Ligand-binding properties of three odorant-binding proteins of the diamondback moth *Plutella xylostella*

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Abstract

Strategies for insect population control are currently targeting chemical communication at the molecular level. The diamondback moth *Plutella xylostella* represents one of the most serious pests in agriculture, however detailed information on the proteins mediating olfaction in this species is still poor. This species is endowed with a repertoire of a large number of olfactory receptors and odorant binding proteins (OBPs). As a contribution to map the specificities of these chemical sensors in the moth and eventually unravel the complexity of chemodetection, we have measured the affinities of three selected OBPs to a series of potential odorants. Three proteins are highly divergent in their amino acid sequences and show markedly different expression profiles. In fact, PxylOBP3 is exclusively expressed in the antennae of both sexes, PxylOBP9 is male specific and present only in antennae and reproductive organs, while PxylOBP19, an unusual OBP with nine cysteines, is ubiquitously present in all the organs examined. Such expression pattern suggests that the last two proteins may be involved in non-chemosensory functions. Despite such differences, the three OBPs exhibit similar binding spectra, together with high selectivity. Among the 26 natural compounds tested, only two proved to be good ligands, retinol and coniferyl aldehyde. This second compound is particularly interesting being part of the chemical pathway leading to regeneration of lignin, one of the defense strategies of the plant against insect attack, and might find applications as a repellent for *P. xylostella* and other pests.

Keywords: odorant-binding protein, *Plutella xylostella*, ligand-binding, tryptophan quenching, molecular docking

1. Introduction

The insect’s antenna is an extremely sensitive and so-
of cysteines have been reported in insects of different orders, notably in Hymenoptera and Diptera, but in all cases a conserved structural core can be recognised to confidently assign such proteins to the family of OBPs (Zhou et al. 2004; Pelosi et al. 2006; Lagarde et al. 2011).

From a functional perspective, not all OBPs are involved in chemodetection, as suggested by the expression of several members in non-sensory organs. Some of them solubilise pheromones in the appropriate glands and assist their release in the environment (Li et al. 2008; Sirot et al. 2008; Dani et al. 2011; Lovinella et al. 2011; Sun et al. 2012; Zhou et al. 2013). Other OBPs are engaged in roles unrelated to chemical communication, such as the protein expressed in the oral disk of *Phormia regina*, suggested to solubilise important nutrients (Ishida et al. 2013) or those of the mosquito *Aedes aegypti* mediating embryonic development and anti-inflammatory response (Calvo et al. 2009; Costa-da-Silva et al. 2013; Marinotti et al. 2014).

The microlepidopteran *Plutella xylostella* is a major agricultural pest feeding on cabbage and other cruciferous plants. Its short life cycle and the voracity of the larvae account for large losses to crops. An antennal transcriptome project has identified 53 genes encoding ORs and 24 encoding OBPs (data not shown), these latter include three PBP and three GOBPs (Zhang et al. 2009; Sun et al. 2013b).

As a contribution to understanding the role of OBPs in chemical ecology and physiology of *P. xylostella*, we have investigated the expression profile and ligand-binding properties of three unusual members of this family, each belonging to a different sub-class based on the number of cysteines.

2. Results

2.1. Choice of the proteins

Based on the number of cysteines, as well as on preliminary information on tissue expression, we selected, among the predicted 24 OBPs, three genes, encoding structurally different proteins, *PxyOBP3*, *PxyOBP9* and *PxyOBP19*, for a comparative study of ligand-binding properties of the bacterially expressed proteins. *PxyOBP9* is a classic OBP with six conserved cysteines, *PxyOBP3* presents an additional cysteine besides the conserved pattern, while *PxyOBP19* contains nine cysteines, which only in part reproduce the classic motif.

A BLAST search using both protein and nucleotide collection, as well expressed sequence tags (EST) databases, only returned few Lepidopteran protein sequences with significant similarity with the three OBPs of *P. xylostella* to be considered as orthologues. Fig. 1 reports the amino acid sequences of the three *PxyOBPs* and a phylogenetic tree showing the relationships between the three *Plutella* OBPs and their orthologues. All the sequences used to build the
tree are aligned in Appendix A. In particular, for PxylOBP3 we found nine orthologues sharing 35–55% of their amino acids with the sequence of *P. xylostella*, six orthologues for PxylOBP9 (25–35% identity) and only three for PxylOBP19 with 56–65% of identical residues. In each group of aligned sequences, the patterns of cysteines were fully conserved.

The phylogenetic tree shows three well separated clusters, reflecting the poor similarity between the three OBPs of *Plutella*, which share only 7–13% of their amino acids between each pair of proteins.

Fig. 1 Amino acid sequences of the three *Plutella xylostella* odorant-binding proteins (OBPs) used in the present work. Only the mature proteins are reported and aligned. The three OBPs share only 7–13% of their residues between each pair. The few orthologues identified in Lepidoptera, using current data bases are aligned with the three *P. xylostella* OBPs in Appendix A. A phylogenetic tree indicates three distinct groups of sequences. Accession numbers for all the sequences reported are: PxylOBP3, KR706376; PxylOBP9, KR706377; PxylOBP19, KR706378; BmorOBP7, CAS90131; CmedPBP3, AGI37367; CsupOBP4, AGK24580; CsupOBP612, AGM38612; DhouOBP, AII00976; DkikOBP993, AII00993; DkikOBP998, AII00998; DpleABP5, EHJ78968; DpleOBP, EHJ67714; DpleOBP5, EHJ67764; HarmOBP1, AEB54580; HarmOBP16, AF57165; LstiPBP1, ACF48467; MsexABP5, AF393498; SexiOBP10, GH70106; SexiOBP14, AGP03460; SinfOBP10, AGS36751.

2.2. Gene expression

To get a first clue on their functions, we analysed the expression pattern of the genes encoding the three OBPs in different parts of the adult body, using semi-quantitative PCR (Fig. 2). The three OBPs, besides being divergent in sequences are also different from each other in their tissue expression. The *PxylOBP3* gene is almost exclusively and abundantly present in antennae. *PxylOBP9* is mainly expressed in male reproductive organs, while *PxylOBP19* was detected in all the tissues examined.

2.3. Bacterial expression

We therefore expressed the three OBPs in a bacterial system in order to provide the proteins for ligand-binding characterisation.

We cloned the genes into a pET-30a to produce fusion proteins bearing an His-tag at the N-terminus, followed by an enterokinase digestion site. All three proteins were expressed in high yields (around 30 mg L⁻¹ of culture) and were mostly found as insoluble inclusion bodies after sonication of the cells. After solubilisation with urea, the proteins were purified by affinity chromatography on Ni-columns. Denaturation was then completed by treatment with DTT and renaturation was accomplished by extensive dialysis against Tris buffer. Removal of His-tag was done by enzymatic digestion with enterokinase. Fig. 3 reports the...
electrophoretic analysis (SDS-PAGE) relative to relevant steps of expression and purification for the three OBPs.

2.4. Ligand-binding assays

Affinities of the three proteins towards low-molecular weight ligands were measured using a collection of 35 pure chemicals in competitive binding assays, with 1-NPN (N-phenyl-1-naphthylamine) as the fluorescent reporter. Therefore, we first verified that 1-NPN could be used as a probe and then measured good dissociation constants with PxylOBP3, PxylOBP9 and PxylOBP19 of 2.9, 4.0 and 5.5 µmol L⁻¹, respectively (Fig. 4-A).

Selected results of the binding experiments are reported in Figs. 4-B and 5, while the names of all the chemical utilised with the values of 1-NPN displacement at the maximum concentration tested are listed in Appendix B.

The first group of potential ligands included the three sex pheromone components of *P. xylostella*, (Z11)-hexadecenal, (Z11)-hexadecenol and (Z11)-hexadecenyl acetate. None of these compounds binds any of the three OBPs, excluding a potential role in sex pheromone detection or release. In particular, we have observed a concentration-dependent increase in fluorescence with the acetate. This phenomenon has been previously described (Sun et al. 2012a; Leal and Leal 2014).

Next we used a series of benzoates, which were synthesised with the aim of exploring size and structural requirements for a good fitting of a ligand into the binding site (Qiao et al. 2009). The results obtained with these chemicals were broadly similar among the three proteins, with the largest structures being also the best ligands (Fig. 5).

Then we tested a number of natural compounds occurring in plants and known to act as semiochemicals for insects. These included terpenoids, aromatic derivatives and other related structures, spanning a range of sizes from 8 to 20 carbon atoms. The actual displacement curves for the ligands which showed a significant binding activity are reported in Fig. 5, while the calculated dissociation constants are graphically compared in Fig. 4-B. All the data relative to the compounds tested are reported in Appendix B.

We can observe that:

a) Only few of the tested chemicals exhibited dissociation constants lower than 10 mmol L⁻¹, indicating high selectivity for the three proteins.

b) Rather surprisingly, the three OBPs showed the best affinities for the same set of ligands, despite their markedly different structural differences.

c) Only 2 among the 26 natural compounds qualified as ligands good enough to suggest that these OBPs might be involved in their detection, coniferyl aldehyde and retinol.

Given the presence of an aromatic ring in the best ligands or, as in the case of retinol, of an extended conjugation of double bonds, we addressed the question of how these molecules could fit into the binding pockets of the proteins, measuring the quenching caused by the ligand on the intrinsic fluorescence of the protein due to tryptophan, and comparing the results with predictions obtained in docking simulations.

We selected PxylOBP9 for such study, as it contains two tryptophan residues, one (Trp35) located in the binding site, the other (Trp122) exposed to solvent, according to a structural model of the protein. In PxylOBP3, instead, the only tryptophan is located outside the binding pocket, while PxylOBP19 contains three such residues, only one of which could interact with ligands.

Therefore, we titrated solutions of PxylOBP9 with three among the best ligands, retinol, *p*-tert-butylibenzoephene and octyl benzoate, recording the fluorescence spectra obtained after exciting the tryptophan at 295 nm. We have chosen this wavelength, rather than 280 nm, corresponding to the maximum of absorbance of tryptophan, to reduce the effect of tyrosines. The actual fluorescence spectra are reported in Fig. 6 and show strong quenching with

![Fig. 3 Bacterial expression and purification of *P. xylostella* PxylOBP3, PxylOBP9 and PxylOBP19. M, molecular weight markers; Bf, cell pellet before induction with IPTG; Af, cell pellet 2 h after induction; SN, supernatant after sonication; P, pellet after sonication; Pf, protein purified by affinity chromatography; Dg, purified protein after digestion with enterokinase.](image-url)
the first two compounds, a much weaker effect with octyl benzoate, though all three chemicals are good ligands for PxyOBP9. The reason for such behaviour could be due to different modes of interaction between the ligands and Trp35. To explore this hypothesis, we performed docking simulations. First, we built a model of the protein, using the on-line programme Swiss Model (Guex and Peitsch, 1997; Schwede et al. 2003; Arnold et al. 2006), based on the three-dimensional structure of ASP1 of Apis mellifera (Lartigue et al. 2004). Although the two proteins share only 10% of their residues, the model was rated with a score of 17, when analysed by the FUGUE programme (Shi et al. 2011), corresponding to a confidence better than 99%. Then we simulated binding of the three ligands, using the on-line programme Swiss Docking (Grosdidier et al. 2011) and visualised the output with the software Chimera (Pettersen et al. 2004). The relative images are reported in Fig. 6. We observed that p-tert-butylbenzophenone can easily establish interactions with Trp35, whatever its orientation in the binding pocket, having two benzene rings. For octyl benzoate, instead, structurally similar but endowed with a single benzene ring, the orientation is important. In fact, in the simulation reported, Trp35 is close to the aliphatic chain and can poorly interact with the benzene ring, justifying the small effect observed on the fluorescence of tryptophan, despite a good affinity for the protein. The situation is reversed with retinol because it is the chain with its long conjugated double bond system which can affect the tryptophan fluorescence. In fact, the chain is closer to Trp35, producing a marked quenching.

3. Discussion

As a first contribution to understand the function of the large repertoire of proteins involved in chemical communication in the serious agricultural pest P. xylostella, we have characterised three OBPs different in amino acid sequences and expression profiles, but similar in their binding properties. The first, PxyOBP3, is selectively expressed in antennae, strongly suggesting a role in detecting volatile chemicals. On
Fig. 5 Binding assays of recombinant PxylOBP3, PxylOBP9 and PxylOBP19 with a selection of ligands. Solutions of OBP and 1-NPN, both at 2 µmol L⁻¹ in Tris buffer were titrated with aliquots of 1 mmol L⁻¹ ligands in methanol. Decreases in fluorescence of the complexes were normalised.

the basis of the binding results we can exclude sex pheromones and common floral and plant odours, terpenoids such as geraniol, linalool, citronellal, menthol, borneol terpineol and others, but also aromatic compounds, like eugenol and carvacrol.

The second protein, PxylOBP9, is mainly expressed in male reproductive organs and, to a lower level, in male antenna. It could act as a pheromone carrier, although not a sex pheromone, both in delivering and detecting some specific semiochemicals. The occurrence of the same OBP in antennae and reproductive organs has been described in Helicoverpa species (Sun et al. 2012b) and the same CSP in locusts (Zhou et al. 2013), while honeybees secrete a number of OBPs in their pheromone glands including some also expressed in the antennae (lovinella et al. 2011). In mammals, however, this phenomenon is much better and more widely documented, where OBPs of the nasal mucosa are also produced in different pheromone secretory glands (Pelosi et al. 2014).

The third protein object of this study, PxylOBP19, seems to be ubiquitously expressed at least in all the tissues examined and, on the basis of our results it is difficult to formulate hypotheses on its function.

From the point of view of the ligands, we first observed that the three OBPs, despite their structural diversity, are surprisingly similar in their binding specificities. This fact is related to the presence in their binding pockets of a set of common amino acids, including few aromatic residues, a large number of aliphatic chains and only one or two polar groups. A detailed discussion of this kind structure requires the construction of better models, more preferably the availability of experimentally solved structures of the proteins. However, such cases of convergent evolution towards a common function have been described by Yu et al. (2009).

We also noticed that the three OBPs are highly selective in their binding, when we compared the structures of the two best ligands, retinol and coniferyl aldehyde, with structurally similar compounds, farnesol and β-ionone for retinol and eugenol for coniferyl aldehyde, all of which did not exhibit significant affinity for any of the three proteins (Fig. 5-C).

We can finally try and guess what ecological significance might be attributed to two best natural ligands. Retinol and its derivatives are important molecules in the metabolism of all animals. In insects, 3-hydroxyretinol is involved in
the visual cycle (von Lintig 2012), while retinoids promote regeneration processes (Halme et al. 2010). Being highly hydrophobic, these molecules need a carrier protein for their transport within the cell and across the body.

As for coniferyl aldehyde, this molecule takes part in the production of lignin, being first reduced to coniferyl alcohol, then converted to different derivatives to end-up in the structure of lignin (Moura et al. 2010). These processes represent a defense mechanism against pathogens attacks, and have been reported to occur also in the Chinese cabbage, a common host plant for *P. xylostella* (Zhang et al. 2007; Eynck et al. 2009). Therefore, coniferyl aldehyde and coniferyl alcohols may act as volatile chemical messages for a regeneration process following a wound in the plant.

Coniferyl alcohol has also been reported as a semiochemical. In fact, it is a component of the retinue queen pheromone of the honey bee (Keeling et al. 2003) and is also produced by orchids to attract fruit flies (Tan et al. 2006). Finally, coniferyl alcohol has been found in the rectal pheromone gland of the guava fruit fly, *Bactrocera correcta* (Tokushima et al. 2010).

Such information indicate that coniferyl derivatives can act as chemical messages, also produced by cabbage plants under stress. They might act as deterrent for *P. xylostella* when the female is selecting host plants for oviposition, as often in the event of an attack plants produce toxic compounds, but other hypotheses can be formulated and more research is needed before discussing this aspect any further.

4. Conclusion

As part of a wider research based on a transcriptome project and aimed at characterising proteins of chemical communication in the agricultural pest *P. xylostella*, we have measured the affinity and selectivity of three OBPs to a series of volatile compounds. The three proteins are markedly different from each other in their amino acid sequences, as well as in their expression patterns, but quite similar under their functional aspects. Among the natural compounds tested, only two are good ligands, with strict requirements for a correct fitting into the binding pockets.

The affinity of all three OBPs for coniferyl aldehyde and possibly for structurally related compounds, suggests that *Plutella* can detect processes linked to damage and lignin regeneration in host plants in order to avoid potentially dangerous situations. Such aspects can be further investigated and, if this is the case, lead to strategies of population control based on the use of oviposition deterrents.

Fig. 6 The quenching caused by the ligand and docking simulations of PxyLOBP9. A, quenching of intrinsic tryptophan fluorescence of PxyLOBP9 by the presence of three selected ligands. The protein (2 μmol L⁻¹ in Tris buffer) was titrated with 1 mmol L⁻¹ aliquots of ligands in methanol to final concentrations of 0, 2, 4, 8, 12 and 16 μmol L⁻¹. The top curves correspond to the spectrum of the protein. Strong quenching was observed with *p*-tert-butylbenzophenone and with retinol, but a much weaker effect was recorded with octyl benzoate, despite the fact that all three ligands exhibited good affinity for the protein. B, molecular docking showed that in the case of octyl benzoate, Trp35 does not establish good interactions with the benzene ring of the ligand.
5. Materials and methods

5.1. Insects

Adults of diamondback moth, *P. xylostella*, were collected from suburbs in Beijing in 2001 and reared on Chinese cabbage in the Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, China, at (27±1)°C, under a photoperiod of 16:8 light/dark and (65±5)% relative humidity. Antennae, heads (without chemosensory appendages), thoraxes and reproductive organs were dissected from 1- to 3-day-old adults and stored at –70°C.

5.2. Reagents

All enzymes, unless otherwise stated, were from Thermo Scientific (USA). Oligonucleotides were custom synthesised and plasmids were sequenced at Sangon Biotech (Shanghai) Co., Ltd., China. Some of the ligands for binding assays were synthesized along with standard procedures (Qiao et al. 2009). All other chemical reagents and ligands, unless stated otherwise, were from Sigma-Aldrich (USA) and with reagent grade.

5.3. RNA extraction and cDNA synthesis

Total RNA was extracted from antennae and other tissues using Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions. The first-strand cDNA was synthesized from 2 mg of total RNA using an oligo-dT primer and the Revert Aid First Strand cDNA Synthesis Kit (Fermentas, Glen Burnie, MD) following the manufacturer’s protocol. The product was either used directly for PCR amplification or stored at –70°C.

5.4. Phylogenetic analysis

The amino acid sequences of *P. xylostella* OB3, OB9 and OB19 were used as templates to perform a sequence similarity search using BLAST ver. 2.2.25 (utility BLASTp or tBLASTn, standard penalties for DNA substitutions) against protein, nucleotide and EST databases and limiting the search to Lepidoptera. Alignments of significantly similar sequences were performed with ClustalW using a Blosum62 matrix and the following values: gap open 5, gap extension 0.05, and NJ (neighbor-joining) clustering, and a phylogenetic tree was constructed based on such alignments.

5.5. Expression profiles of three OBPs

Tissue expression patterns of three OBPs were evaluated by RT-PCR with cDNA templates from antennae and other tissues. Specific primers were designed at both ends of the cDNA sequences. For testing the integrity of the cDNA templates, a pair of control primers from the coding region of the *P. xylostella* actin gene (GenBank accession no. AB282645) was used. The sequences of all primers used are listed Appendix C. After a first denaturation step at 95°C for 5 min, we performed 35 amplification cycles (30 s at 95°C, 30 s at 50°C, 1 min at 72°C) followed by a final step of 7 min at 72°C. PCR products were analyzed on 2.0% agarose gels.

5.6. Bacterial expression and purification of proteins

The coding region of mature protein sequence of each PxylOBP was amplified by PCR using specific primers at both ends, carrying restriction sites EcoRI and XhoI, digested with both enzymes and ligated into pET30a (+) vector (Novagen, Madison, WI), previously linearised with the same enzymes. After transformation in Trans-T1 Escherichia coli competent cells (Tiangen, China) and plating on LB kanamycin (25 mg mL–1) agar, individual colonies were analysed for the presence of the insert by PCR, using T7 and the specific gene reverse primer. Positive colonies were grown in 5 mL liquid LB kanamycin (30 mg mL–1) overnight at 37°C. Plasmids were extracted from the cell pellets and used to transform BL21 *E. coli* competent cells (Tiangen). The crude transformation product was grown in 5 mL LB/kanamycin overnight at 37°C to prepare a pre-culture, that was used to inoculate 1 L of LB/kanamycin. Cells were grown to OD (600 nm) of 0.6–0.8, then protein synthesis was induced by the addition of 0.4 mmol L–1 IPTG and the culture was further incubated for 2 h in the same conditions. Cells were harvested by centrifugation (15 min at 3 500 r min–1) and suspended 50 mmol L–1 Tris buffer, 0.5 mol L–1 NaCl, 1 mmol L–1 PMSF. After sonication and centrifugation (1 h at 12 000 r min–1), most of the protein was present in the pellet. This was dissolved in 8 mol L–1 urea and the solution applied to HisTrap affinity columns (GE Healthcare Biosciences, Uppsala, Sweden). Bound protein was step-wise eluted using 50 mmol L–1 Tris containing 50, 250 and 500 mmol L–1 imidazole, respectively. After electrophoretic analysis, the fractions containing the protein were pooled and treated with DTT (final concentration 1 mmol L–1) for 2 h at room temperature. Renaturation was accomplished by dialysing three times against 2 L Tris buffer overnight at 4°C. His-tag was removed by digestion with recombinant enterokinase (rEK) (Novagen, USA), following the manufacturer’s protocol and the product was purified again by HisTrap affinity columns to remove the excised fragment as well as any undigested protein.
5.7. Fluorescence measurements

Emission fluorescence spectra were recorded on a Horiba scientific Fluoromax-4 spectrofluorometer at room temperature in a right-angle configuration, with a 1-cm light path quartz cuvette and 5-nm slits for both excitation and emission. The protein was dissolved in 50 mmol L⁻¹ Tris-HCl buffer, pH 7.4, while ligands were added as 1 mmol L⁻¹ methanol solutions.

5.8. Ligand-binding experiments

The affinity of the fluorescent reporter 1-NPN to each protein was measured by titrating a 2 μmol L⁻¹ solution of the protein with aliquots of 1 mmol L⁻¹ ligand in methanol to final concentrations of 2–16 μmol L⁻¹. The probe was excited at 337 nm and emission spectra were recorded between 380 and 450 nm. The affinities of other ligands were measured in competitive binding assays, where a solution of the protein and 1-NPN, both at the concentration of 2 μmol L⁻¹, was titrated with 1 mmol L⁻¹ methanol solutions of each competitor to final concentrations of 2–16 μmol L⁻¹. Dissociation constant for 1-NPN and the stoichiometry of binding was obtained by processing the data with Prism software. Dissociation constants of the competitors were calculated from the corresponding IC₅₀ values (concentrations of ligands halving the initial fluorescence value of 1-NPN), using the following equation:

$$K_d = \frac{[1\text{-NPN}]}{1+[1\text{-NPN}]}K_{1\text{-NPN}}$$

Where, [1-NPN] is the free concentration of 1-NPN and $K_{1\text{-NPN}}$ is the dissociation constant of the complex protein/1-NPN.

5.9. Intrinsic fluorescence

The intrinsic fluorescence of tryptophan was measured for a 2 μmol L⁻¹ solution of the protein, using an excitation wavelength of 295 nm and recording the emission spectrum between 310 and 380 nm. Quenching of intrinsic fluorescence by ligands was measured in the same condition and in the presence of 0–16 μmol L⁻¹ of each ligand.

5.10. Molecular modeling and docking

Three-dimensional models were generated using the online program SWISS MODEL (Peitsch 1995; Arnold et al. 2006; Kiefer et al. 2009) and the following templates: for PxylOBP3: AaegD7 (acc. 3dx1, Calvo et al. 2009); for OBP9: AmelASP1 (acc. 1fr5, Lartigue et al. 2004); for OBP19: AgamOBP47 (acc. 3pm2, Lagarde et al. 2011). Models were verified with the programme FUGUE at http://tardis.nibio.go.jp/fugue/ (Shi et al. 2001) obtaining scores of about 17 for the first two proteins and 11 for the third. In all cases, confidence was evaluated as better than 99%. Docking was performed by the on-line programme SWISS DOCK using default parameters (Grosdidier et al. 2011). Models were visualised with the UCSF Chimera package. Chimera is developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIGMS P41-GM103311) (Pettersen et al. 2004).

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Appendix associated with this paper can be available on http://www.ChinaAgriSci.com/V2/En/appendix.htm

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