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Mutagenesis of *Ptdss1* Gene, Using CRISPR/Cas9 System in *Drosophila*

초파리 CRISPR/Cas9 시스템을 이용한

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Abstract

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Due to the increase in average life expectancy, there is growing interest in research on aging-related diseases, especially neurodegenerative diseases. Phospholipids are a major component of the cell membrane and are also highly concentrated in nerve cells. Phospholipid composition is altered in the brain in neurodegenerative disease. Previous studies have shown that *phosphatidylserine synthase1 (ptdss1)* mutant *Drosophila* has characteristics of neurodegenerative disease. However, existing strains of *ptdss1* mutants are knockdown mutations created by P-element insertion at regulatory sites, which limits their use in researching the specific role of *ptdss1* in neurodegeneration. To determine the more precise function of *ptdss1*, knockouts with mutations in the coding sequence are

necessary. In this study, knockout mutations were constructed using the CRISPR/Cas9 system. For *ptdss1* mutagenesis, target site gRNA expression mutations were produced and crossed with a germline-specific Cas9 expression line. In germ cells of the flies carrying both the Cas9 transgene and the gRNA transgene, a Cas9-gRNA complex was formed, making an indel mutation. These flies were crossed with wild type flies to construct heterozygous mutants. Mutant flies were characterized by T7 Endonuclease I assay and DNA sequencing. Three types of knockout mutations were constructed, reducing RNA expression levels to about 70% of wild type flies. They are expected to using model animal to configure out the function of *ptdss1*.

Keywords : *Drosophila, ptdss1,* CRISPR/Cas9, mutagenesis, phosphatidylserine

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CONTENTS

LIST OF FIGURES

LIST OF TABLES

I. Introduction

1. Phospholipids in neuron

Phospholipids, composed of a hydrophilic phosphate group and hydrophobic fatty acid groups, are major components of biological membranes. Because of the amphiphilic characteristic of phospholipids, the plasma membrane forms a lipid bilayer structure. The phosphate group of the phospholipid can be modified with simple molecules and these modifications determine the characteristics of the phospholipid. There are several types of phospholipid, including phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS) that are characterized by their different head groups. Phospholipids interact with proteins to regulate cell signaling and metabolism

Different cell types and different cellular organelles have different phospholipid composition (Jones, Harwood, Bowen, & Griffiths, 1992; Vance & Steenbergen, 2005). In nervous tissue, there is a high phospholipid concentration and phospholipid metabolism is active (Sastry, 1985). Phospholipids compose the backbone of the neural membrane and provide it with fluidity and permeability (Farooqui & Horrocks, 1991).

Alteration of phospholipid composition and loss of phospholipid function can occur in neurological disorders. In the Alzheimer's disease (AD) brain, the phospholipid composition is altered and this induces abnormal signal transduction and neurodegeneration (Ginsberg, Atack, Rapoport, & Gershfeld, 1993 ; Kosicek &

Hecimovic, 2013; Wells, Farooqui, Liss, & Horrocks, 1995). Altered lipid composition is also observed in Parkinson's disease (PD) (Fabelo et al., 2011). PD occurs due to abnormal homeostasis of α-synuclein, inhibiting the synthesis of PE from PS in the mitochondria (Buccoliero, Bodennec, Echten-Deckert, Sandhoff, & Futerman, 2004; Wang et al., 2014). Furthermore, in *Drosophila* model systems, phospholipid-related gene mutations have neurodegenerative effects. For example, mutation of *eas*, which effects the phospholipid synthesis pathway, results in seizure, neuronal failure, and paralysis (Pavlidis, Ramaswami, & Tanouye, 1994).

Other phospholipids are also receiving attention regarding their metabolism and function in the brain. PS is mainly distributed in nerve tissue and is the major acidic phospholipid in the brain. PS is localized to the inner leaflet, facing the cytoplasm through the action of flippase (Svennerholm, 1968). Because of this special location, PS interacts with many proteins. PS acts as an apoptosis signal when it moves to the outer leaflet, but it forms a docking site for several signaling proteins when located at the cytoplasmic leaflet. Akt, protein kinase C, and Raf-1 signaling are all activated via PS and are important for neuronal survival, differentiation, and proliferation (Figure 1) (H. Y. Kim, Huang, & Spector, 2014; Mozzi, Buratta, & Goracci, 2003; Tung et al., 2013).

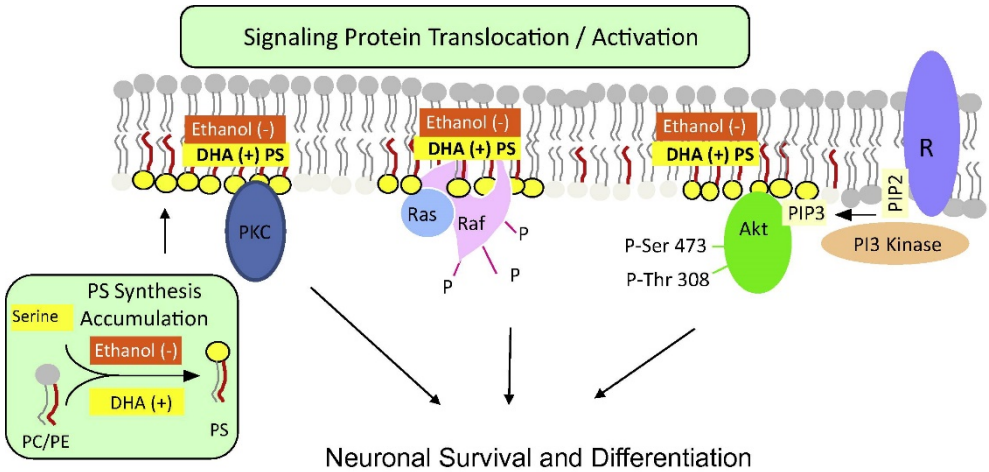


Figure 1 Activation of neuronal signaling pathways facilitated by PS (H. Y. Kim et al., 2014)

PS is participated in the activation of Akt, protein kinase C and Raf-1 signaling. It is important for neuronal differentiation and survival.

2. Phosphatidylserine synthase 1(ptdss1)

Phosphatidylserine synthase is the enzyme that catalyzes the synthesis of PS. In mammalian cells, there are two phosphatidylserine synthases, PSS1 and PSS2, which are complementary to each other. PSS1 synthesize PS from PC and PSS2 synthesizes PS from PE (Arikketh, Nelson, & Vance, 2008). In *Drosophila,* there is only PSS1-homologous gene, *CG4825(ptdss1).*

In human, Lenz-Majewski syndrome is a *Ptdss1* related disease, resulting from gain-of-function mutations in which amino acids are changed. When amino acids at certain sites are changed, the PSS1 protein can be over-activated (Figure 2) (Sousa et al., 2014; Whyte et al., 2015). In mouse, only *PSS1* and *PSS2* deletion mutations have been characterized but they have no significant characteristics. Both deletion mutations cause embryonic lethality (Arikketh et al., 2008; Bergo et al., 2002).

In general, *Drosophila* models of neurodegenerative disease have a reduced life span, impaired behavioral abilities, and degeneration of brain tissue (Lu & Vogel, 2009). Heterozygous *ptdss1* mutants of *Drosophila* have been shown to have neurodegenerative characteristics in previous research (M. C. Jo, 2014). In *Drosophila* models of neurodegenerative disease, there have been many studies on genes that affect synapses (Estes et al., 2011). Reducing the expression level of *ptdss1* in glial cells, results in abnormal synapse formation (T. Y. Kim, 2017). However, the exact function of *ptdss1* is not yet defined.

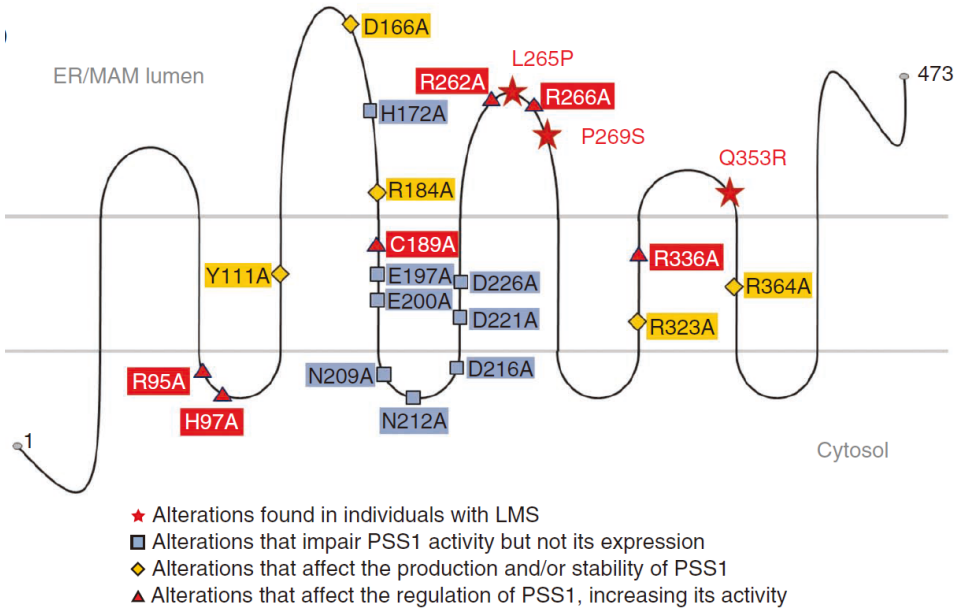


Figure 2 Topology of PSS1 protein in human (Sousa et al., 2014)

3. CRISPR/Cas9 System

To study the function of a gene, mutations are typically used and the resulting phenotype is examined. In *Drosophila,* numerous mutations were constructed before the whole genome sequence of *Drosophila* was published, which greatly facilitated genetics research.

In *Drosophila*, a many techniques have been used to change the expression level of certain genes and induce mutations in target genes. The most common method is the insertion of transposon P-elements in a regulatory site. To create a deletion, the P-element is excised. Gene replacement systems using homologous recombination have also been developed. However, these techniques are limited by low specificity and low efficiency (Rong & Golic, 2000; Ryder & Russell, 2003).

Because of their accuracy, designed zinc-finger (ZFNs) and TALE nucleases (TALENs) are generally used for targeted gene editing. These nucleases bind to target loci and induce double-stranded breaks. An indel mutation then occurs when the DNA is repaired by nonhomologous end joining. Despite their efficiency, the difficulty to microinject nuclease mRNA makes this technique difficult to use in *Drosophila* (Bibikova, Golic, Golic, & Carroll, 2002; Liu et al., 2012).

Recently, the CRISPR/Cas9 system was discovered as part of the bacterial immune system and was subsequently developed as a genome editing tool. The guide RNA (gRNA) recognizes a 20 bp DNA sequence and the Cas9 protein, which is bound to the gRNA, makes a double stranded break (Cong et al., 2013). The CRISPR/Cas9 system has been used in various applications, including gene regulation and replacement (Figure 3) (Sander & Joung, 2014; Shalem, Sanjana, & Zhang, 2015).

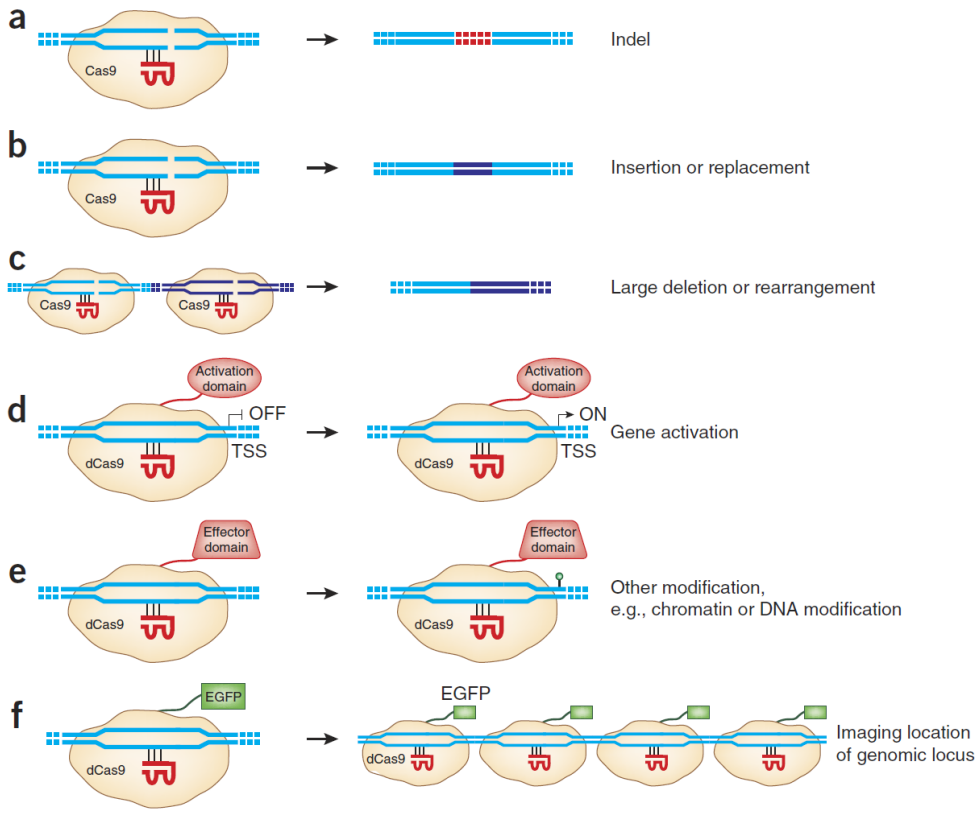


Figure 3 Various Cas9 based applications (Sander & Joung, 2014)

4. Purpose of this research

Phosphatidylserine is thought to be involved in neurodegeneration and *ptdss1* knock-down mutants of *Drosophila* have neurodegeneration in the brain, reduced life span, and loss of behavioral abilities. Although these neurodegenerative characteristics have been shown in *ptdss1* mutants, the specific role of *ptdss1* in neurodegeneration is unknown. Therefore, research using knockout mutants is needed to determine the precise mechanism of *ptdss1* in neurodegenerative disease.

The purpose of this research is to study the effects of *ptdss1* on neurodegeneration by constructing *ptdss1* knockout mutants using the CRISPR/Cas9 system. Coding sequence was selected to make gRNA expression flies. The gRNA strains were crossed with the germline-specific Cas9 protein expression strains to make an indel mutation of *ptdss1*. The *ptdss1* knockout mutants are not expected to express normal PTDSS1 protein due to a shift in amino acid sequence. These mutants are available for further research and will be valuable tools to help characterize the role of *ptdss1* in neurodegeneration.

II. Materials and Methods

1. Plasmid construction

pBFv-U6.2 (NIG-fly) was used to construct a gRNA vector. The pBFv-U6.2 vector has a *Bbs*I cleavage site, followed by a U6 promoter sequence. It has an attB sequence for site-specific integration by phiC31 integrase. The *vermillion (v)* gene of *Drosophila* was cloned into the pBFv-U6.2 vector as a visible marker. A 399 bp promoter sequence from the *Drosophila snRNA:U6:96Ab* gene was used for gRNA expression (Figure 4).

Five 20 bp target sites on the *Ptdss1* exon were selected to construct gRNA expression vectors. Two oligonucleotides complementary to the target sequences were designed. The top oligonucleotide contained 'CTTC' and 20 bp of the target sequence and the bottom oligonucleotide contained 'AAAC' and 20 bp of complementary sequence to the top target sequence. 'CTTC' and 'AAAC' are the cleavage sites of *Bbs*I. Two oligonucleotides of each target site were annealed to double stranded DNA and cloned into a pBFv-U6.2 vector digested with *Bbs*I (New England Biolabs, Ipswich, MA, USA). The annealed DNA and *Bbs*I-digested vector were ligated with T4 DNA ligase (New England Biolabs). The constructed gRNA expression vectors were named according to their location (pBFv-U6.2-CG4825-ex2-1, pBFv-U6.2-CG4825-ex2-2, pBFv-U6.2-CG4825-ex3-1, pBFv-U6.2-CG4825-ex3-2, and pBFv-U6.2-CG4825-ex4-1). The sequences of the oligonucleotides used to construct of each of the gRNA vectors are as follows (Table 1) (Kondo & Ueda, 2013).

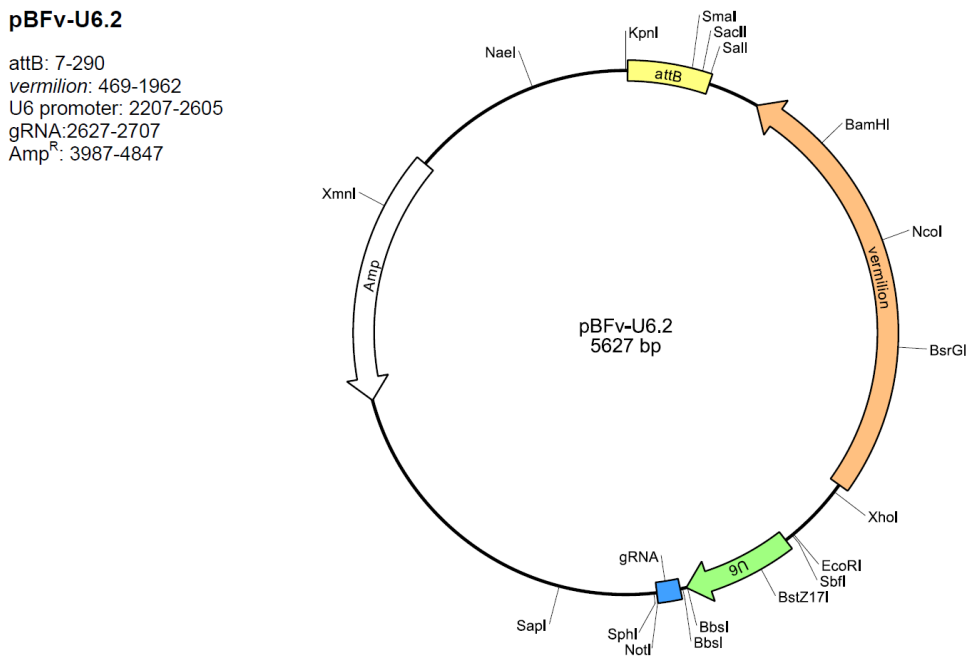


Figure 4 The map of vector used to construct gRNA expression vector

The vector pBFv-U6.2 was used to construct gRNA expression vector. It has the attB sequence for specific integration. The *vermilion (v)* gene is the visible marker for sgRNA line selection. U6 promoter in the vector express gRNA. The double strand DNA of gRNA is cloned into vector and followed U6 promoter sequence. The vector has a resistance for ampicillin (Kondo & Ueda, 2013).

Table 1 Oligonucleotide used to construct gRNA expression vector

gRNA ID	Top oligonucleotide	Bottom oligonucleotide
Ex2-1	CTTCGAACGTGGATGAGAATCTCT	AAACAGAGATTCTCATCCACGTTC
Ex2-2	CTTCGACCCCATCCCGCCGTCTGG	AAACCCAGACGGCGGGATGGGGTC
Ex3-1	CTTCGGATCGCGTTAAAGGCCACT	AAACAGTGGCCTTTAACGCGATCC
Ex3-2	CTTCGGAGATGCGCGAGTACAAGT	AAACACTTGTACTCGCGCATCTCC
Ex4-1	CTTCGGTGGCATTTGTCTACCTCG	AAACCGAGGTAGACAAATGCCACC

2. *Drosophila* genetics

All *Drosophila* lines were incubated in vials containing cornmeal/yeast medium with dry yeast at 25℃, 60% humidity and exposed to a cycle of 12 hours of light and 12 hours of darkness.

Three gRNA expression vectors were selected for *Drosophila* transformation. These vectors were individually microinjected into the embryos of y^1 v^1 *P{nos-phiC31\int.NLS}X ; attP40(II)* (NIG-fly) (Bischof, Maeda, Hediger, Karch, & Basler, 2007). PhiC31 integrase integrated all vectors into the *att P40* landing site on the second chromosome (Markstein, Pitsouli, Villalta, Celniker, & Perrimon, 2008). G_0 males that survived to adulthood were individually mated with y^2 cho^2 v^1 (NIG-fly). When a v^+ transgene in the gRNA expression vector was expressed in a cho^2 v^1 background, eye color changed from light orange to dark brown. Male offspring of the first cross showing dark brown eye color was crossed with y^2 cho^2 v^1 *; Sp/CyO* (NIG-fly) virgins. Offspring in which the transgene was balanced were collected to establish a stock. The resulting genotype was y^2 cho^2 v^1 *;attP40{U6-gRNA}/CyO,* named after the integrated gRNA (Figure 5) (Kondo & Ueda, 2013).

All the U6-gRNA lines had the U6-gRNA gene on the second chromosome. Males carrying a U6-gRNA transgene were crossed with y^2 cho^2 v^1 *; attP40{nos-Cas9}/CyO*(NIG-fly) virgins carrying the *nos-Cas9* transgene, which express the Cas9 protein specifically in the germ line, directed by the *nanos* gene promoter. The male founders with both the U6-gRNA and the *nos-Cas9* transgene were mated with y^2 cho^2 v^1 *; Pr Dr/TM6C, Sb Tb* flies. Offspring balanced with *TM6C, Sb Tb* were collected to establish a stock and were used for molecular characterization (Figure 6).

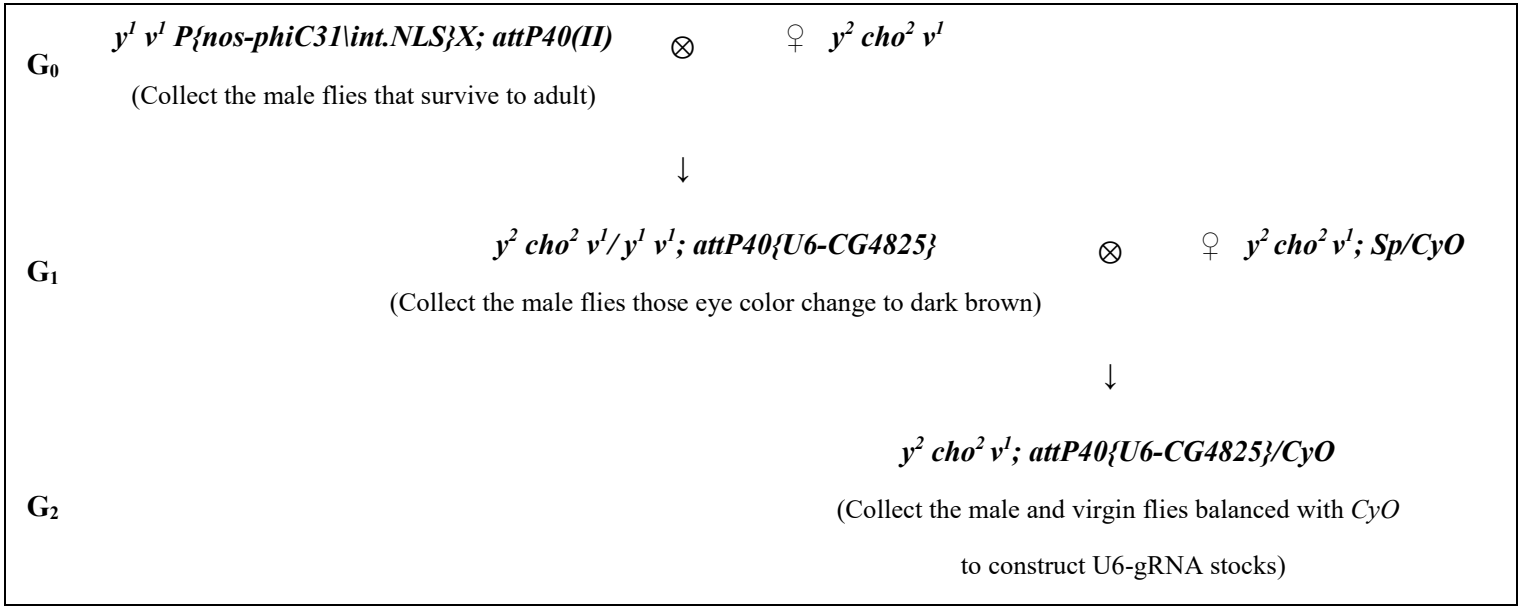


Figure 5 The schematic overview to construct the U6-gRNA stocks

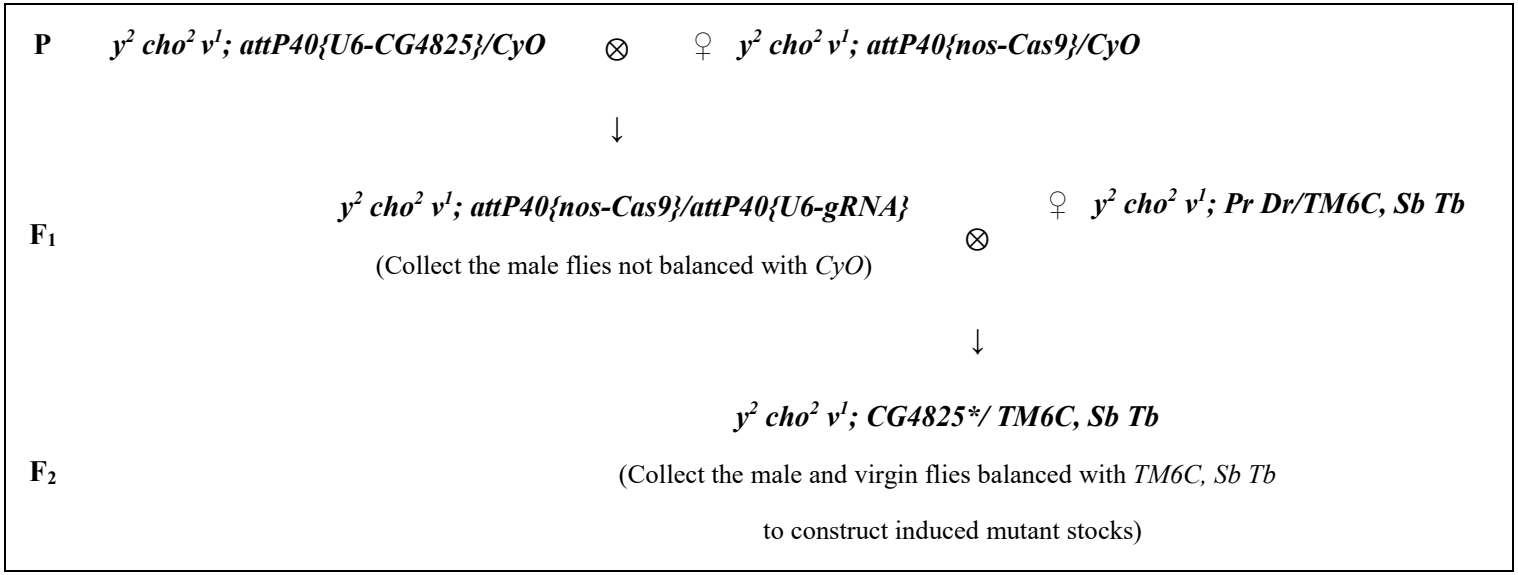


Figure 6 The schematic overview to construct the *ptdss1* mutant

3. PCR amplification and T7EI Assay

To characterize the induced mutations, target loci were amplified by PCR. First, adult flies were collected in a microcentrifuge tube and frozen at -80℃ for more than 1 hour. They were then homogenized in 200 μL of 1X PBS buffer. Genomic DNA (gDNA) was extracted from mutant flies using the DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany). The extracted gDNA was quantified and 100 ng was used as a template for PCR amplification of the surrounding target sequence. PCR was performed for 40 cycles using 12.5 μL of Q5 Hot Start High-Fidelity 2X Master Mix (New England Biolabs). The sequences of the PCR primers used to amplify the target site are as follows (Table 2).

The T7EI assay was used to identify heterozygous mutations. T7 endonuclease I (T7EI) is a structure-specific nuclease that cleaves at mismatch sites of DNA hetero-duplexes. If there is a heterozygous mutation at the target site, the PCR product is a mixture of mutant and wild type DNA. For reannealing to make hetero-duplex DNA, 5 μL of PCR product was mixed with 2 μL of 10X NEBuffer 2 (New England Biolabs) and nuclease-free water added to make 19 μL. The PCR product mixture was incubated at 95℃ for 5 minutes and ramped down -1.5℃/second to 85℃, then -0.1℃/second to 25℃. The reannealed DNA was treated with 1 U of T7 endonuclease I (New England Biolabs) and incubated for 1 hour at 37℃. After incubation, the sample was treated with 1.5 μL of 0.25M EDTA to inactivate T7 endonuclease I. Since the cleaved DNA is easily degraded, samples were immediately analyzed by electrophoresis in 2.5% Tris-Borate-EDTA (TBE) buffer. All the samples were also identified DNA sequence with DNA sequencing.

Table 2 Sequence of the primer used for PCR amplification and DNA sequencing

Primer ID	Forward primer	Reverse primer
PCR#1	CATCGTGCCATTGACCCC	CAGCACTCGATGAAGTTGGG
PCR#2	TTACAGAAACGAGGCGAACG	CGGTGACCTGCCAGAAGA
PCR#3	CCTGTTAAGTCCCTTATCGCC	GAAGTGACCCCAGGCAAAG
Seq#1	GTGCCTTCTTAGTTCACACATCA	-
Seq#2	AGCCAAGATCATCCATTTAGCA	-

4. Quantitative real-time PCR

Quantitative real-time PCR was used to estimate the *ptdss1* RNA expression level of the induced mutations. Thirty flies were collected in a 1.5 mL microcentrifuge tube within a day of hatching and frozen at -80℃ for more than 1 hour. They were homogenized in 350 μL of RA1 buffer from the Nucleospin RNA extraction kit (Macherey-Nagel, Duren, Germany) and 3.5 μL of 1 M DTT. RNA was extracted using a Nucleospin RNA extraction kit (Macherey-Nagel). The extracted RNA was quantified. RNA with a purity between 1.8 and 2.1 was used as a template for cDNA synthesis using a 5X All-In-One RT MasterMix (Applied Biological Materials, Richmond, Canada). cDNA was used in quantitative real-time PCR with the TOPreal qPCR 2X PreMix (SYBR Green with low ROX, Enzynomics, Daejeon, South Korea). The fluorescence of amplified DNA was detected with the Rotor-Gene Q instrument (QIAGEN). Expression of *ptdss1* was normalized to the housekeeping gene, *rp49* and analyzed by delta-delta CT relative quantitation analysis. The primer sequences used for *rp49* and *ptdss1* were as follows (Table 3) (T. Y. Kim, 2017).

Table 3 Sequence of the primer used for qRT-PCR

Gene	Forward primer	Reverse primer
rp49	TACAGGCCCAAGATCGTGAA	TCTCCTTGCGCTTCTTGGA
ptdss1	CATCAGTTGGGATCGCGTT	CACAGAAATGGCCCACAGA

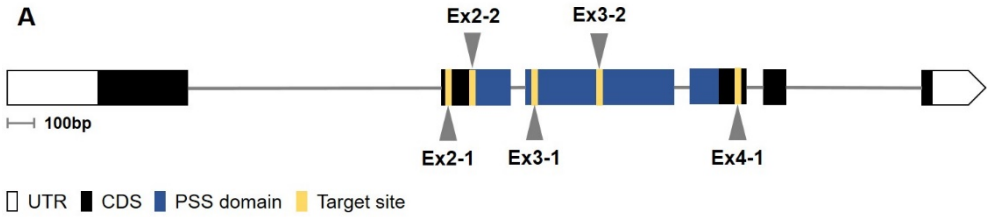
III. Results

1. Target Loci and gRNA strain construction

To construct the gRNA expression vector, five target sites were selected and cloned into the pBFv-U6.2 vector. All target sites were on the coding sequence to mutate the phosphatidylserine synthase 1 (PSS1) protein, which is expressed from *ptdss1*. Each site was 20 bp and was upstream of the NGG sequence, known as NGG PAM, and the recognition site of Cas9. Two sites were in the 2nd exon of the gene, two were in the 3rd exon and the fifth site was in the 4th exon. Three of the gRNA target sites were in the conserved PSS domain (Figure 7).

Three gRNA expression vectors, pBFv-U6.2-CG4825-ex2-1, pBFv-U6.2-CG4825-ex2-2, and pBFv-U6.2-CG4825-ex3-2 were selected for use. Vectors were selected based on the GC content of the target sites and the location of sites. The GC content of all target sites was under 80%. The *ex2-1* site is located upstream of the coding sequence and therefore, the mutation at this site was expected to greatly damage the protein. The *ex3-2* site was selected because it is located at the center of the PSS domain, which is expected to have an important role in the function of the protein. The *ex2-2* site is located in both the upstream and PSS domain regions.

Vectors were injected into the embryos of y^1 v^1 *P{nos-phiC31\int.NLS}X; attP40(II)* flies and crossed with y^2 cho^2 v^1 flies to construct gRNA expression line stock. A change in eye color from light orange to dark brown was assessed to confirm the mutation (Figure 8)



B

Target loci name	Target sequence	Size	Exon	PSS domain
Ex2-1	GAACGTGGATGAGAATCTCTGGG	20bp	2nd	No
Ex2-2	GACCCCATCCCGCCGTCTGGCGG	20bp	2nd	Yes
Ex3-1	GGATCGCGTTAAAGGCCACTTGG	20bp	3rd	Yes
Ex3-2	GGAGATGCGCGAGTACAAGTGGG	20bp	3rd	Yes
Ex4-1	GGTGGCATTTGTCTACCTCGTGG	20bp	4th	No

Figure 7 The information of target loci

(A) The schematic transcript of *ptdss1* gene. It has six exon and PSS domain is located on 2nd, 3rd and 4th exon. (B) The information of target site. Five 20bp sites that NGG PAM (blue) is followed are selected.

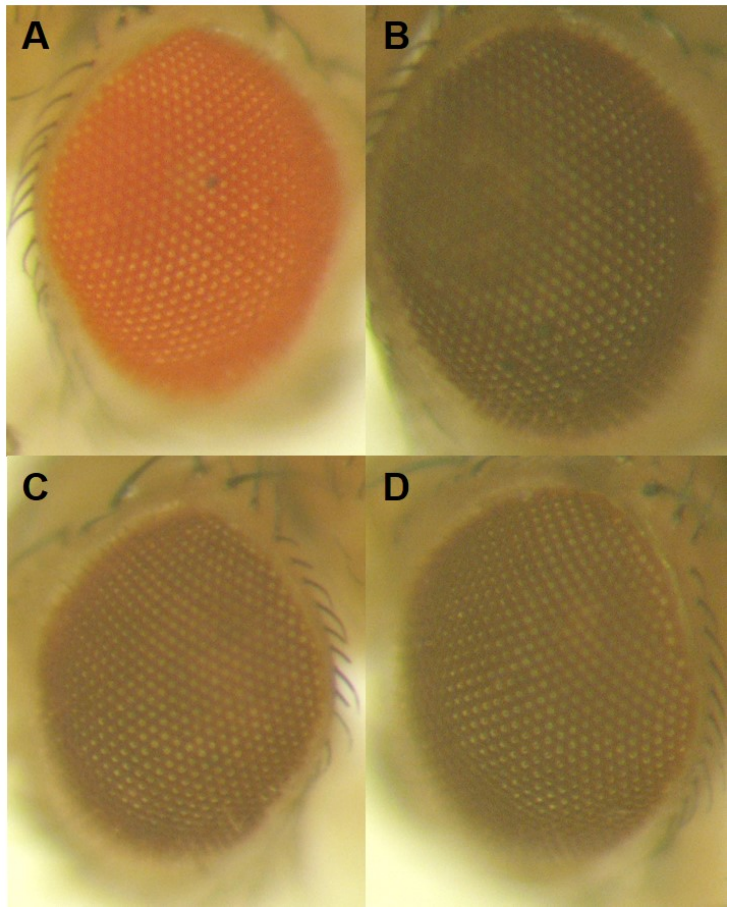


Figure 8 The eye colors of gRNA expression lines

(A) The eye color of y^2 cho^2 v^1 flies, which is shown as light orange because of *vermillion (v)* gene mutation. (B) The eye of y^2 cho^2 v^1*; attP40{U6-CG4825-ex2-1}/CyO* (C) The eye of y^2 cho^2 v^1*; attP40{U6-CG4825-ex2-2}/CyO* (D) The eye of y^2 cho^2 v^1*; attP40{U6-CG4825-ex3-2}/CyO* (B-D) The eye colors of gRNA expressed lines changed to dark brown because the v^+ gene on the transgene is expressed.

2. Indel mutation of *Ptdss1*

The *ptdss1* mutations induced using the germline-specific CRISPR/Cas9 system were characterized by T7EI assay and DNA sequencing. Three gRNA-expressing lines, *y^2 cho v^1 ; attP40{U6-CG4825-ex2-1}/CyO, y^2 cho^2 v^1 ; attP40{U6-CG4825-ex2-2}/CyO*, and *y^2 cho^2 v^1 ; attP40{U6-CG4825-ex3-2}/CyO* were crossed individually with the *y^2 cho^2 v^1 ; attP40{nos-Cas9}/CyO* line. Their offspring were founder animals carrying both the *Cas9* transgene and gRNA transgene. In the germ cell of the founder animals, the *Cas9*-gRNA complex made double-strand breaks at the target sites and induced indel mutations. Founder animals were crossed with *y^2 cho^2 v^1 ; Pr Dr/TM6C, Sb Tb*, which has the wild-type *ptdss1* sequence. Six lines with mutations induced at the *ex2-1* gRNA site and eleven lines with mutation at *ex2-2* were molecularly characterized. However, the founder animals carrying the *U6-CG4825-ex3-2* transgene have no offspring.

2.1. T7EI assay

First, to characterize heterozygous mutations, PCR-amplified DNA fragments of target loci were treated with T7 endonuclease. The T7EI assay can detect indel mutations ≥ 2 bp and therefore, some induced mutations cannot be detected by this assay. PCR products were 642 bp, with the cleavage sites of the ex2-1 and ex2-2 loci located 150 bp and 245 bp from the 5′ end, respectively. Five ex2-1 mutant lines were analyzed using the T7EI assay and mutations were detected in two of those lines (Figure 9). Out of ten ex2-2 mutant lines tested with the T7EI assay, DNA fragments were cleaved in six lines (Figure 10).

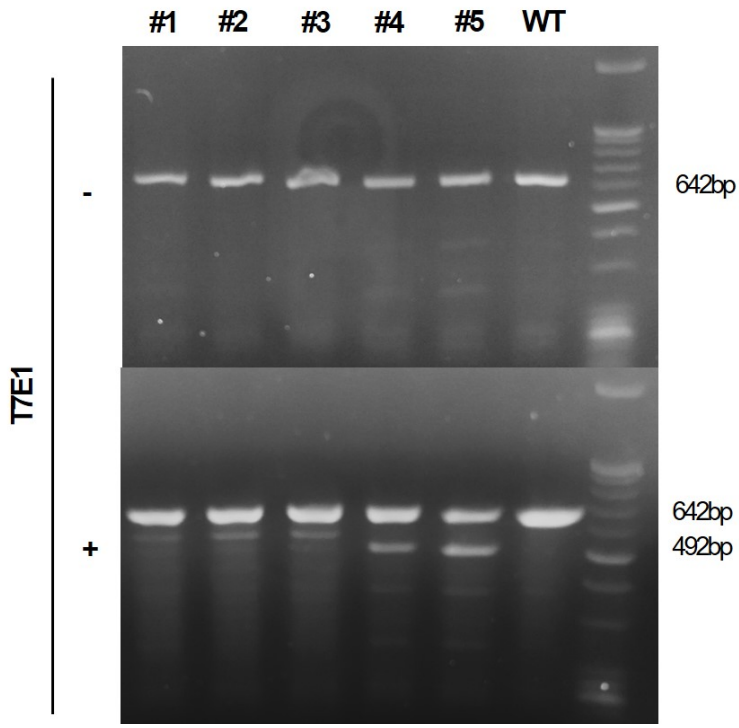


Figure 9 The T7EI assay result of the mutation induced at ex2-1 site

642bp size DNA fragment containing target site of five induced mutations at *ex2-1* were amplified by PCR amplification and treated with T7 endonuclease I. Untreated PCR products and the amplified DNA fragment of wild type flies were used for negative control. The size of cleaved PCR product is about 492bp. Then the PCR products of #4 and #5 mutation were cleaved but those of #1, #2 and #3 weren't.

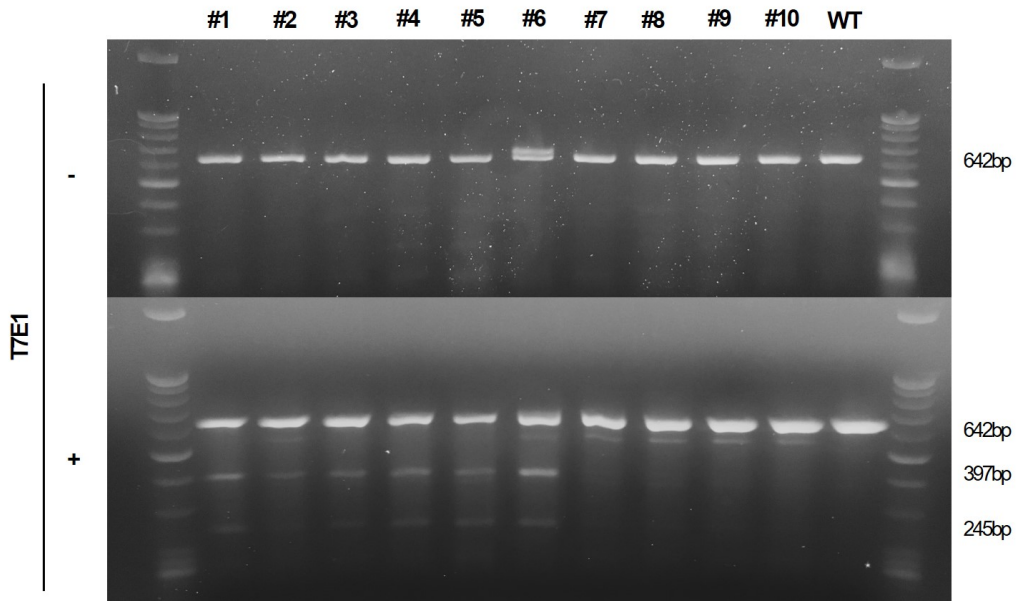


Figure 10 The T7EI assay result of the mutation induced at ex2-2 site

642bp size DNA fragment containing target site of ten induced mutations at ex2-2 were amplified by PCR amplification and treated with T7 endonuclease I. Untreated PCR products and the amplified DNA fragment of wild type flies were used for negative control. The size of cleaved PCR product is about 397bp and 245bp. Then the PCR products of #1 to #6 mutation were cleaved but those of #7 to #10 weren't.

2.2. DNA sequencing

Six lines with induced mutations at *ex2-1* were sequenced and three types of mutation were detected (Figure 11). Sequencing data showed that mutant lines #1 to #3 had 1 bp deletions that were not detected by T7EI assay. Mutant lines #4 and #5 had 15 bp and 14 bp deletions, respectively. Only mutant line #4 could survive as a homozygous mutant. Mutant line #6 wasn't tested with the T7EI assay, but sequencing analysis of this line detected the same mutation as lines #1 to #3 (Table 4).

Eleven lines with induced mutations at *ex2-2* were sequenced and four types of mutation were detected (Figure 11). In mutant lines #1 to #4 2 bp deletions and 2 bp insertions were detected. A 5 bp deletion and a 5 bp insertion were detected in line #5 and in mutant line #6, a 5 bp deletion was detected. Mutant lines #7 to #10 had 1 bp deletions and 1 bp insertions that were not detected by the T7EI assay. Mutant line #11 wasn't tested with the T7EI assay, but sequencing analysis detected the same mutation as line #6. Only the mutants homozygous for a 5 bp deletion did not survive (Table 5)

sn

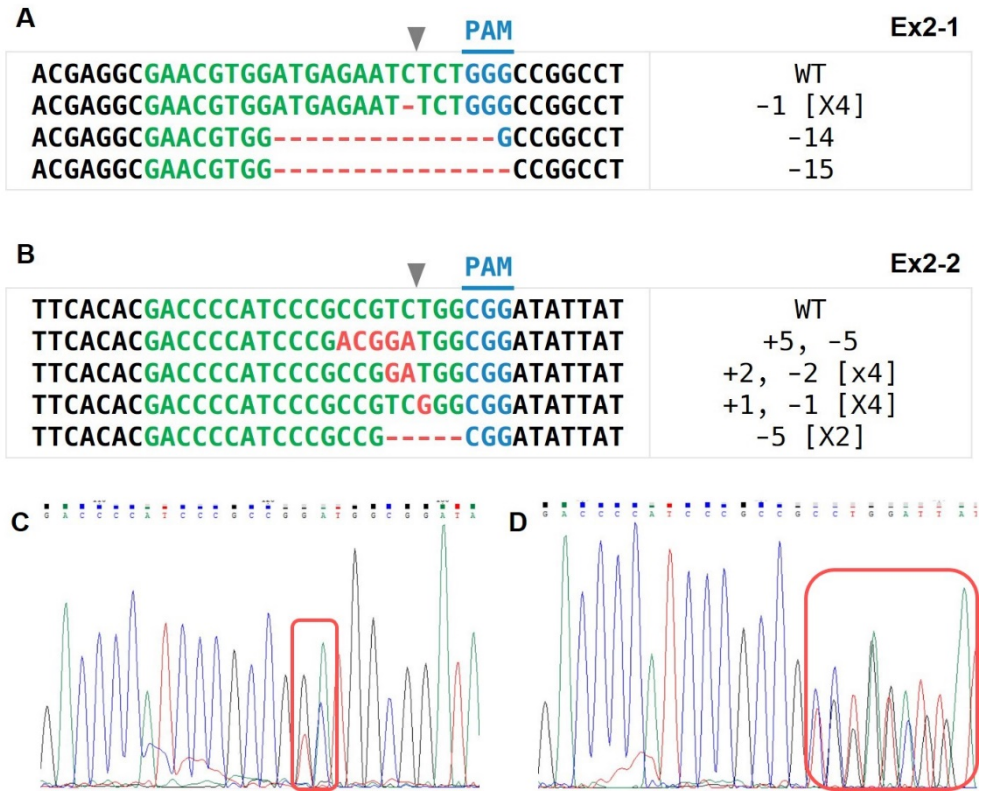


Figure 11 The DNA sequencing result of the induced mutation

(A) The sequence of induced mutation at *ex2-1* (B) The sequence of induced mutation at *ex2-2* (A-B) The wild-type sequence is shown at the top. The target sequence of gRNA is indicated in green. The PAM is indicated blue. Deleted nucleotides are indicated in red dash and inserted nucleotides are indicated red. (C) The sequencing chromatogram of mutation at *ex2-2* which is [+2, -2] indel mutation. (D) The sequencing chromatogram of mutation at *ex2-2* which is [-5] deletion mutation.

Table 4 Sequence and viability of induced mutation at *ex2-1*

Line No.	Mutation sequence	Indel size	Homo-viable /Homo-lethal
***Ex2-1* #1**	GAACGTGGATGAGAAT-TCTGGG	-1	Homo-lethal
***Ex2-1* #2**	GAACGTGGATGAGAAT-TCTGGG	-1	Homo-lethal
***Ex2-1* #3**	GAACGTGGATGAGAAT-TCTGGG	-1	Homo-lethal
***Ex2-1* #4**	GAACGTGG---------------	-15	Homo-viable
***Ex2-1* #5**	GAACGTGG--------------G	-14	Homo-lethal
***Ex2-1* #6**	GAACGTGGATGAGAAT-TCTGGG	-1	Homo-lethal

Table 5 Sequence and viability of induced mutation at *ex2-2*

Line No.	Mutation sequence	Indel size	Homo-viable /Homo-lethal
***Ex2-2* #1**	GACCCCATCCCGCCGgaTGGCGG	+2, -2	Homo-viable
***Ex2-2* #2**	GACCCCATCCCGCCGgaTGGCGG	+2, -2	Homo-viable
***Ex2-2* #3**	GACCCCATCCCGCCGgaTGGCGG	+2, -2	Homo-viable
***Ex2-2* #4**	GACCCCATCCCGCCGgaTGGCGG	+2, -2	Homo-viable
***Ex2-2* #5**	GACCCCATCCCGacggaTGGCGG	+5, -5	Homo-viable
***Ex2-2* #6**	GACCCCATCCCGCCG-----CGG	-5	Homo-lethal
***Ex2-2* #7**	GACCCCATCCCGCCGTCgGGCGG	+1, -1	Homo-viable
***Ex2-2* #8**	GACCCCATCCCGCCGTCgGGCGG	+1, -1	Homo-viable
***Ex2-2* #9**	GACCCCATCCCGCCGTCgGGCGG	+1, -1	Homo-viable
***Ex2-2* #10**	GACCCCATCCCGCCGTCgGGCGG	+1, -1	Homo-viable
***Ex2-2* #11**	GACCCCATCCCGCCG-----CGG	-5	Homo-lethal

3. Predicted amino acid sequences of induced mutations

There are two transcripts encoded by *ptdss1* in *Drosophila.* These are translated into 498 aa and 497 aa polypeptides, which differ in the presence or absence of glutamine (Q) at the 459th amino acid. They both contain a conserved PSS domain from amino acid 150 to 425. Mutations induced by Cas9 were expected to change the amino acid sequence of the translated proteins.

Polypeptides translated from induced mutations at *ex2-1* had differences between the 121st and 126th amino acids. In the 1 bp deletion, the 124th amino acid, tryptophan (W), is changed. In the 14 bp deletion, amino acids after the glutamic acid (E) residue at amino acid 121 are changed. The 15 bp deletion did not induce major changes like the other mutations. Amino acid residue 121 was changed into an alanine (A) and five amino acids from 122–126 were deleted as a result of the 15 bp deletion (Figure 12A).

Polypeptides translated from the induced mutation at *ex2-2* have differences from amino acid residue 153 to 155, which is a part of the PSS domain. Two amino acids are changed by the 5 bp deletion, 5 bp insertion mutation. The alanine (A) and valine (V) at amino acid positions 153 and 154 are changed to aspartic acid (D) and glycine (G), respectively. In the 1 bp insertion, 1 bp deletion and 2 bp insertion, 2 bp deletion mutations, the valine (V) at position 154 or tryptophan (W) at position 155 were changed to glycine (G). The amino acid sequence after the 154th amino acid was changed as a result of the 5 bp deletion (Figure 12B)

The amino acids deleted or altered are near the 2nd and 3rd transmembrane regions of the *Drosophila* PTDSS1 protein (Figure 13).

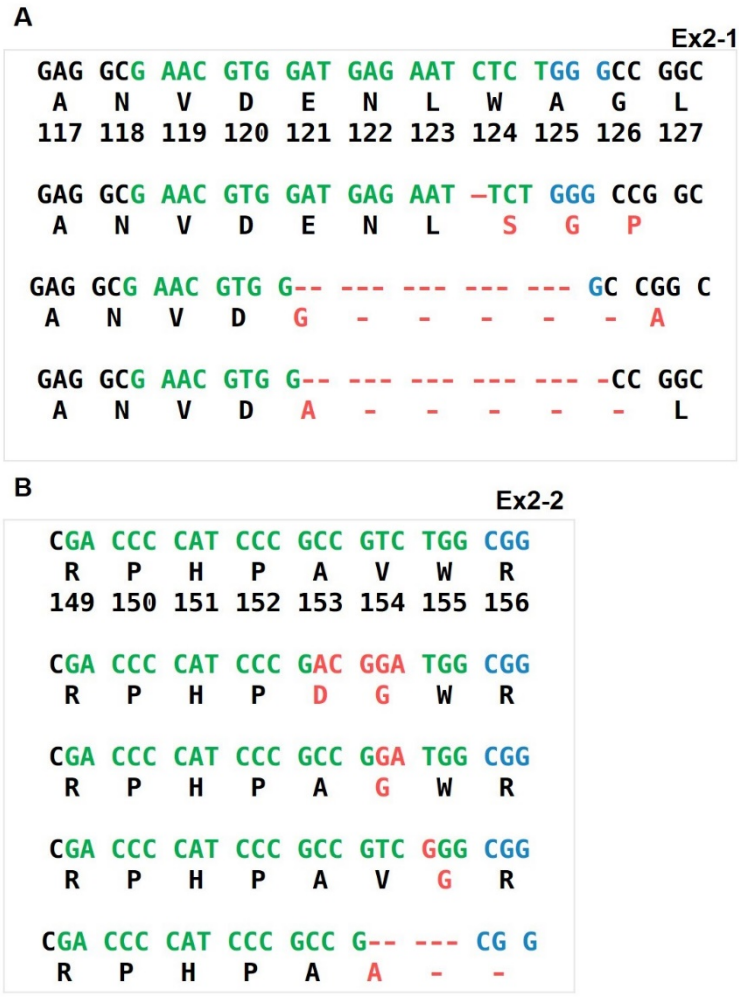


Figure 12 Predicted amino acid sequences of induced mutations

(A-B) The wild-type sequence is shown at the top. The changed amino acids are indicated in red. (A) The amino acid sequence of induced mutation at *ex2-1*. (B) The amino acid sequence of induced mutation at *ex2-2*.

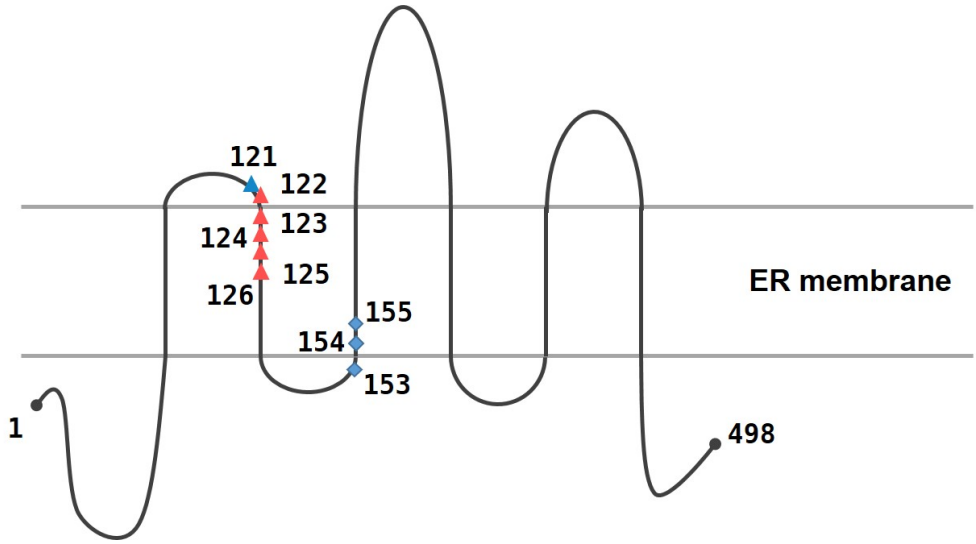


Figure 13 Topology of *Drosophila* PTDSS1 protein (Uniprot)

The triangles are the amino acids which are mutated by induced mutation of *ex2-1* site. The diamonds are the amino acids which are mutated by induced mutation of *ex2-2.* Although the transmembrane regions of *Drosophila* PTDSS1, the exact topology of protein is unknown

4. *Ptdss1* mRNA expression level of mutants

Using quantitative real-time PCR, *ptdss1* mRNA expression levels were measured in flies with Cas9-induced mutations. Expression analysis was performed in three types of mutants: $y^2\ cho^2\ v^1$; $CG4825^{ex2\text{-}1\ \#1}$/*TM6C, Sb Tb,* which has a 1 bp deletion; $y^2\ cho^2\ v^1$; $CG4825^{ex2\text{-}1\ \#5}$/*TM6C, Sb Tb,* which has a 14 bp deletion; and $y^2\ cho^2\ v^1$; $CG4825^{ex2\text{-}2\ \#6}$ /*TM6C, Sb Tb*, which has a 5 bp deletion. The *ptdss1* mRNA expression levels in mutant flies reduced to about 70% of the expression level in the control flies, *TM6C, sb Tb*/+ (Figure 14).

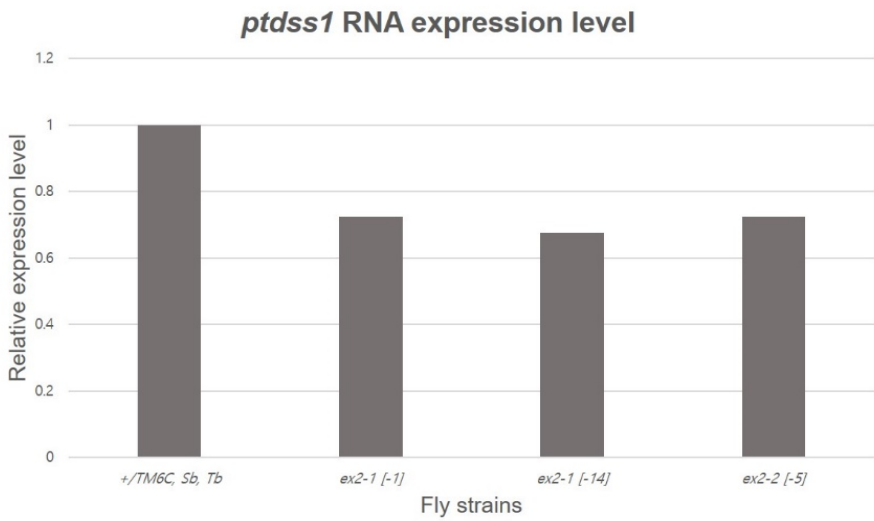


Figure 14 The *ptdss1* RNA expression level of Cas9 induced mutation

IV. Discussion

Ptdss1 knockdown mutants have the characteristics of neurodegenerative disease. They have reduced life span, neurodegeneration in the brain, and abnormal behavior (M. C. Jo, 2014). When *ptdss1* expression is regulated in glial cells, flies show abnormalities in synapse formation (T. Y. Kim, 2017). Since the composition of phospholipids is altered in the neurodegenerative brain, phospholipids are considered to be involved in neurodegeneration (Wells et al., 1995). Although phosphatidylserine is abundant in the neural membrane, phosphatidylserine synthesis has not been studied as much as other phospholipids (Svennerholm, 1968). To study the effect of *ptdss1* on neurodegeneration, knockdown and knockout mutants are needed. Therefore, in this study, *ptdss1* knockout mutants were constructed using the germline-specific CRISPR/Cas9 system.

Three gRNA-expressing strains were constructed using the CRISPR/Cas9 system. However, one strain expressing the *ex3-2* locus gRNA had no progeny when carrying the Cas9 protein expression transgene. In mice, deletion of both *Pss1* and *Pss2* results in embryonic lethality, whereas deletion of either *Pss1* or *Pss2* gives no specific phenotype, with the exception of infertility in some male *Pss2* mutants (Arikketh et al., 2008; Bergo et al., 2002; H. Y. Kim et al., 2014). Male flies carrying both the gRNA transgene and the Cas9 transgene can be infertile.

Seven mutations were induced using the CRISPR/Cas9 system. Four of these were deletions and three were base-change mutations. Three of the base-change mutations and the 15 bp deletion were viable as homozygotes because

their termination codons operated normally. The other deletions produced large changes in amino acid sequence. Therefore, the phenotype of *ptdss1* deficiency was severe in those mutants, even though the mRNA expression level was only reduced to about 70% of wild type flies. The polypeptide regions changed in the viable homozygous mutants were from amino acids 121 to 126 and amino acids 153 to 155. Those regions extend to the transmembrane domain. There are nine transmembrane domains in the *Drosophila* PSS1 protein, which include amino acids 123 to 142 and amino acids 154 to 174.

The mutants with shifted amino acid sequences are difficult to make as knockout mutants. They only have between one and five changed amino acids. In Lenz-Majewski disease, one amino acid change is enough to over-activate the *ptdss1* gene, resulting in increased PS synthesis (Sousa et al., 2014). The mutants in this study with amino acid shifts also had an overactive PTDSS1 protein phenotype.

The mutations produced in this study will facilitate further research on the relationship between *ptdss1* and neurodegenerative diseases. If a more severe mutation is needed, a double gRNA line can be constructed, using the same process, to induce a large deletion (Kondo & Ueda, 2013; Sander & Joung, 2014).

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국문초록

과학기술의 발달에 의해 평균 수명이 증가하면서 노화와 관련된 질병에 대한 관심도 증가하고 있다. 특히 노화에 의해 점진적으로 신경세포의 능력이 저하되고 신경세포 사멸이 나타나는 퇴행성 신경 질환에 대한 연구가 각광받고 있다. 인지질은 생물체를 구성하는 세포막의 주요 구성 요소일 뿐만 아니라 신경세포에 높은 밀도로 존재하고 있다. 퇴행성 신경질환 환자의 뇌에서 인지질 구성 변화가 관찰되는 것과 같이 인지질은 퇴행성 신경 질환과 밀접한 관계를 가지고 있다. 선행 연구에서는 포스파티딜세린 합성효소 유전자 *phosphatidylserine synthase1* (*ptdss1*) 돌연변이 초파리에서 운동 능력 저하, 신경 퇴화 등의 퇴행성 신경 질환의 특성이 나타났다. 하지만 기존의 초파리 *ptdss1* 돌연변이는 유전자 조절 부위에 P 전이 인자가 삽입되어 만들어진 knockdown 돌연변이들로 *ptdss1* 유전자와 퇴행성 신경 질환의 연관성을 밝히는 데에는 한계가 있다. 유전자의 좀 더 정확한 기능을 밝히기 위해서는 단백질 발현 지역에 돌연변이가 생긴 knockout 돌연변이를 이용한 연구가 필요하다. 그래서 본 연구에서는 정교한 유전자 조작 기술인 CRISPR/Cas9 시스템을 이용해 초파리 *ptdss1* knockout 돌연변이를 제작하였다. *ptdss1* 돌연변이 제작을 위해 DNA 를 인식하는 gRNA 발현 돌연변이를 제작하여 생식세포에서만 특이적으로 Cas9 단백질을 발현하는 초파리와 교배하였다. F1 세대 초파리의 생식세포에서 Cas9-gRNA 복합체가 형성되어 유전자 조작이 일어났다. 이 초파리를 야생형 초파리와 교배하여 이형 접합 돌연변이를 제작하고

T7EI 과 DNA 시퀀싱을 통해서 돌연변이를 확인하였다. 3 종류의 knockout 돌연변이가 제작되었으며 qRT-PCR 을 통해 *ptdss1* mRNA 발현량을 확인한 결과 야생형의 *ptdss1* mRNA 발현의 70%만이 발현되었다. 제작된 돌연변이들은 *ptdss1* 유전자의 기능을 연구하는데 효과적으로 이용될 수 있을 것으로 생각된다.

주요어 : 초파리, *ptdss1*, CRISPR-Cas9 시스템, 돌연변이, 포스파티딜세린

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