STORED RICE WITHOUT LIPOXYGENASE-3 RESISTANCE TO A STORED GRAIN PEST IS DRIVEN BY A LACK OF INDUCED SUSCEPTIBILITY, NOT AN INDUCED DEFENSE

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SUMMARY

The angoumois grain moth, Sitotroga cerealella *(Olivier), is one of the most serious pests attacking stored grain all over the world. Based on our earlier work, the relationship between lipoxygenase-3 (LOX-3) and the characteristic of resisting the angoumois grain moth were investigated in order to reduce the losses caused by the moth and to suppress its populations. LOX-3 near-isogenic lines of rice,* Oryza sativa *L., were obtained by backcrossing; they had similar genetic background while the enzyme activities of LOX-3 were different. Results from experiments of development and newly-hatched larvae orientation assay showed the* S. cerealella *development, reproductive success and newly-hatched larvae orientation not to be influenced by rice grain LOX-3 presence. Mated* S. cerealella *females responded positively to volatiles emitted from stored rice with LOX-3 in a choice test between stored rice with and without LOX-3; however, there was no significant difference when choosing between fresh rice with LOX-3 and without LOX-3. Results indicated that the loss of LOX-3 was related to the tropism of mated* S. cerealella *females and that the volatiles hexanal or pentanal emitted by stored rice grains might be attractants to* S. cerealella *females. The analysis found that a lack of induced susceptibility, rather than induced defense, explains the 'resistance' of stored rice without LOX-3 to* S. cerealella.

Introduction

Rice is the staple food for the largest number of people on Earth. It is eaten by nearly half the world's population and is the second largest produced cereal in the world (Renuka *et al*., 2016). But rice grains are often easy to be affected by insects, mouse, deterioration, mildew, etc., which lead to weight loss and poor quality (Rajendran and Sriranjini, 2008). According to the Food and Agriculture Organization, losses of rice grain reserves amount to 6-10% of the total reserves in 50 countries, and in some countries the loss can reach 30%. Among the losses, insect damage accounts for 40-50% of the total. To protect crops from insect damage, tons of insecticides have been applied for pest control, which has resulted in environmental damages, pest resurgence, pest resistance to insecticides, and lethal effects on non-target organisms (Athanassiou *et al*., 2004; Abbas *et al*., 2015). Moreover, because of cost, these pesticides are becoming increasingly inaccessible to farmers, particularly in developing countries (Tang *et al*., 2009). This fact, combined with the consumer's demand for residue-free food, prompted researchers to evaluate other alternative reduced-risk methods in order to control the most serious stored grain pests.

Lipoxygenase (LOX. linoleate oxygen oxidoreductase, E.C.1.13.11.12) catalyzes the oxidation of polyunsaturated fatty acids containing a 1, 4-pentadiene structure, such as linoleic and linolenic acids, into conjugated hydroperoxy fatty acids. LOX activity is nearly ubiquitous in the plant kingdom (Hildebrand, 1989; Siedow, 1991; Gardner, 1995; Beauchamp *et al*., 2005; Suzuki *et al*., 2015). Three isozymes, LOX-1, LOX-2 and LOX-3, were found in rice embryos on a DEAE-Sephacel Column; LOX-3 and LOX-2 were the major components, and LOX-1 is too scant to be purified by Sephadex G-150 chromatography (Ida *et al*., 1983). Many researchers have suggested that the absence of LOX-3 is inherited with a single recessive trait; its absence does not affect rice growth and development, and can reduce deterioration in part (Suzuki *et al*., 1992, 1993, 1995, 1996, 1999, 2015).

The rice seed germplasm of the Chongtui variety (CI-115), which was shown to contain no LOX-3 using monoclonal antibodies, was especially resistant to storage insects: damage by insects in rice varieties without LOX-3 was 2.5-6.5 times lower than in varieties with LOX-3 after 42 months of storage (Suzuki *et al*., 1992; Zhang *et al*., 2007). However, the effect of LOX-3 presence or absence on the resistance of stored rice grain to stored grain pest has not been clearly established, as the genetic background of rice varieties differs greatly. Thus, it is necessary to exclude genetic background effects on resistance of stored rice grain to post-harvest insect pests.

The angoumois grain moth, *Sitotroga cerealella* Olivier (Lepidoptera: Gelechiidae) has been considered a primary colonizer of stored grain in subtropical and warm temperate regions of the world (Fouad *et al*., 2013), especially on stored rice and wheat in China (Yang *et al*., 2012). Losses due to infestation by the angoumois grain moth have been increasing along with the greater yields and amount of cereal grains being stored in farmer households since the 1980s; in some areas, this insect alone can account for over 40% of the total losses in stored grain. The larvae of *S. cerealella* attack both in field or/and in storeroom a variety of kernels, including corn

KEYWORDS / Defense / Grain Moth / LOX-3 / ***Oryza sativa*** **/ Rice /** ***Sitotroga cerealella*** **/ Susceptibility /**

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LA RESISTENCIA DEL ARROZ SIN LIPOOXIGENASA-3 ALMACENADO A UNA PESTE SE DEBE A FALTA DE SUSCEPTIBILIDAD INDUCIDA Y NO A UNA DEFENSA INDUCIDA

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RESUMEN

La polilla de granos Sitotroga cerealella *(Olivier) es una de las pestes de granos almacenados más importantes en todo el mundo. Con base en nuestros trabajos previos, se investigó la relación entre la presencia de lipoxygenase-3 (LOX-3) y la característica de resistir a la mencionada especie, a fin de reducir las pérdidas causadas por la polilla y reducir sus poblaciones. Líneas casi isogénicas de arroz (*Oryza sativa *L.) con LOX-3 fueron obtenidas por retrocruzamiento; todas tenían similar origen genético pero diferentes actividades de LOX-3. Los resultados de experimentos de desarrollo y estudio de la producción de larvas recién nacidas mostraron que el desarrollo de* S. cerealella*, su éxito reproductivo y la orientación de las larvas recién nacidas no fueron influenciados por la presencia de granos de arroz LOX-3. Hembras apareadas de* S. cerealella *respondieron positivamente a sustancias volátiles emitidas por el arroz con LOX-3 almacenado en una prueba de escogencia entre arroz con o sin LOX-3 almacenado. No obstante, no hubo diferencia significativa al escoger entre arroz con o sin LOX-3. Los resultados indicaron que la pérdida de LOX-3 se relacionó al tropismo de hembras apareadas de* S. cerealella *y que hexanal o pentanal emitido por los granos almacenados pudiesen ser atractantes de las hembras de* S. cerealella*. El análisis reveló que la falta de susceptibilidad inducida, en lugar de una defensa inducida, explica la 'resistencia' del arroz sin LOX-3 almacenado a* S. cerealella.

A RESISTÊNCIA DO ARROZ SEM LIPOOXIGENASA-3 ARMAZENADO A UMA PESTE SE DEVE A FALTA DE SUSCEPTIBILIDADE INDUZIDA E NÃO A UMA DEFESA INDUZIDA

QingFeng Tang

RESUMO

A traça dos cereais Sitotroga cerealella *(Olivier) é uma das pestes de grãos armazenados mais importantes em todo o mundo. Baseado em nossos trabalhos prévios, se investigou a relação entre a presença de lipoxygenase-3 (LOX-3) e a característica de resistir à mencionada espécie, com o fim de reduzir as perdas causadas pela traça e reduzir suas populações. Linhas quase isogênicas de arroz (*Oryza sativa *L.) com LOX-3 foram obtidas por retrocruzamento; todas tinham similar origem genética, mas diferentes atividades de LOX-3. Os resultados de experimentos de desenvolvimento e estudo da produção de larvas recém-nascidas mostraram que o desenvolvimento de* S. cerealella*, seu êxito reprodutivo e a orientação das larvas recém-nascidas não foram influenciados pela presença de grãos de arroz LOX-3. Fêmeas cruzadas de* S. cerealella *responderam positivamente a sustâncias voláteis emitidas pelo arroz com LOX-3 armazenado em uma prova de escolha entre arroz com ou sem LOX-3 armazenado. No entanto, não houve diferença significativa ao escolher entre arroz com ou sem LOX-3. Os resultados indicaram que a perda de LOX-3 foi relacionada ao tropismo de fêmeas cruzadas de* S. cerealella *e que o hexano ou o pentano emitido pelos grãos armazenados pudessem ser atraentes das fêmeas de* S. cerealella*. A análise revelou que a falta de susceptibilidade induzida, em lugar de uma defesa induzida, explica a 'resistência' do arroz sem LOX-3 armazenado a* S. cerealella.

(Weston and Rattlingourd, 1999; Ahmed and Raza, 2010). An important factor that contributes to this serious loss of grains is the tendency of the larvae to feed inside the grains, which provides the pest additional protection from direct contact with insecticides (Moreira *et al*., 2009).

Considering the potential of the rice varieties without LOX-3 as a potent insect-resistance material, the present study was initiated to elucidate resistance and resistance mechanisms of near-isogenic lines of rice LOX-3 to *S. cerealella*, in order to provide new information about inheritance of resistance to stored-grain pests and a technical support for the great demand for the green grain storage.

Materials and Methods

Rice test materials

A rice (*Oryza sativa* L.) variety with normal LOX-3, HP98055, and a rice variety without LOX-3, DawDam, were taken from our breeding stock. LOX-3 near-isogenic lines were developed from them by continuous backcrossing. HP98055 was used as the donor parent and crossed with DawDam, selecting individuals without LOX-3 in each backcrossing generation. Several stable BC_4F_3 lines without LOX-3 (HP98055/DawDam) were chosen as near-isogenic lines of HP98055 (Figure 1). Samples were collected from an experimental field managed by Dayang Agricultural Experimental Farm, Anhui Agricultural University, China. In this paper, fresh and stored LOX-3 near-isogenic rice grain lines were used. The latter were naturally stored during 12 months. Before testing, all grains were freed from pests, mildew and rot, and oven dried to a moisture content of 12.6-14.1%. Moisture equilibration at 65-70% r.h. was achieved by placing Petri dishes with rice materials in humidifiers containing a saturated NaCl solution

Determination of LOX-3 enzyme activity in LOX-3 near-isogenic lines

LOX-3 was determined by the rapid method of Wu *et al*. (2001; Chinese patent ZL001 12539.7). This method utilizes LOXs' coupled oxidation to an oxidation reduction indicator to develop a color reaction to

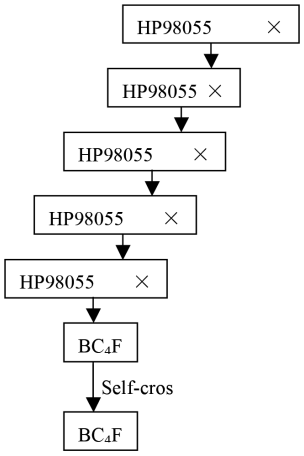

Figure 1. Procedure chart for breeding of rice LOX-3 near-isogenic lines.

determine LOX isoenzymes status. For LOX-3, the redox indicator was β-carotene. Crude enzyme was extracted according to Ohta *et al.* (1986). The LOX-3 reaction was performed in a 200mM phosphate buffer (pH 6.6) containing 10mM Na linoleate, β-carotene and acetone. Optical density (OD) at 454nm was recorded at intervals of 2 min against the buffer alone using an Uvikon 860 spectrophotometer (Kontron Instruments).

Experimental insects

The angoumois grain moth, *S. cerealella*, was raised in a constant temperature and humidity (CTH) room at 25 ±2ºC and 70 ±5% r.h., under a 12:12h photoperiod. All of the experiments were carried out in the same CTH room and wheat was used as food. *S. cerealella* adults used were generally 1-2 days post adult eclosion, except where stated otherwise. Male and female adult moths were sorted out by their size (males are smaller than females) and observing their abdominal tergites under a simple microscope: in males, the abdomen is thinner, pointed and blackish when viewed from the ventral side, whereas in females it is bulky and long, without any blackish coloration (Akter *et al.*, 2013).

Collection of eggs of S. cerealella

For collection of a fresh egg mass, rearing of *S. cerealella* was carried out sing wheat grain as a diet (only for rearing purposes) in a special mass-rearing chamber. Thousands of adults were collected from the chamber and kept in a glass cylinder covered by a 32 mesh net at the top for one day, for mating and subsequent egg laying on the glass. In the following days, the eggs laid on the walls of the cylinder were brushed and sieved to collect fresh eggs along with moth body parts. The latter were then cleaned and fresh eggs were obtained. The collected eggs of *S. cerealella* were kept in labeled glass tubes and stored at 4ºC temperature to ensure a continuous supply for future study.

Development assays of S. cerealella

Assays using the original Dobie method (Dobie, 1974) were performed to determine how the rice LOX-3 near-isogenic lines, both fresh and stored, affect the development and reproductive success of *S. cerealella*. Four 5g replicates per rice line test were weighed and placed in round plastic pots (67×32mm) with screw-top lids. One hundred *S. cerealella* eggs were laid to hatch on the test rice line placed in the plastic pots. Each container was checked regularly before the expected time of emergence of the first filial generation (F1). The date of the first emerging F1 was registered and the number of F1 individuals emerging from each pot was recorded daily during the working week, until all the F1 adults had emerged. The criterion for determining the end of the F1 generation was the absence of emergence for at least six consecutive days, excluding the first week of emergence.

Susceptibility indices were used as a measure of the susceptibility of the test rice lines to infestation by *S. cerealella*. The higher the index, the greater the susceptibility of the test plant species (Gudrups *et al.*, 2001). The index is defined as

$$DI = \frac{LnF \times 100}{DMF}$$

where DI: Dobie index of susceptibility, Ln: natural logarithm, F: total number of F_1 adults, and DME: date of median emergence of F_1 (days).

Choice experiments of adult oviposition and newly-hatched larvae orientation

Four replicates, each consisting of ten grains of the tested rice lines were homogeneously spaced in 30cm dessicators. Eight newly emerged mated female adults were placed in the center of every dessicator to lay their eggs. On day 4 thereafter the laid eggs were counted. Fifteen of these eggs were then placed to hatch in the center of the dessicator and the newly hatched larvae counted.

Behavioural assays

A two-way olfactometer (Figure 2) was used, similar to that used by Havill and Raffa (2000) for behavioral tests. For each test, an adult mated female was introduced into the olfactometer arena by aspiration through a small slit in the screen covering the top. The moths were allowed to walk freely in the olfactometer arena and make short flight attempts.

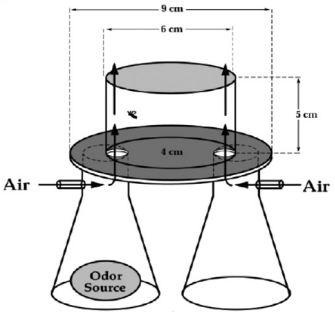

Figure 2. Schematic of two-choice olfactometer for evaluating effects of odors on behavior of insects.

A choice was scored when an adult moved upwind and entered the neck of either odor source flask, at which time it was removed quickly, before encountering the odor source itself. If no choice was made after 5min, the moth was removed and considered as a 'no response'. Each adult was used only once. In order to remove any spatial bias, the positions of the odor source flasks were exchanged after each individual was tested. Evaluations were performed in a CTH room at 25 ±2ºC and 70 ±5% r.h.

Volatiles from LOX-3 near-isogenic rice lines were used to compare the responses of *S. cerealella* adults. About 200g of the rice grain with normal LOX-3 to be tested were weighed and placed in one of the flasks, while in the other one there was rice without LOX-3. Suzuki *et al.* (1999) reported that the peroxidation products of unsaturated fatty acids were lower in the DawDam bran fraction during storage than in cultivars with LOX-3 in their seeds; the amounts of hexanal, pentanal, and pentanol in normal raw rice markedly increased during storage at 35ºC, whereas in rice that did not contain LOX-3, the levels did not increase to the same extent (Figure 3). For the behavioral tests in which

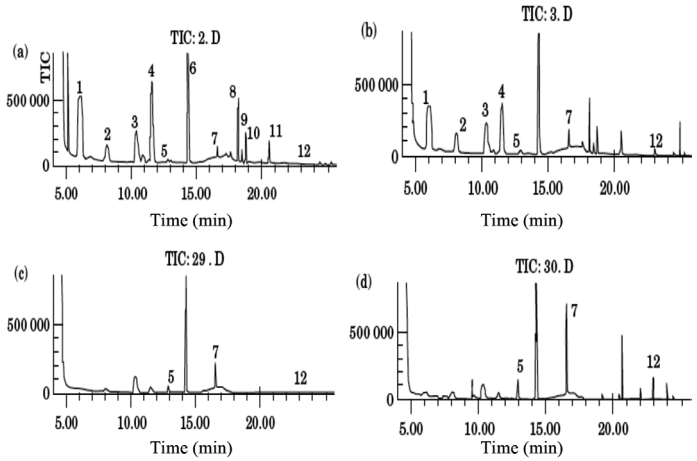

Figure 3. Typical gas chromatographic profiles of headspace volatiles in raw brown rice seeds with and without LOX-3 before and after 8 weeks of storage. Profiles are shown for fresh brown rice (variety lacking LOX-3) before (a) and after storage at 35ºC (c); normal brown rice before (b) and after storage at 35ºC (d). Peaks are identified as 1: acetaldehyde, 2: acetone, 3: methanol, 4: ethanol, 5: pentanal, 6: chloroform, 7: hexanal, 8-11: unknown, 12: pentanol.

TABLE I
REPRODUCTIVE SUCCESS OF *S. cerealella* REARED ON RICE LOX-3 NEAR-ISOGENIC LINES (n=4)

Near-isogenic lines	Adult emergence rate (%)	Development time (days)	Dobie Index	Grain loss rate (%)
LOX-3 lacking (fresh)	58.25 a	38.46 a	10.57 a	12.94 a
LOX-3 present (fresh)	60.00 a	39.11 a	10.47 a	12.47 a
LOX-3 lacking (stored)	60.25 a	38.98 a	10.51 a	12.85 a
LOX-3 present (stored)	57.75 a	37.94 a	10.69 a	13.11 a

Numbers within columns followed by different letters are significantly different at P<0.05.

these volatile substances were used, hexanal, pentanal, and pentanol (>99% pure; Sigma Chemical Company, St. Louis, MO, USA) were dissolved into liquid paraffin to obtain 1, 10 and 50µl·l^{-1} solutions that were kept at -20ºC until used. For the tests, 1ml of one of the solutions was applied to a filter paper placed in one of the flasks; the controls consisted of 1ml liquid paraffin applied on the filter paper.

Statistical analysis

Data from the development assays were analyzed using SAS PROC MIXED (SAS Institute, 2001). Before data analysis, counts data were transformed using log(x+1), in order to satisfy the assumptions of normality and homogeneity of variance (Zar 1999). Tukey's Studentized range test was used to separate means (Tukey 1953). Data on progeny production and development time on different rice lines were analyzed as a one-factor experiment, while data for number of moths emerged was analyzed as a two-factor experiment in which the main factors were rice variety and number of moths emerged.

The olfactometer choice assays were analyzed using a χ^2 test (df=1; P=0.05) to determine differences between observed and expected values for each combination of odor sources.

Results

Development assays of S. cerealella

The results of reproductive success of *S. cerealella* reared on fresh and naturally stored for 12 months rice LOX-3 near-isogenic lines are given in Table I. There were no significant differences between the means of emerged *S. cerealella* F_1 adults reared on the different test rice lines, and a similar development time of *S. cerealella* was observed on the different rice lines tested. The Dobie indexes and grain loss rate of the test rice lines were similar, as were the grain loss rates. The test of fresh or stored rice lines (with LOX-3 lacking or present) did not significantly affect the development and reproductive success of *S. cerealella*.

Choice of adult oviposition and newly-hatched larvae orientation

The effect of fresh and naturally stored for 12 months rice LOX-3 near-isogenic lines on adult oviposition and newly-hatched larvae orientation are given in Table II. There were no significant differences between the mean of newly-hatched larvae orientation on different test rice lines. When mated female adults could choose to lay eggs between the fresh rice with LOX-3 and without LOX-3, there was no preference. However, the results of adult oviposition preference experiments showed that when mated female adults could choose between fresh and stored rice, the stored rice was preferred. Furthermore, the stored rice with LOX-3 was preferred for *S. cerealella* laying eggs when the stored rice without LOX-3 was the alternative choice.

Behavioural assays of S. cerealella

The percentage of mated *S. cerealella* females responding was generally high (70-95%) in the trials in which the flasks of the two-choice olfactometer were filled with rice grain. The results of preference experiments showed that, when *S. cerealella* chose between the odor of fresh rice with LOX-3 and without LOX-3, there was no preference for either source (Figure 4). However, in the case of stored rice, the moths preferred the odor emerging from the flask with stored rice with LOX-3 as the alternative choice (Figure 5).

In the two-choice olfactometer, the results of volatile products preference experiments (Figure 6) showed that hexanal

TABLE II
EFFECT OF RICE LOX-3 NEAR-ISOGENIC LINES ON ADULT OVIPOSITION AND NEWLY-HATCHED LARVAE ORIENTATION (n=4)

Near-isogenic lines	Mean oviposition (N)	Mean of newly-hatched larvae
LOX-3 lacking (fresh)	62.50 c	6.50 a
LOX-3 present (fresh)	65.25 c	5.75 a
LOX-3 lacking (stored)	124.00 b	6.25 a
LOX-3 present (stored)	210.75 a	6.50 a

Numbers within columns followed by different letters are significantly different at P<0.05.

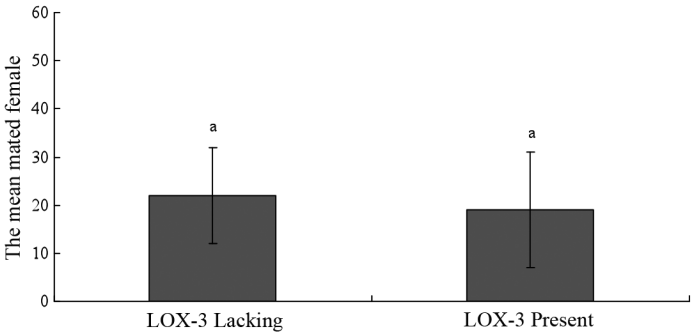

Figure 4. Preference of mated *S. cerealella* females in the two-choice olfactometer to the odor of fresh rice LOX-3 near-isogenic lines. The error bars represent standard error of the mean (SE). Bars with different letters are significantly different from each other P<0.05.

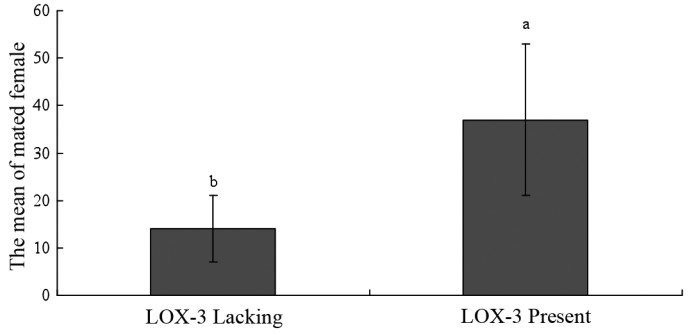

Figure 5. Preference of mated *S. cerealella* females in the two-choice olfactometer to odor of rice LOX-3 near-isogenic lines stored for 12 months. The error bars represent standard error of the mean (SE). Bars with different letters are significantly different from each other P<0.05.

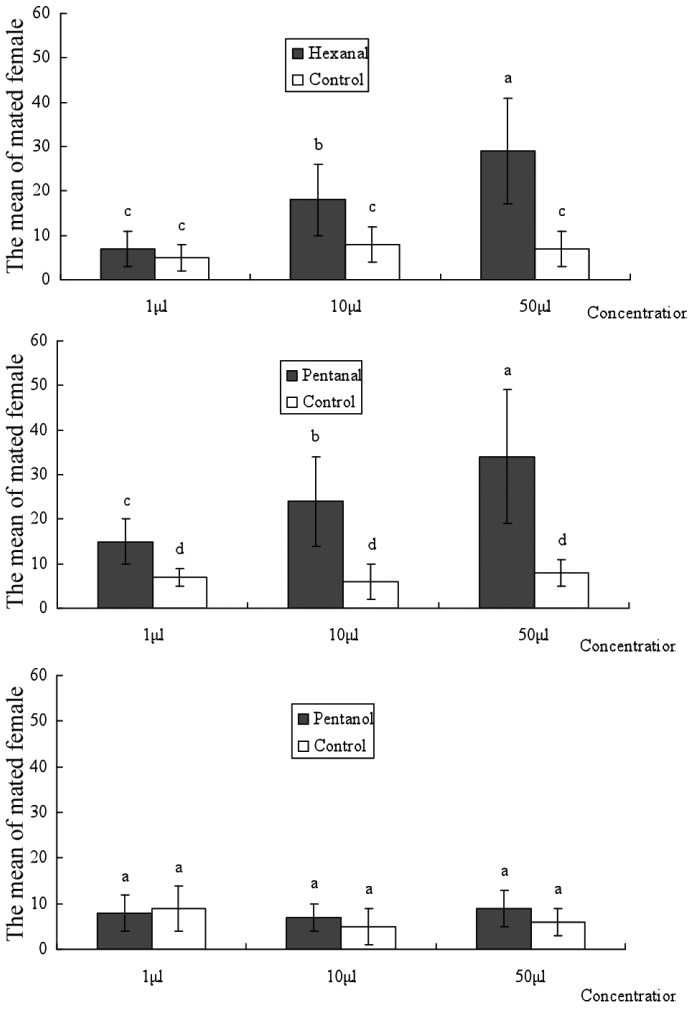

Figure 6. Preference of mated *S. cerealella* females in dual choice tests with volatile compounds produced by stored rice dissolved in liquid paraffin. The error bars represent standard error of the mean (SE). Bars with different letters are significantly different from each other P<0.05.

and pentanal are specific chemical cues that would affect the tropism of *S. cerealella* mated females, which are attracted in a dose-dependent manner. On the other hand, the moths showed no statistically significant difference in their choice between the two cylinders of the olfactometer when they contained pentanol and liquid paraffin.

Discussion and Conclusions

Plant defense responses against herbivores, pathogens and mechanical wounding involve global changes in gene expression mediated by multiple signaling pathways. Plant LOX-derived oxylipins regulate defense against biotic and abiotic stress (Ryan, 1990). The genes belonging to the LOX pathway in plants are activated by signaling molecules and then trigger resistance when the plant is subjected to biotic and abiotic stresses. The defense pathways are mainly mediated by small molecules such as salicylic acid, jasmonic acid and ethylene. Generally, the jasmonic acid pathway is considered to be required for defense against necrotrophic pathogens and chewing insects, while the salicylic acid pathway is involved in a wide range of plant defense responses (Kunkel and Brooks, 2002; Peng and Huang, 2005; Qiu *et al*., 2007).

From the results, however, it can be can inferred that there is something that exists in the rice grain, produced by LOX-3 during storage, that is important to the tropism of moths. From the findings by Suzuki *et al.* (1999), we can also infer that LOX-3 is involved in the production of volatile constituents in stored rice and that the development of stale flavor is delayed in LOX-3-less rice. By summing up the above results, it can be concluded that rice grain volatiles produced by LOX-3 during storage are attractant to storage insects.

LOXs plays protective roles against biotic and abiotic stress during plant growth and development (Ryan, 1990). However, the absence of LOX-3 can alleviate the deterioration of seeds (Lima *et al*., 2010; Gayen *et al*., 2015) and insect damage during the seed stage. It is important to elucidate the mode of action of host plant secondary metabolites with insect attractant or repellent properties, because this may provide useful information on the most appropriate formulation and means of delivery for insect control. The choice of the oviposition site by female insects is a principal factor that determines species proliferation, population densities and dispersion in different geographical areas (Waliwitiya *et al*., 2009). Considering the biological properties of the grain moth causing such losses, it is an important measure to control grain moth by manipulating the behavior of adult oviposition. Based on the experimental results of Suzuki *et al.* (1999), we evaluated the behavioral influence and attractant ovipositional activities of hexanal, pentanal and pentanol toward mated *S. cerealella* females. The results showed that hexanal and pentanal possess high activities of behavioral deterrence and oviposition attractant toward *S. cerealella* in olfactometer bioassay tests.

Under the condition that some specific chemical cues produced by rice LOX-3 metabolism are evolutionally used in the communications between insect pests and their hosts, the strategy of screening rice varieties with the absence of LOX-3 not only can alleviate seed deterioration, but can also reduce insect damage, a very important consideration for farmer's open storage. This question is being further investigated by gas chromatography combined with electroantennogram detection and bioassays to determine the attractiveness of components to the stored grain pest. The effects of the absence of LOXs should be studied further in the future. Our ultimate goal is to be able to develop an environmentally friendly method to control the pest resurgence on a crop and reduce the currently heavy dependence on pesticides.

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