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Transient expression of organophosphorus hydrolase to enhance the degrading activity of tomato fruit on coumaphos*

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Abstract: We constructed an expression cassette of the organophosphorus pesticide degrading (*opd*) gene under the control of the E8 promoter. Then *opd* was transformed into tomato fruit using an agroinfiltration transient expression system. β-Glucuronidase (GUS) staining, reverse transcription-polymerase chain reaction (RT-PCR), wavelength scanning, and fluorescent reaction were performed to examine the expression of the *opd* gene and the hydrolysis activity on coumaphos of organophosphorus hydrolase (OPH) in tomato fruit. The results show that the agroinfiltrated tomato fruit-expressed OPH had the maximum hydrolysis activity of about 11.59 U/mg total soluble protein. These results will allow us to focus on breeding transgenic plants that could not only enhance the degrading capability of fruit and but also hold no negative effects on pest control when spraying organophosphorus pesticides onto the seedlings in fields.

Key words: Bioremediation, E8 promoter, Organophosphorus hydrolase (OPH), Transient expression
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INTRODUCTION

Organophosphorus pesticides are used worldwide to control major pests. Their residues not only exist on the surface of agricultural products, but also can be soaked into tissues based on contact absorption. In addition, plants absorb and enrich these residues through vascular bundles from surrounding soil and water, leading to a high risk of residue exceeding limits in food and vegetables. Organophosphorus pesticide residues have been detected above residue limits in several commercial grape juices (Picó and Kozmutza, 2007), fresh apples (Nie *et al*., 2005), and tomato juice or paste (Aysal *et al*., 2004). It seems that degradation was slower in fruit juices than in water due to natural antioxidants (Picó and Kozmutza, 2007).

Because organophosphorus pesticides are potent inhibitors of acetylcholinesterase (AchE) that is also present in all vertebrates, non-target organisms will be damaged by residues of this kind of agents (Zhang *et al*., 2006). As reported, organophosphorus pesticide poisoning has become a major global health problem, with hundreds of thousands of deaths each year, although the majority of deaths occurred in developing countries (Eddleston and Phillips, 2004). To solve this problem, there is intense interest for us to breed fruit that could rapidly detoxify pesticide residues spontaneously to minimize the potential damage to people, but, meanwhile, would not affect pest control when spraying organophosphorus pesticides onto seedlings.

Organophosphorus hydrolase (OPH), encoded by the organophosphorus pesticide degrading (*opd*) gene, can hydrolyze a wide range of organophosphorus compounds including pesticides, chemical warfare agents, and herbicides (Dumas *et al*., 1989;

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Kolakowski *et al*., 1997; Di Sioudi *et al*., 1999). This enzyme has received considerable attention on its use as a bioremediation agent. The *opd* gene was first isolated from *Pseudomonas diminuta* and *Flavobacterium* spp. (Munnecke and Hsieh, 1976; Harper *et al*., 1988), and has been cloned and expressed in *Escherichia coli* (Lan *et al*., 2006; Shimazu *et al*., 2001), *Moraxella* spp. (Shimazu *et al*., 2001), *Saccharomyces cerevisiae* (Takayama *et al*., 2006) and other microbial strains.

The researches mentioned above focused on the production of stable and efficient hydrolase by genetically modified microorganisms for environmental remediation. Recently, *opd* has been also expressed in transgenic maize for establishing a new selection marker in transgenic research (Pinkerton *et al*., 2008). In the current study, we studied the feasibility of transient expression of OPH to enhance the degrading activity of tomato fruit on coumaphos.

MATERIALS AND METHODS

Promoter clone and plasmids construction

A 1.1-kb E8 promoter that is specific for fruit expression was polymerase chain reaction (PCR) amplified from tomato DNA using LA Taq DNA polymerase with the primer pairs designed based on the sequence (GeneBank accession No. X13437) described by Deikman *et al*.(1992). The 5′- and 3′-primer sequences were 5′-CCG GGT ACC AAG CTT AGG AAT TTC ACG AAA TCG-3′ and 5′-GCC GTC GAC GGA TCC TCT TTT GCA CTG TGA ATG-3′, respectively.

The amplified segment was cloned into pGEM-T (Promega, USA) for sequencing and other manipulation. The fragment from pGEM-T was then cut by *Hin*dIII/*Bam*HI and subcloned into pSH (constructed by Prof. Zhao Degang and conserved in Guizhou University) to exchange the double 35S promoter to generate plasmid pSE8. The *opd* gene (GeneBank accession No. AX384799) of p9137 kindly gifted by Prof. John A. Howard, Cal Poly State University of USA, was subcloned into the *Eco*RI site of pSH and pSE8. Then the plant expression vectors pSOP and pSE8OP were obtained and transformed to *Agrobacterium tumefaciens* LBA4404. The cauliflower mosaic virus 35S (CaMV 35S) promoter or E8 promoter drives the expression of the *opd* gene preceded by a fragment of the ubiquitin intron. The transferred DNA (T-DNA) regions of the two expression cassettes are depicted as Fig.1.

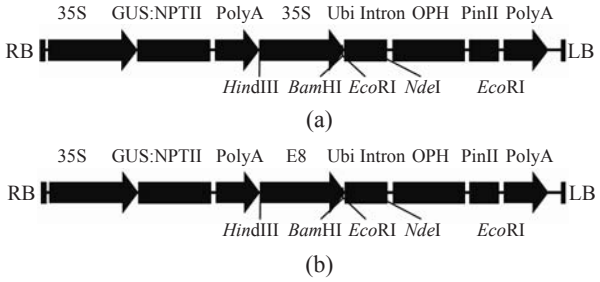


Fig.1 T-DNA regions of the two expression cassettes of pSOP (a) and pSE8OP (b)

Agrobacterium-based transient transformation

Growth and induction of *Agrobacterium* were carried out mainly according to the methods of Spolaore *et al*.(2001) and Orzaez *et al*.(2006). *Agrobacterium tumefaciens* LBA4404 harboring one of the two vectors was grown separately overnight at 28 °C in 5 ml of induction medium (5 g/L beef extract, 1 g/L yeast extract, 5 g/L peptone, 5 g/L sucrose, 2 mmol/L $MgSO_4$, and 20 μmol/L acetosyringone), and then buffered with 10 mmol/L 2-(*N*-morpholino) ethanesulphonic acid (MES) to pH 5.6, followed by the addition of rifampicin (20 mg/L) and kanamycin (100 mg/L). Then, the sample was transferred to 50-ml induction medium. When the culture reached an optical density at 600 nm (OD_{600}) of about 0.8, it was recovered by centrifugation, then resuspended in infiltration medium (10 mmol/L $MgCl_2$, 10 mmol/L MES (pH 5.6), 20 g/L sucrose, and 200 μmol/L acetosyringone) up to a final OD_{600} of 2.0, and incubated at room temperature with gentle agitation (20 r/min) for a minimum of 2 h. *Agrobacterium* cultures were collected with a syringe when required, and then fruit agroinjection was performed next.

Commercially ripe tomato fruit was rinsed thoroughly in distilled water before injection. The *Agrobacterium* suspensions harboring one of the two expression cassettes were evenly injected throughout the entire fruit using a 10-ml sterile syringe with a 4-cm needle. The needle was inserted 2~3 cm deep into the fruit tissue and injected the infiltration solutions gently into the fruit. A total of about 6 ml of the infiltration solutions was injected into commercially ripe tomatoes,

the volume injected varying according to the size of the fruit. Then the fruit was covered with preservative film and placed at 25 °C for 3 d with a 16-h photoperiod before assaying the expression of *opd*.

Evaluation of OPH activity

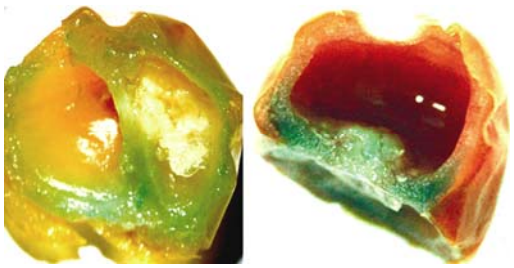
The injected fruit was preliminarily frozen at −80 °C and then taken out and ground to a meal. The meal was mixed overnight with 50 mmol/L Tris-HCl (pH 8.0) complemented with 10 mmol/L $CoCl_2$. The next day the extract was spun at 12000 r/min for 20 min and the supernatant obtained was used for enzyme analysis. Protein concentration was determined according to Bradford (1976) methods. Prior to evaluating *opd* expression, the histochemical assay was performed. The evaluation of the *opd* expression was carried out by detecting OPH activity with coumaphos (3-chloro-4-methyl-7-coumarinyl diethyl phosphorothioate) as substrate. Stock solution of coumaphos (96% purity; Fenghuang Silkworm Pharmaceutical, China) was prepared in methanol at 10 mmol/L. The hydrolysis of coumaphos was determined by wavelength scanning and fluorescence detecting in a 1-cm quartz curette and a 40-well tissue culture plate, respectively, during the incubation of 50 μl protein extract with 1 ml of 0.1 mmol/L coumaphos in 50 mmol/L Tris-HCl (pH 7.0, plus 10% (v/v) methanol) at 37 °C for 30 min. One unit of hydrolysis activity was defined as the production of 1 nmol of chlorferon (3-chloro-4-methyl-7-hydroxycoumarin) per minute under the above conditions based on a calibration curve of chlorferon (98% purity; Sigma-Aldrich, USA) measured at 348 nm (Dumas *et al*., 1989; Harcourt *et al*., 2002) by a spectrophotometer (Specord 50; Analytik Jena AG, Germany). The fluorescence was examined and photographed by a gel documentation system (Gel Doc 2000; Bio-Rad, USA) under UV light (ultraviolet rays, approx. 340 nm).

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA isolated from the injected fruits was used in RT-PCR analysis with a one-step RNA PCR kit (TaKaRa, China) and the PCR primers used were 5′-AGA TCC AGT ACG GCA TCG AG-3′ and 5′-GTC GTT GGA CAC GAG GAT CT-3′. The segment cloned was about 446 bp in length. Total RNA isolated from non-injected fruits was used as a control to check for possible genomic DNA contamination.

RESULTS

In order to test the expression of the *opd* gene under the control of the E8 promoter in fruit quickly, transient expression of foreign genes in fruits is a valuable tool for plant biotechnology. In the current study, histochemical staining results indicate that high levels of β-glucuronidase (GUS) activity driven by CaMV 35S promoter were detected in transformed fruit 3 d after agroinfiltration (Fig.2). Tissues with higher GUS expression levels were mainly around the placenta. It was found that no matter whether the tomato materials were at developmental stages beyond breaker, the histochemical staining results were consistently successful. Thereafter, GUS activity decreased.



(a) (b)

Fig.2 Transient expression of a *35S-GUS* gene in tomato fruit

Fruit was agroinfiltrated with pSOP (a) or pSE8OP (b) for 3 d, and then part of it was histochemical GUS stained

In this study, coumaphos was used as the hydrolysis substrate of OPH. Coumaphos is one kind of organophosphorus pesticide which can be hydrolyzed to the less toxic chlorferon (Pinkerton *et al*., 2008). Chlorferon can be detected by its fluorescence under UV light (excitation at 355 nm, emission at 460 nm) or absorbance at 348 nm, whereas coumaphos exhibits no significant absorbance under 348 nm (Harcourt *et al*., 2002). The absorbance peak of coumaphos at 320 nm is demonstrated by wavelength scanning from 190 nm to 1100 nm (Fig.3a). This approach has been used for high throughput screening and characterizing of soil microorganisms capable of hydrolyzing

coumaphos (Harcourt *et al*., 2002). It was also applied for establishing a selectable marker for transgenic plants (Pinkerton *et al*., 2008). When scanning the hydrolysis reaction mixture from 300 to 400 nm to detect the OPH activity, we found that the absorbance curve decreased at 320 nm and then lifted to a peak at 348 nm if the mixture was supplemented with agroinfiltrated extracts, compared with non-agroinfiltrated one (Fig.3b). This indicates that the agroinfiltrated tomatoes had significant organophosphorus hydrolysis activity.

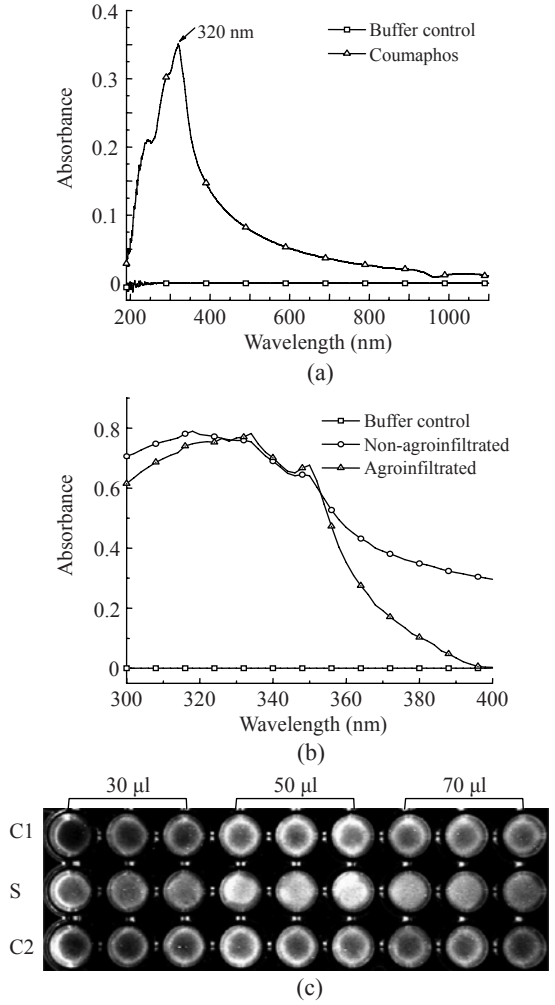


Fig.3 Detecting the coumaphos degradation by wavelength scanning and fluorescence illumination
(a) Wavelength scanning the absorbance peak of coumaphos; (b) Wavelength scanning the reaction mixture to compare the degradation capability of agroinfiltrated extracts with that of non-agroinfiltrated ones; (c) Fluorescence emission from coumaphos-containing wells supplemented with tomato extracts in three levels (30 μl, 50 μl, and 70 μl) in triplicate under UV light. C1: agroinfiltrated extract of heat inactivation; C2: non-agroinfiltrated extract; S: agroinfiltrated extract

To further test the activity of crude extracts from pSE8OP-agroinfiltrated tomato, the fluorescence of reaction mixture supplemented with three amounts (30 μl, 50 μl, and 70 μl) of protein extracts at an identical concentration, was scored under UV light in triplicate. Fig.3c showed that the reaction mixtures supplemented with the extracts from agroinfiltrated tomatoes had significant fluorescence emission. This confirms the results tested by wavelength scanning. The maximum hydrolysis activity by agroinfiltrated fruit extracts was about 11.59 U/mg total soluble proteins. Assays of RT-PCR further demonstrated the transcription of *opd* gene in tomato fruit (Fig.4).

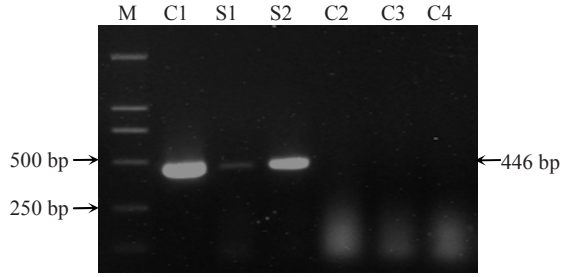


Fig.4 RT-PCR analysis of the transcription of *opd* gene in agroinfiltrated tomato
M: marker LD2000; S1, S2: different transformed samples with *opd*; C1: plasmid; C2: amplification system without RNA template; C3: amplification system without reverse-transcriptase; C4: amplification system with RNA isolated from non-transformed tomato

DISCUSSION

Tomato is easily subjected to multiple diseases and pests both in farm and post-harvest, but the pesticide control always focuses on farm in situ treatment. The E8 promoter can drive the exotic gene expression in different tissues of fruit during its growth. As reported, the E8 promoter could drive GUS expression in vascular bundles but not in intervascular parenchyma cells in unripe fruit (mature green 1 stage), in pericarp except columella at the mature green 4 stage, and at a uniformly high level throughout the fruit at the red ripe stage (Kneissl and Deikman, 1996; Deikman *et al*., 1998). In view of that tomato fruit is at harvest usually during mature green stage in order to prolong the marketable life, it would be thought that the E8 promoter would not drive the OPH expression enough to fade away the efficacy of pesticide before tomato harvest, while the pesticide degrading function of OPH would flow out maximally in storage stage.

In the present study, OPH transiently expressed in tomato fruit was detected and demonstrated by means of UV wavelength scanning and fluorescence emission. These findings can be valuable for starting the breeding of transgenic plants that could not only enhance the detoxifying capability of fruit but also hold no negative effects on pest control when spraying organophosphorus pesticides onto the seedlings in fields. To our knowledge, we have firstly brought forward this idea; however, further studies are needed to examine the stable expression of *opd* by transgenic plants.

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