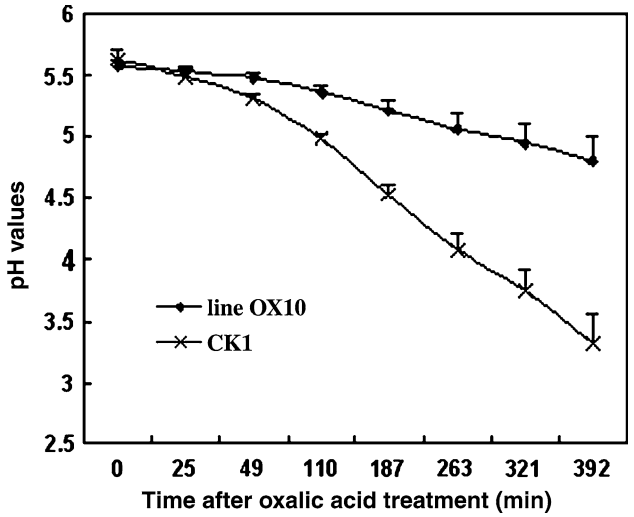

Fig. 3 In-situ and real-time detection of the pH values in the wheat *oxalate oxidase* (*OXO*) transgenic oilseed rape lines. Following application of exogenous oxalic acid (OA), the plants of line OX10 and untransformed 84039M (CK1) were analysed using a pH micro-sensor as described in "Materials and methods". The data were obtained from three independent experiments (three different plants and for each plant, three leaves were measured) and expressed as mean ± SD

at more than 40 mM OA (Fig. 5a). At the same time point, the changes in yellowing areas of the transgenic lines were very similar to those in brown lesion sizes over the concentrations of OA (Fig. 5b); however, changes in yellowing areas of two controls were different and they started to rapidly increase at very low concentrations of OA and kept the increase over the concentrations of OA (Fig. 5b). At 200 mM OA, all of the transgenic lines tested, OX1, OX6, OX9, OX10 and OX14, exhibited significantly ($P < 0.05$) smaller lesions than 84039M and Zhongyou 821 84 h after OA treatment (Fig. 5a). At the same concentration of OA, all of these transgenic lines exhibited significantly ($P < 0.05$) smaller yellowing areas than 84039M, and OX9 and OX14 were significantly ($P < 0.05$) smaller in yellowing areas than Zhongyou 821 (Fig. 5b). Eighty-four hours after OA treatment, differences in yellowing area were greater than those in brown lesion size between transgenic lines and the controls (Fig. 5a, b). The correlation between yellowing area (mean over seven concentrations of OA) and brown lesion size (mean over seven concentrations of OA) of five transgenic lines and two controls was highly significant ($r = 0.933$, $P < 0.01$).

Enhanced resistance of transgenic oilseed rape lines to *S. sclerotiorum*

The *OXO* transgenic lines (sixth generation) were evaluated for resistance to *S. sclerotiorum* by detached leaf inoculation tests and field disease nursery tests. In the detached leaf inoculation, all five transgenic lines, OX1, OX6, OX9,

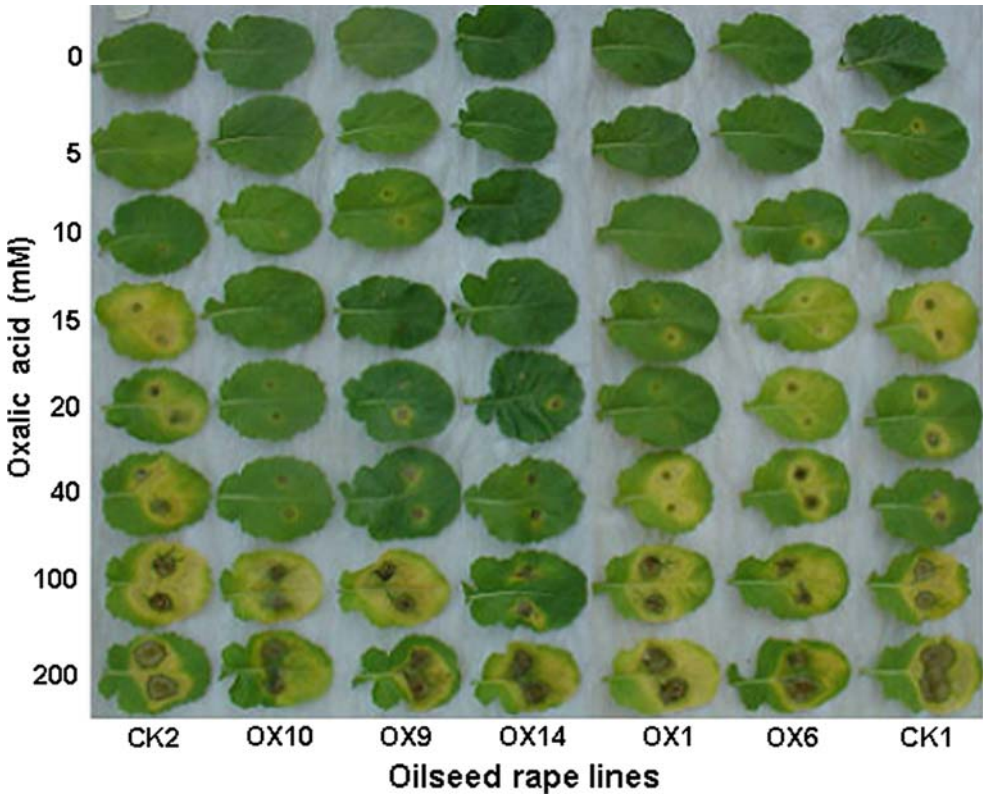

Fig. 4 Detached leaf assays for resistance to oxalic acid (OA) in the wheat *oxalate oxidase* (*OXO*) transgenic oilseed rape lines. Twenty microlitres of OA of eight different concentrations were applied to leaves of plants. Brown lesion and yellowing area surrounding the lesion were statistically analysed 84 h after OA treatment (see Fig. 5). Detached leaves tested were photographed 96 h after OA treatment and just one of four replicates is presented in the figure. CK1 (*cv.* 84039M) was used as an untransformed control and CK2 (cv. Zhongyou 821) as a resistant control; OX1, OX6, OX9, OX10 and OX14 are the transgenic lines tested

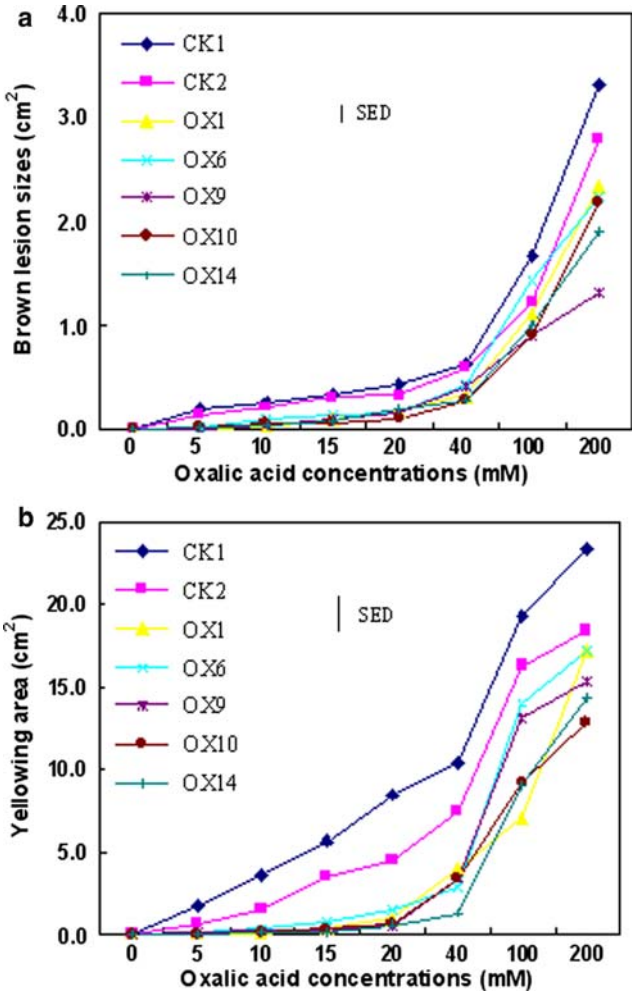

Fig. 5 Sensitivity of the wheat *oxalate oxidase* (*OXO*) transgenic lines of oilseed rape to oxalic acid (OA) in detached leaf assays. Twenty microlitres of OA of eight different concentrations were applied to leaves of plants. Brown lesion size (**a**) and yellowing area surrounding the lesion (**b**) were measured as length by width 84 h after OA treatment and expressed as means for four replicates of each OA concentration. CK1 (cv. 84039M) was used as an untransformed control and CK2 (cv. Zhongyou 821) as a resistant control; OX1, OX6, OX9, OX10 and OX14 are the transgenic lines tested. The *vertical bar* indicates standard error of difference (SED; SED is 0.14 for brown lesion sizes and 2.18 for yellowing areas) between means

OX10 and OX14, had significantly ($P < 0.05$) smaller lesion sizes than did untransformed 84039M 76 h after inoculation although no line was considered to be immune (Fig. 6; Table 1). Lesion sizes of lines OX10, OX14, OX6, OX1 and OX9 were reduced by 44.4, 42.8, 38.3, 35.0, 34.6 and 34.2%, respectively, compared with that of 84039M (Table 1). There were no significant ($P = 0.05$) differences between the transgenic lines or between each of them and Zhongyou 821 (Table 1).

In the nursery tests, disease indices of all five transgenic lines tested were significantly ($P < 0.05$) smaller than those of untransformed 84039M and Zhongyou 821 (Table 1). Disease indices of Zhongyou 821 and 84039M were 57.7 and 68.5%, respectively (Table 1). Disease indices of the transgenic lines tested, including OX14, OX1, OX10, OX6 and OX9, were no more than 20%, a reduction of 88.4, 84.5, 83.4, 80.2 and 65.3% when compared with that of Zhongyou 821, and of 90.2, 87.0, 86.0, 83.4 and 70.8% when compared with that of 84039M (Table 1). Thus all the transgenic lines tested exhibited increased resistance to *S. sclerotiorum*. These transgenic lines tested can be divided into two resistance groups: one included OX14, OX10 and OX1 with smaller indices (6.7, 8.9, and 9.6%, respectively) and another included OX6 and OX9 with greater indices (11.4 and 20.0%, respectively). There were significant ($P < 0.05$) disease resistance differences between these two groups (Table 1). The correlation between lesion size and disease index of five transgenic lines and two controls was significant ($r = 0.803$, $P < 0.05$).

Relationships between OXO activity and resistance to OA and *S. sclerotiorum*

In the *OXO* transgenic plants, OXO activity increased significantly (Fig. 2), resulting in elevation of H_2O_2 through OA degradation. OA degradation prevented pH decrease and tissue acidification. There was a significant correlation between OXO activity and resistance of the transgenic lines to OA. The correlation coefficients were -0.817 ($P < 0.05$) for OXO activity and yellowing area (mean over seven concentrations of OA and at 84 h after OA treatment) and -0.770 ($P < 0.1$) for OXO activity and brown lesion size (mean over seven concentrations of OA and at 84 h after OA treatment). Furthermore, OXO activity was highly correlated with Sclerotinia lesion size in the detached leaf inoculation tests ($r = -0.783$, $P < 0.1$) and with disease index in the nursery tests ($r = -0.677$) although the latter correlation was statistically not significant.

In further analysis, we found that increased resistance to OA was significantly ($P < 0.05$ or 0.01) and positively correlated with enhanced resistance to *S. sclerotiorum*. The correlation coefficients were 0.949 ($P < 0.01$) between yellowing area and Sclerotinia lesion size, 0.818 ($P < 0.05$) between brown lesion size and Sclerotinia lesion size, 0.914 ($P < 0.01$) between yellowing area and disease index, and 0.877 ($P < 0.01$) between brown lesion size and disease index. These values indicated a close association between OXO activity and resistance to OA and to *S. sclerotiorum*.

Discussion

Genetic transformation with a gene encoding OXO is an effective strategy for improvement of resistance to *S. sclerotiorum* in oilseed rape. Previously, transgenic oilseed

Fig. 6 Detached leaf inoculation tests for resistance to *S. sclerotiorum* in the wheat *oxalate oxidase* (*OXO*) transgenic oilseed rape lines. Detached leaves were inoculated with mycelial plug of *S. sclerotiorum*, and photographed 20 h after inoculation. Lesion sizes were statistically analysed 76 h after inoculation (see Table 1). CK1 (*cv.* 84039M) was used as an untransformed control and CK2 (*cv.* Zhongyou 821) as a resistant control. OX1, OX6, OX9, OX10 and OX14 are the transgenic lines tested. The *white scale bars* stands for 0.8 cm in length

Table 1 Detached leaf inoculation tests and field disease nursery tests for resistance to *Sclerotinia sclerotiorum* in the wheat *oxalate oxidase* (*OXO*) transgenic lines of *Brassica napus*

Detached leaf inoculation		Field disease nursery test	
Line[a]	Lesion size[b] (cm^2)	Line[a]	Disease index[b] (%)
CK1	24.3 a	CK1	68.5 a
CK2	16.0 b	CK2	57.7 b
OX1	15.8 b	OX1	8.9 de
OX6	15.0 b	OX6	11.4 c
OX9	15.9 b	OX9	20.0 c
OX10	13.5 b	OX10	9.6 de
OX14	13.9 b	OX14	6.7 e

[a] CK1 (*cv.* 84039M) was used as an untransformed control and CK2 (*cv.* Zhongyou 821) as a resistant control

[b] In the detached leaf inoculation, lesion size was measured as length by width 76 h after inoculation with *S. sclerotiorum*, and expressed as mean for four replicates (each with eight leaves). In the field nursery tests, disease index was calculated from the scores of a disease score scale recorded 7 days before harvest, and expressed as mean for two replicates (each with 25–28 plants). Different lowercase letters indicate the significant differences in the Duncan's multiple range tests at the 5% level

rape expressing barley OXO showed it has a role in OA degradation (Thompson et al. 1995); however, no laboratory or field assessments of resistance to *S. sclerotiorum* have been reported to date. In this study, laboratory bioassays and field disease nursery tests showed clearly that the stably inherited *OXO* transgenic oilseed rape lines (sixth generation) exhibit significantly increased resistance to *S. sclerotiorum*. In the field disease nursery tests, the disease indices of OX14, OX10 or OX1 were less than one seventh of that of the untransformed 84039M (Table 1), a moderately resistant cultivar which served as a recipient for the transgene, and no more than one sixth of that of Zhongyou 821, one of the most Sclerotinia-resistant cultivars of oilseed rape available (Liu et al. 2005). The improvement in resistance achieved using transgenic biotechnology was in this study greater during a period of 4 years (six generations) than that achieved by a single plant or population selection (Zhou et al. 1994) or recurrent selection (Zhou et al. 1998b) during a period of 7 or 9 years. Genetic gains of improvement for Sclerotinia disease resistance have been slow and small using traditional breeding practices during the past 40 years. Over the last two decades, more than 6,000 *Brassica* oilseed rape accessions (some from other countries) in the China Rapeseed Germplasm Pool have been evaluated for their resistance to *S. sclerotiorum* and some resistant lines have been selected (Zhou et al. 1994). However, none of these resistant lines exhibited greater resistance to *S. sclerotiorum* than OX1, OX10 or OX14. Among these resistant lines selected, ZhongRS083 had the highest resistance to *S. sclerotiorum*. In the field nursery tests, the disease index of ZhongRS083 was reduced by less than 75% when compared with that of the resistant control Zhongyou 821, whereas those of OX1, OX10 or OX14 were reduced by more than 83%.

The enhanced resistance of the *OXO* transgenic lines to *S. sclerotiorum* can be attributed to the significantly increased resistance to OA. The increased resistance to OA is likely due to the increased capability for OA degradation in the *OXO* transgenic lines. OA degradation is valuable to protect infected tissues from acidification and damage caused by excess OA. We employed a novel method to show in situ and in real-time that the *OXO* transgenic plants can immediately degrade OA and rapidly prevent pH values from decreasing upon exogenous OA application. The increased resistance to *S. sclerotiorum* is likely to result from one or more of the following effects of constitutively

expressed OXO in the transgenic lines: preventing plant tissue acidification, inhibiting the activity of fungal cell wall-hydrolytic enzymes (Lumsden 1979; Noyes and Hancock 1981; Godoy et al. 1990), reducing chelation of divalent cations by OA (which may serve to weaken cell walls) (Bateman and Beer 1965), enhancing plant defence by counteracting the OA-mediated suppression of the oxidative burst (Cessna et al. 2000), and impeding OA inhibition of other plant oxidases like *0*-diphenol oxidase (Ferrar and Walker 1993) and polyphenol oxidase (Marciano et al. 1983; Liu et al. 1998).

Oilseed rape can tolerate OA to a quite high level as it has an endogenous ability to metabolize OA (Liu et al. 1998; Liu et al. 2005). Such ability varies with variety and this variation is consistent with known varietal resistances to *S. sclerotiorum*. 84039M has a lower ability to metabolize OA than the resistant control Zhongyou 821 (Liu et al. 1998). Interestingly, after 84039M was transformed with wheat *OXO*, its ability to metabolize OA was increased, and as a consequence, its resistance to OA became significantly greater than that of Zhongyou 821 (Figs. 4, 5). These results suggest that enhancing the OA metabolism in this way will be an effective strategy for improving resistance to *S. sclerotiorum* in oilseed rape.

OXO-generated H_2O_2 may also play an important role in contributing to the enhanced resistance of the *OXO* transgenic plants to *S. sclerotiorum*. Wheat and barley germins have been proposed to possess both OXO and superoxide dismutase activities, leading to production of the defence-inducing molecule H_2O_2 (Lane et al. 1993; Kotsira and Clonis 1997; Woo et al. 2000). There is increasing evidence showing that OXO is involved in disease defence responses associated with H_2O_2 (Lane 1994; Wei et al. 1998; Zhou et al. 1998a; Dunwell et al. 2000). In our case, the *OXO* transgenic oilseed rape exhibited obviously elevated levels of H_2O_2 (Fig. 2b), which may contribute to the enhanced resistance of the transgenic lines to *S. sclerotiorum*.

In this study, we noted two features that distinguish line OX6 from the other *OXO* transgenic lines. One is that lesion development in line OX6 was slightly quicker than in the other transgenic lines from 84–96 h after OA treatment and OX6 was more sensitive to OA at high concentrations of OA (40–200 mM). Another is that the transgene segregation in OX6 detected by PCR did not have a typical 3:1 ratio although Southern-blot analysis indicated a single copy of the 35S promoter present in OX6. The *OXO* transgene was expressed in OX6 at the RNA level (data not presented) and functioned at the protein level. The reasons for the exceptions need to be studied. Whether there is an association between the two phenomena remains unknown.

In summary, we demonstrate that expressing a gene encoding wheat OXO in oilseed rape can efficiently improve resistance to *S. sclerotiorum* although oilseed rape has considerably high endogenous OA metabolism activity. The efficacy of constitutively expressed OXO in enhancing the Sclerotinia resistance may be a consequence of enhanced OA detoxification and H_2O_2 production. Future work is necessary to develop a novel oilseed rape cultivar highly resistant to *S. sclerotiorum* by hybridizing OX1, OX10 or OX14 with some commercially available cultivars having superior agronomical traits such as Zhongshuang 10, and to further investigate the role of H_2O_2 in contributing to enhanced resistance of the *OXO* transgenic plants to *S. sclerotiorum*.

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