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Planarian D-amino acid oxidase is involved in ovarian development during sexual induction

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ABSTRACT

To elucidate the molecular mechanisms underlying switching from asexual to sexual reproduction, namely sexual induction, we developed an assay system for sexual induction in the hermaphroditic planarian species *Dugesia ryukyuensis*. Ovarian development is the initial and essential step in sexual induction, and it is followed by the formation of other reproductive organs, including the testes. Here, we report a function of a planarian D-amino acid oxidase, Dr-DAO, in the control of ovarian development in planarians. Asexual worms showed significantly more widespread expression of *Dr-DAO* in the parenchymal space than did sexual worms. Inhibition of *Dr-DAO* by RNAi caused the formation of immature ovaries. In addition, we found that feeding asexual worms 5 specific D-amino acids could induce the formation of immature ovaries that are similar to those observed in *Dr-DAO* knockdown worms, suggesting that Dr-DAO inhibits the formation of immature ovaries by degrading these D-amino acids. Following sexual induction, *Dr-DAO* expression was observed in the ovaries. The knockdown of *Dr-DAO* during sexual induction delayed the maturation of the other reproductive organs, as well as ovary. These findings suggest that Dr-DAO acts to promote ovarian maturation and that complete sexual induction depends on the production of mature ovaries. We propose that Dr-DAO produced in somatic cells prevents the onset of sexual induction in the asexual state, and then after sexual induction, the female germ cells specifically produce Dr-DAO to induce full maturation. Therefore, Dr-DAO produced in somatic and female germline cells may play different roles in sexual induction.

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1. Introduction

Asexual freshwater planarians reproduce by dividing their body into 2 parts and regenerating the lost parts. However, depending on the environmental conditions, some of these planarians can develop hermaphroditic reproductive organs to undergo sexual reproduction (Curtis, 1902; Kenk, 1937; Hyman, 1939; Vowinckel, 1970; Vowinckel and Marsden,

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1971a,b). Methods to induce sexual worms from asexual worms have contributed to our understanding of the mechanisms involved in this switch from asexual to sexual reproduction (Kenk, 1941; Okugawa, 1957; Grasso and Benazzi, 1973; Sakurai, 1981). We have found that an asexual population of *Dugesia ryukyuensis* (OH strain) develops reproductive organs when fed minced bodies of sexually mature *Bdellocephala brunnea* worms (Kobayashi et al., 1999). This result clearly indicates that sexually mature worms contain certain substance(s) that stimulate sexual induction in the OH worms. In this sexual induction, a pair of ovaries, a copulatory apparatus, testes, a genital pore, and yolk glands developed in this order within 5 weeks (Fig. S1) (Kobayashi and Hoshi, 2002, 2011).

D-Amino acids play various physiologically important roles in animals (Khoronenkova and Tishkov, 2008). For example, D-aspartate modulates hormone secretion in the neuroendocrine systems of vertebrates (Ota et al., 2012). In animals, D-amino acids are primarily degraded by D-amino acid oxidase (DAO) and D-aspartate oxidase (DDO) (Pollegioni et al., 2007; Schell, 2004; Yamamoto et al., 2010). In the case of *Caenorhabditis elegans*, inhibition of these enzymes profoundly affects egg-laying and the development of germ cells (Saitoh et al., 2012).

In the present study, we examined DAO/DDO activity in homogenates of OH worms to find only the DAO activity. We then cloned a *D. ryukyuensis DAO* homolog (*Dr-DAO*). The gene was expressed as a fusion protein in *Escherichia coli* to confirm that the encoded protein degrades D-amino acids. Using *in situ* hybridization, RNA interference (RNAi), and a feeding system to stimulate sexual induction, we analyzed the role of Dr-DAO in the sexual induction of *D. ryukyuensis*, and showed that Dr-DAO is involved in ovarian development.

2. Results

2.1. DAO activity in the asexual and sexual worms of D. ryukyuensis

We examined the D-amino-acid-degrading activity in homogenates of whole asexual and sexual *D. ryukyuensis* OH strain worms. D-Amino acid oxidases (DAOs) oxidize various D-amino acids into their corresponding 2-oxo acids (Tanaka et al., 2007). When we incubated D-alanine with the homogenates, pyruvate was formed in a time-dependent manner. Pyruvate production was strongly inhibited in the presence of 100 mM benzoate, indicating that the production of pyruvate from D-alanine is dependent on DAO. As shown in Fig. 1A, homogenates of asexual worm bodies (0.66 ± 0.08 U/g protein) contained about 2-fold higher DAO activity than homogenates of sexual worm bodies (0.30 ± 0.03 U/g protein).

2.2. Cloning of the D. ryukyuensis DAO gene

To characterize the DAO found in *D. ryukyuensis* worms, we cloned its cDNA and expressed *D. ryukyuensis* DAO (Dr-DAO) in *E. coli*. Fig. 1C shows the nucleotide sequence of the cloned cDNA and the deduced amino acid sequence. The cDNA has a 20-bp 5′ untranslated region and a 94-bp 3′ untranslated region. A polyadenylation signal (AATAAA) is located 14 bp upstream from the beginning of the poly A tail. The cDNA is predicted to encode a protein of 332 amino acids. The GAGING sequence in the N-terminal region of Dr-DAO (indicated by the box in Fig. 1C) is predicted to be a GXGXXG dinucleotide binding motif, suggesting that these residues are responsible for FAD binding (Pollegioni et al., 2007). The C-terminal AKL sequence (indicated by the solid underline in Fig. 1C) is a predicted type 1 peroxisomal targeting signal, indicating that Dr-DAO is a peroxisomal enzyme (Pollegioni et al., 2007). The amino-acid identity shared between Dr-DAO and other animal DAOs from *C. elegans* and pig was 35%. Dr-DAO was overexpressed in *E. coli* BL21 (DE3) cells and purified by Ni-affinity chromatography to homogeneity (Fig. S2A). The recombinant Dr-DAO showed strong D-amino acid-degrading activity against various D-amino acids (Fig. S2B). We compared *Dr-DAO* mRNA expression levels in asexual and sexual worms using qRT-PCR (Fig. 1B). The asexual worms had significantly higher *Dr-DAO* mRNA expression than the sexual worms, consistent with the results found for DAO activity (Fig. 1A). When the expression of *Dr-DAO* in the worms was knocked down by RNAi (Fig. S3A), DAO activity could not be detected (Fig. S3B).

2.3. Expression pattern of Dr-DAO in asexual and sexual worms

To examine where *Dr-DAO* is expressed in the worms, we performed *in situ* hybridization on whole-mount asexual worms. We observed strong *Dr-DAO* expression throughout the mesenchymal tissues (Fig. 2A). As shown in transverse sections of the head (Fig. 2I) and trunk (Fig. 2L) regions, *Dr-DAO* transcripts were widely distributed in the parenchyma.

Pluripotent stem cells (neoblasts) exist in the parenchyma of the worm and can be selectively destroyed by X-ray irradiation (Wolff and Dubois, 1948). After 2 days of irradiation, expression of *Drpcna*, a neoblast marker gene (Nakagawa et al., 2012), was barely detectable by qRT-PCR (Fig. S4A), whereas the expression levels of *Dr-DAO* transcript did not change (Fig. S4B). These results suggest that the parenchymal *Dr-DAO*-expressing cells are not neoblasts.

In situ hybridization of whole-mount sexual worms showed that *Dr-DAO* transcripts were strongly expressed in the head and tail tip regions (Fig. 2D). The expression pattern of *Dr-DAO* in the sagittal sections of sexual worms was similar to that of asexual worms in the head region (Fig. 2I, O); however, it was significantly different in the trunk region (Fig. 2L, R).

We performed whole-mount *in situ* hybridization for *Dr-DAO* to analyze its expression pattern at different stages of sexual induction. High expression levels of *Dr-DAO* were observed throughout the parenchyma during sexual induction (Fig. 2B, C). In addition, we detected transient *Dr-DAO* expression in the developing ovaries at stage 3/4 (Fig. 2B, C, F and G). This coincides with the frequent differentiation of oogonia into oocytes and their movement from the periphery to the center of the developing ovaries. By performing *in situ* hybridization experiments on sections, we found that *Dr-DAO* was expressed in oogonia within the periphery of the ovaries at stage 4 (Fig. 2U).

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