



# DNA aptamers against the Cry j 2 allergen of Japanese cedar pollen for biosensing applications



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## ABSTRACT

Sensing pollen allergens is required to prevent allergic disorders such as pollinosis. Aptamers, which bind to specific molecules, offer great potential as useful tools for detecting pollen allergens as measures against allergic disorders. Here, we report the identification of DNA aptamers binding to Cry j 2, one of the major allergens in Japanese cedar pollen, and the histochemical sensing of Cry j 2 in ruptured Japanese cedar pollen. DNA aptamers were selected by systematic evolution of ligands by exponential enrichment (SELEX) using nitrocellulose membranes. Through four rounds of SELEX, we identified aptamers binding to Cry j 2. The aptamers generated staining in ruptured Japanese cedar pollen on glass slides without extraction, similar to anti-Cry j 2 antibodies. The staining was compatible with starch localization, in which Cry j 2 is present. An aptamer, CJ2-06, which had high and specific binding ability to Cry j 2 ( $K_d = 24$  nM), detected an amount of Cry j 2 equivalent to that in several tens of micrograms of pollen. Cry j 2 contained in house dust was detected in a spike test. The aptamers identified in this study can be powerful tools for allergen recognition in the practical biosensing of Cry j 2, leading to preventive measures against allergic disorders caused by Japanese cedar pollen.

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## 1. Introduction

Pollen derived from various plants causes allergic disorders worldwide (Suphioglu, 2000). In Japan, many people suffer from allergic disorders induced by pollen from the Japanese cedar *Cryptomeria japonica* during the pollen dispersal season between February and April of each year. Japanese cedar pollinosis, which is a typical type I allergy with symptoms such as sneezing, sniffing, and nasal congestion has become one of the most prevalent disorders, afflicting 26.5% of the population in Japan (Baba and Nakae, 2008). In addition, atopic dermatitis, also known as allergic skin disease with itching, is exacerbated by exposure to Japanese cedar pollen (Aihara et al., 1999; Nishie et al., 2010; Yokozeki et al., 2006). Patients use a forecast of airborne Japanese cedar pollen based on information collected by automatic pollen counters installed nationwide to avoid exposure to pollen. Thus, outdoor detection of airborne Japanese cedar pollen has become common in Japan. However, the detection of only outdoor pollen is insufficient to avoid exposure to pollen because Japanese cedar pollen is also present indoors during the pollen dispersal season

(Enomoto et al., 2001, 2004; Takahashi et al., 2008). Thus, it is desirable to detect Japanese cedar pollen anywhere in the living environment. Moreover, because allergens contained in pollen induce allergic disorders, detection of the allergens is effective for preventing allergic disorders.

The major allergens of Japanese cedar pollen have been identified as Cry j 1 and Cry j 2 (Sakaguchi et al., 1990; Yasueda et al., 1983). Cry j 1 is a pectate lyase (Taniguchi et al., 1995) located at pollen cell walls (Miki-Hirosige et al., 1994). Cry j 2 is a polymethylgalacturonase (Ohtsuki et al., 1995) located with starch granules in amyloplasts of pollen (Nakamura et al., 2004; Nakamura, 2007). Given that serum from > 90% of patients with Japanese cedar pollinosis showed immunoreactivity to both Cry j 1 and Cry j 2 (Hashimoto et al., 1995), they are considered as major allergens inducing allergic disorders. Wang et al. (2008) reported the need to detect these allergens because the allergens attached to fine particles in the air were observed even when pollen dispersal was low.

To detect Cry j 1 and Cry j 2, the use of antibodies raised in mice and rabbits as molecular recognition elements has been reported (Homma et al., 1995; Kawashima et al., 1992; Suzuki et al., 1996; Watanabe et al., 1992; Yasueda et al., 1991). Enzyme-linked immunosorbent assays (ELISAs) have been developed to detect

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the allergens using these antibodies (Saito and Teranishi, 1999; Sawatani et al., 1994; Yasueda et al., 1991). Antibodies have been used mainly as research tools, such as in forestry for selection of plus trees with pollen containing fewer allergens, but not applied to other uses aimed at avoiding exposure to the allergens. The difficulty of antibody production limits their application in allergen detection. To overcome this restriction, Yamada et al. (2009) developed single-chain variable fragment (scFv) antibodies against Cry j 1 and Cry j 2 using phage display, which is superior to the hybridoma method for production of monoclonal antibodies with respect to time and cost. Both scFv antibodies revealed high specificity to each target, but anti-Cry j 2 scFv displayed insufficient binding ability compared with an anti-Cry j 2 monoclonal antibody (Yamada et al., 2009). Easily producible molecular recognition elements are desirable for detection of Japanese cedar pollen allergens, particularly Cry j 2.

Aptamers are oligonucleotides binding to target molecules with high affinity and specificity comparable to that of antibodies. Given that aptamers are selected by *in vitro* selection by systematic evolution of ligands by exponential enrichment (SELEX) (Ellington and Szostak, 1990; Tuerk and Gold, 1990), and can be easily synthesized, modified, and labeled by chemical processes, various aptamer-based sensing systems have been developed (Abe et al., 2014; Tombelli et al., 2005). In addition, aptamers are appropriate for environmental monitoring of allergens indoors and outdoors, given their stability at a wide range of temperatures. Recently, aptamers against mold and food allergens have been reported (Low et al., 2009; Nadal et al., 2012; Tran et al., 2013). However, aptamers against pollen allergens have not been developed. Given that Japanese cedar pollen allergens spread throughout the country during the pollen dispersal season, development of aptamers against Cry j 2 would be helpful for many people to detect Cry j 2 around them.

Here, we describe DNA aptamers recognizing Cry j 2 in Japanese cedar pollen and the histochemical staining of Cry j 2 in ruptured Japanese cedar pollen as a simple sensing method not requiring extraction. Furthermore, we note the possibility of detecting Cry j 2 in house dust for the development of a practical biosensor.

## 2. Material and methods

### 2.1. Materials

All oligonucleotides were purchased from Operon Biotechnology (Tokyo, Japan) or Fasmac (Kanagawa, Japan). Cry j 1, Cry j 2, anti-Cry j 2 monoclonal antibody T27, anti-Cry j 2 polyclonal antibody, and Japanese cedar pollen were purchased from Hayashibara (Okayama, Japan).

### 2.2. Selection of aptamers by SELEX

A DNA library was designed according to the method described by Tsukakoshi et al. (2012) with slight modifications. A single-stranded (ss) DNA containing a 24-mer randomized region, 18-mer primer-binding sequences at both ends, and 3-mer thymine linkers between the randomized region and each primer-binding region was used as a library (5'-TCGTCTTCGTCACCTAC-ttt-N<sub>24</sub>-ttt-CTCAATAGCGACACCGTA-3'). The library (1  $\mu$ M) was heated in the presence of a complementary strand of forward (Fw) primer (5'-GTAGTTGACGGAAGACGA-3') and a biotinylated reverse (Rev) primer (5'-TACGGTGTGCTATTGAG-3'; 5'-biotinylated) in TBS (10 mM Tris/HCl pH 7.4, 150 mM NaCl, 5 mM KCl) at 95 °C for 10 min and gradually cooled to 25 °C for 30 min to fold the structures. Strands complementary to the primer regions were

mixed to avoid their hybridization with the randomized region. The folded DNA library was diluted in TBS with 0.05% Tween 20 (TBST). On a nitrocellulose membrane, 1  $\mu$ g of Cry j 2 was spotted and air-dried. The membrane was blocked with 4% (w/v) low-fat milk in TBST at room temperature (RT) for 1 h, and then incubated with the folded DNA library at RT for 1 h. After washing with TBST, the Cry j 2 immobilized area of the membrane was cut out and oligonucleotides bound to the area were eluted by phenol–chloroform extraction. The eluted oligonucleotides were purified by ethanol precipitation and amplified by PCR using PrimeSTAR GXL (TaKaRa, Shiga, Japan) with Fw (5'-TCGTCTTCGTCACCTAC-3') and biotinylated Rev primers. All PCR amplifications were performed as follows: 22–28 cycles of denaturation at 98 °C for 10 s, annealing at 59 °C for 15 s, and extension at 68 °C for 30 s. The number of PCR cycles for each round of SELEX was determined at a point of saturation monitored by real-time PCR.

An ssDNA library for the following round was prepared by removing biotinylated reverse strands using streptavidin agarose (Thermo Fisher Scientific, MA, USA). The binding ability of DNA libraries at each round of SELEX was confirmed by aptamer blotting assay. After incubation with the DNA library, the membrane was incubated with 500 ng/mL of HRP-conjugated NeutrAvidin (NeutrAvidin-HRP) (Thermo Fisher Scientific, MA, USA) at RT for 1 h. The binding between the DNA library and Cry j 2 was detected with Immobilon Western chemiluminescent HRP substrate (Merck Millipore, MA, USA).

After four rounds of SELEX, the DNA library amplified by PCR using PrimeSTAR GXL was purified using a Gel/PCR Extraction kit (Nippon Genetics, Tokyo, Japan). The purified PCR products were adenylated using AmpliTaq Gold (Life technologies, CA, USA), subcloned into pGEM-T vectors (Promega, WI, USA), amplified by illustra Templiphi DNA Amplification kit (GE Healthcare, Chalfont St Giles, UK), and sequenced. Phylogenetic analysis of the sequences was performed using CLUSTAL X ver. 2 (Larkin et al., 2007) and the neighbor-joining method (Saitou and Nei, 1987).

### 2.3. Aptamer blotting assay

Binding ability and specificity for Cry j 2 was investigated using an aptamer blotting assay (Hasegawa et al., 2008). The aptamers used here were labeled indirectly via primer-binding sequences in the same manner as SELEX (CJ2-X<sub>p</sub>) or directly with biotin at a 5' end (BioCJ2-X), where X denotes a sequence identification number. These aptamers were heated and folded at 1  $\mu$ M or 5  $\mu$ M under the same conditions as described above. Cry j 2 as a target and Cry j 1 as a nontarget were separately immobilized on a nitrocellulose membrane. The membrane was blocked with 2% (w/v) bovine serum albumin (BSA) at RT for 1 h, and incubated with 100 nM of each aptamer at RT for 1 h. After washing in TBST three times, the membrane was incubated with NeutrAvidin-HRP, and chemiluminescence was detected as described above.

### 2.4. Dot blotting assay using antibodies

Dot blotting assays using antibodies were performed in almost the same manner as the aptamer blotting assay. The membrane immobilizing target or nontarget molecules were incubated with the anti-Cry j 2 monoclonal antibody T27 (50 ng/mL) or anti-Cry j 2 polyclonal antibody (50 ng/mL) as an initial antibody at RT for 1 h. Mouse IgG (Sigma-Aldrich, MO, USA) and rabbit IgG (Sigma-Aldrich) were used as negative controls. After washing in TBST, the membrane was then incubated with HRP-conjugated anti-mouse Igs (Dako A/S, Glostrup, Denmark) (1:1000) or HRP-conjugated anti-rabbit Igs (Dako A/S) (1:2000) as a secondary antibody at RT for 1 h. Detection was performed in the same manner as the aptamer blotting assay.

## 2.5. Histochemical staining using aptamers

Japanese cedar pollen was smeared on glass slides and ruptured in 5  $\mu$ L of TBS. The ruptured pollen grains were dried at RT for more than 30 min and fixed with 95% ethanol/acetic acid (99:1, v/v) on ice for 15 min. The samples were blocked with 1% (w/v) BSA and 100  $\mu$ g/mL sonicated salmon sperm DNA (BioDynamics Laboratory Inc., Tokyo, Japan) in TBS at RT for 15 min. The sonicated salmon sperm DNA was heated at 95 °C for 3 min and maintained on ice before use. The aptamers were indirectly labeled with biotinylated 24-mer thymine (T<sub>24</sub>) through 24-mer adenine (A<sub>24</sub>) at the 3' end as an additional sequence (CJ2-X\_A<sub>24</sub>) or BioCJ2-06. After washing with TBS, the ruptured pollen was incubated with 5  $\mu$ M of the folded aptamers at RT for 1 h. As negative controls, 48-mer adenine (A<sub>48</sub>) and biotinylated 24-mer thymines (BioT<sub>24</sub>) were used. The pollen was washed with TBST and then incubated with 5  $\mu$ g/mL NeutrAvidin-HRP (Thermo Fisher Scientific, MA, USA) at RT for 30 min. Detection was performed using a Peroxidase Stain DAB kit (brown staining) (Nacalai Tesque, Kyoto, Japan). Counterstaining was performed with Mayer's hematoxylin (Sigma-Aldrich). After washing with distilled water, the slides were dehydrated and mounted with coverslips using Mount-Quick (Daido Sangyo, Tokyo, Japan). The pollen on the slides was observed with a digital microscope, VHX-1000 (Keyence, Osaka, Japan).

## 2.6. Immunostaining using antibodies

Japanese cedar pollen grains were ruptured and fixed on glass slides in the same manner as histochemical staining using aptamers. The pollen grains on glass slides were blocked with 5% normal goat serum (Sigma-Aldrich) and 1% BSA in TBS at RT for 15 min. After washing with TBS, the pollen was incubated with anti-Cry j 2 monoclonal antibody T27 (2  $\mu$ g/mL) or anti-Cry j 2 polyclonal antibody (2  $\mu$ g/mL) at RT for 1 h. Mouse IgG and rabbit IgG were used as negative controls. After washing in TBST, the pollen was incubated with HRP-conjugated anti-mouse Igs (1:500) or HRP-conjugated anti-rabbit Igs (1:500) at RT for 1 h. Detection, counterstaining, and mounting were performed as described above.

## 2.7. Circular dichroism measurement

The aptamers were folded at 5  $\mu$ M under the same conditions as described above. Circular dichroism (CD) spectra were measured using a J820 CD spectrophotometer (JASCO, Tokyo, Japan) in a microcell with 10-mm optical path length. Scanning was performed from 220 nm to 320 nm at 25 °C for each aptamer with a step size of 1 nm, a time per point of 1 s, and a bandwidth of 5 nm. Each scan was repeated 10 times and the values were averaged. The spectrum obtained with the buffer sample was subtracted to eliminate the background.

## 2.8. Enzyme-linked oligonucleotide assay

An enzyme-linked oligonucleotide assay (ELONA) was employed to evaluate the binding capacity of BioCJ2-06 and BioT<sub>24</sub> to Cry j 2. Cry j 2 (1  $\mu$ g/mL, 100  $\mu$ L/well) was immobilized in each well of a Maxisorp 96 well microtiter plate (Nunc, NY, USA) using PBS (1.47 mM KH<sub>2</sub>PO<sub>4</sub>, 2.7 mM KCl, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 137 mM NaCl) at 4 °C overnight. The wells were washed with TBST three times and blocked using 150  $\mu$ L of 1% (w/v) BSA in TBS at RT for 1 h. After washing in TBST three times, BioCJ2-06 or BioT<sub>24</sub> (0–250 nM, 100  $\mu$ L/well) were added to the wells and incubated at RT for 1 h. The wells were washed with TBST five times, and NeutrAvidin-HRP (500 ng/mL, 100  $\mu$ L) was then added to each

well and incubated at RT for 1 h. After the wells were washed seven times, 100  $\mu$ L of BM chemiluminescence enzyme-linked immunosorbent assay (ELISA) substrate (POD) (Roche, Basel, Switzerland) was added to each well, and chemiluminescence was measured using an Arvo MX 1420 multilabel counter (Perkin-Elmer, MA, USA). The measurement was performed in triplicate and the same experiments were repeated seven to 10 times for each concentration of BioCJ2-06 and three times for BioT<sub>24</sub>. Average of background signal without Cry j 2 was subtracted from each signal, and the average of the signal without oligonucleotides was also subtracted. The data are presented as mean  $\pm$  SE. One-way analysis of variance was used for statistical analysis at the 5% level of significance using R (Ihaka and Gentleman, 1996).

## 2.9. Detection of Cry j 2 in house dust

To detect Cry j 2 contained in house dust, a spike test was performed using house dust collected from an air conditioner filter. Cry j 2 diluted in Dulbecco's PBS (–) (Nissui, Tokyo, Japan) (0, 0.3, and 0.7 pmol/ $\mu$ L) was added to the collected house dust (150  $\mu$ L/mg). The mixture was vortexed at 4 °C for 10 min and centrifuged at 1,500  $\times$  g at 4 °C for 5 min, and the supernatant was collected in a microtube. The supernatant (2  $\mu$ L) was spotted on a nitrocellulose membrane for an aptamer blotting assay to detect Cry j 2.

# 3. Results and discussion

## 3.1. Screening of aptamers against Cry j 2 by SELEX

SELEX using a nitrocellulose membrane was performed to obtain DNA aptamers against Cry j 2. The initial library was 66-mer oligonucleotides containing a 24-mer randomized region. Binding assay of the library to Cry j 2 in each round of SELEX showed that chemiluminescence signals increased as the rounds of SELEX progressed (Fig. S1A), indicating that the oligonucleotides were successfully enriched on immobilized Cry j 2.

After four rounds of SELEX, the oligonucleotides bound to Cry j 2 were sequenced, and eight different clones were obtained (Table 1). Phylogenetic analysis shows that these clones were classified into two large groups, I (CJ2-01, -03, and -07) and II (CJ2-02, -04, -05, -06, and -08) (Fig. 1A). Group II showed more specific binding to Cry j 2 than group I in the aptamer blotting assay (Fig. 1A). The signal of CJ2-06 in group II was the highest against Cry j 2 on the membrane (Fig. 1A). Detection by dot blotting using antibodies showed that T27 monoclonal antibody bound specifically to Cry j 2 in comparison with the polyclonal antibody (Fig. 1B). The specificity of the aptamers, especially in group II, appeared to be higher than that of the anti-Cry j 2 polyclonal antibody and similar to that of the T27 monoclonal antibody (Figs. 1 and S1B and C).

**Table 1**

Oligonucleotide sequences identified from the 24-nt randomized region of the library through four rounds of SELEX against Cry j 2.

| Clone  | Sequence (5' $\rightarrow$ 3') | Length |
|--------|--------------------------------|--------|
| CJ2-01 | TGGGCTGTCTCGGGGGGGTGGGTTTC     | 24 mer |
| CJ2-02 | GGCAGGGAAGGGGTGTGGCCAGA        | 24 mer |
| CJ2-03 | GGGTGGTGGCCGGGAGGGGGGGG        | 24 mer |
| CJ2-04 | GGCGAGGGAAGGGGGGCGGACGA        | 24 mer |
| CJ2-05 | GGCAGGGAAGGGGGGCGGCCGA         | 24 mer |
| CJ2-06 | GGTGCAGGGAAGGGGGGGGCCAG        | 24 mer |
| CJ2-07 | GGGGTGGGTCGTCTGGGGGGGT         | 24 mer |
| CJ2-08 | GGTAGGGAAGGGGTGTGGCCTGG        | 24 mer |

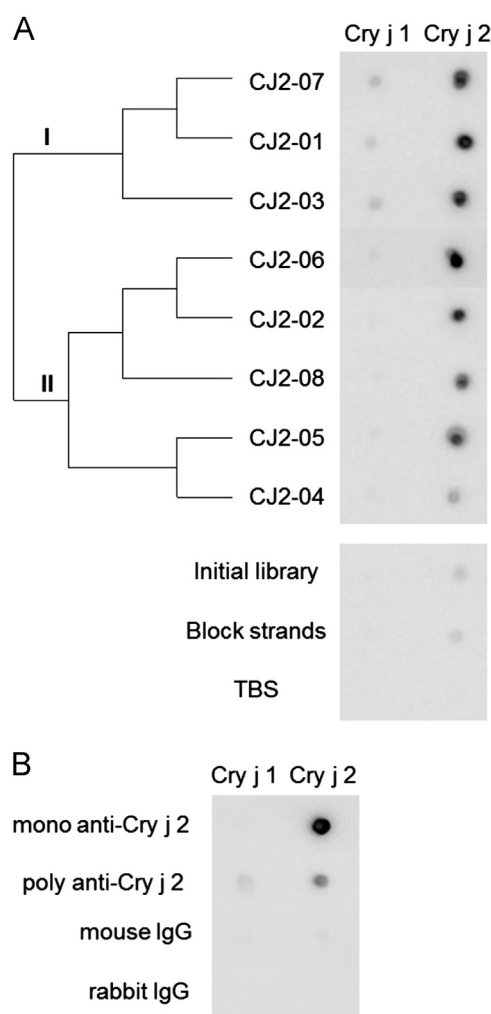


**Fig. 1.** Binding ability of aptamers compared to anti-Cry j 2 antibodies. (A) Aptamers were classified by phylogenetic analysis based on oligonucleotide sequences and their binding ability was assessed by aptamer blotting assay. (B) Binding ability of antibodies to Cry j 2 was evaluated by dot blotting assay.

The aptamers obtained here were predicted to form a G-quadruplex structure using QGRS mapper (Kikin et al., 2006) (Table S1). CD spectra were measured to confirm the G-quadruplex structure of the folded aptamers (Fig. 2), given that guanine-rich sequences tend to form G-quadruplexes showing characteristic CD spectroscopic features indicating their structures (Balagurumoorthy et al., 1992). The aptamers can be divided into four patterns based on the CD spectra.

- i) Group I aptamers showed a positive peak at 260 nm and a negative peak at 240 nm, characteristic of parallel-type G-quadruplexes (Fig. 2A).
- ii) In group II, CJ2-02, -05, and -08 showed a positive peak at 295 nm and a negative peak at 260 nm, characteristic of anti-parallel-type G-quadruplexes (Fig. 2B).
- iii) CJ2-06 showed positive peaks at 260 nm and 295 nm and a negative peak at 240 nm, possibly indicating a hybrid structure or a mixture of parallel and anti-parallel G-quadruplexes (Fig. 2C).
- iv) CJ2-04 showed a positive peak at 295 nm and negative peak at 240 nm, which were different patterns from CJ2-05 in the same clade (Fig. 2D), suggesting that CJ2-04 and -05 may fold into different types of anti-parallel G-quadruplexes or CJ2-04 may fold non-G-quadruplex structures.

These results indicate that Cry j 2 tends to recognize G-quadruplexes.

### 3.2. Histochemical staining of ruptured Japanese cedar pollen

To assess whether the aptamers obtained by SELEX recognize native Cry j 2 in Japanese cedar pollen, histochemical staining was performed using the indirectly labeled aptamers via biotin. Because a biotinylated Rev primer used in SELEX generated a higher background in ruptured Japanese cedar pollen (Fig. S2), the aptamers were labeled with BioT<sub>24</sub> through A<sub>24</sub> (CJ2-X<sub>A24</sub>). The pollen grains were ruptured in advance and fixed on a glass slide, given that Cry j 2 has been reported to localize in starch granules of pollen cytosol (Nakamura et al., 2004; Nakamura, 2007). When pollen was ruptured, isolation of protoplasts with intine 3 from exine with intines 1 and 2 was observed (Nakamura, 2007). The ruptured pollen were initially probed with the biotin-labeled aptamers and then incubated with NeutrAvidin-HRP and a DAB substrate for visualization. Under this condition, all eight aptamers generated staining in expanded intine 3 of the ruptured pollen (Fig. S3). CJ2-01, -03, and -06 showed high stainability in the ruptured pollen (Figs. 3 and S3), in agreement with the results of the aptamer blotting assay against Cry j 2 (Fig. 1A).

To compare staining patterns with those of anti-Cry j 2 antibodies, immunostaining of the ruptured pollen was conducted. The ruptured pollen was initially probed with anti-Cry j 2 antibodies, and Cry j 2 in the pollen was visualized by a reaction using HRP-conjugated secondary antibody and a DAB substrate. Anti-Cry j 2 antibodies (monoclonal and polyclonal antibodies) generated staining in expanded intine 3 of the pollen, similar to the results using the aptamers against Cry j 2 (Figs. 3 and S3).

In addition, staining of starch in the pollen was performed using KI-I<sub>2</sub> solution to localize starch (Fujishita, 2002). The staining of starch in the ruptured pollen was observed inside intine 3, indicating that the staining generated by anti-Cry j 2 aptamers and antibodies was similar to that of starch in the ruptured pollen (Figs. 3 and S4B). These results suggest that the aptamers could recognize Cry j 2 in the ruptured pollen. The sensing method for Cry j 2 using the ruptured pollen described here is easy because it does not require extraction of Cry j 2 from the pollen. As a sample preparation, rupturing of the pollen on a glass slide requires only a drop of TBS buffer and drying at RT for approximately 30 min. This method enables us to detect Cry j 2 in Japanese cedar pollen more rapidly than with ELISA, conventionally used in forestry to select plus trees with the pollen containing reduced amounts of Cry j 2.

### 3.3. Binding ability and specificity of CJ2-06 to Cry j 2

In view of its high and specific binding to Cry j 2 (Figs. 1 and 3), CJ2-06 was further investigated. In place of the indirectly labeled CJ2-06, CJ2-06 directly labeled with biotin at the 5' end (BioCJ2-06) was used for detection. BioCJ2-06 recognized Cry j 2 in the aptamer blotting assay (Fig. S5A) and generated staining in the ruptured cedar pollen (Fig. S5B). To evaluate the affinity of CJ2-06 for Cry j 2, ELONA was performed, in which BioCJ2-06 was added to Cry j 2 immobilized in each well on a 96-well plate. BioT<sub>24</sub> was used as a negative control. To detect the BioCJ2-06–Cry j 2 complex, NeutrAvidin-HRP and a chemiluminescence substrate were added to each well, and chemiluminescence signals were measured. BioCJ2-06 showed a dose-dependent increase of chemiluminescence signals and saturation ( $p < 0.05$ ), whereas the assay with BioT<sub>24</sub> showed no significant change in signal ( $p > 0.05$ ) (Fig. 4). Scatchard analysis based on ELONA showed that BioCJ2-06 exhibited a high affinity for Cry j 2 with a dissociation constant of 24 nM (Fig. S6). The affinity appears to be equivalent to that of typical antibodies.

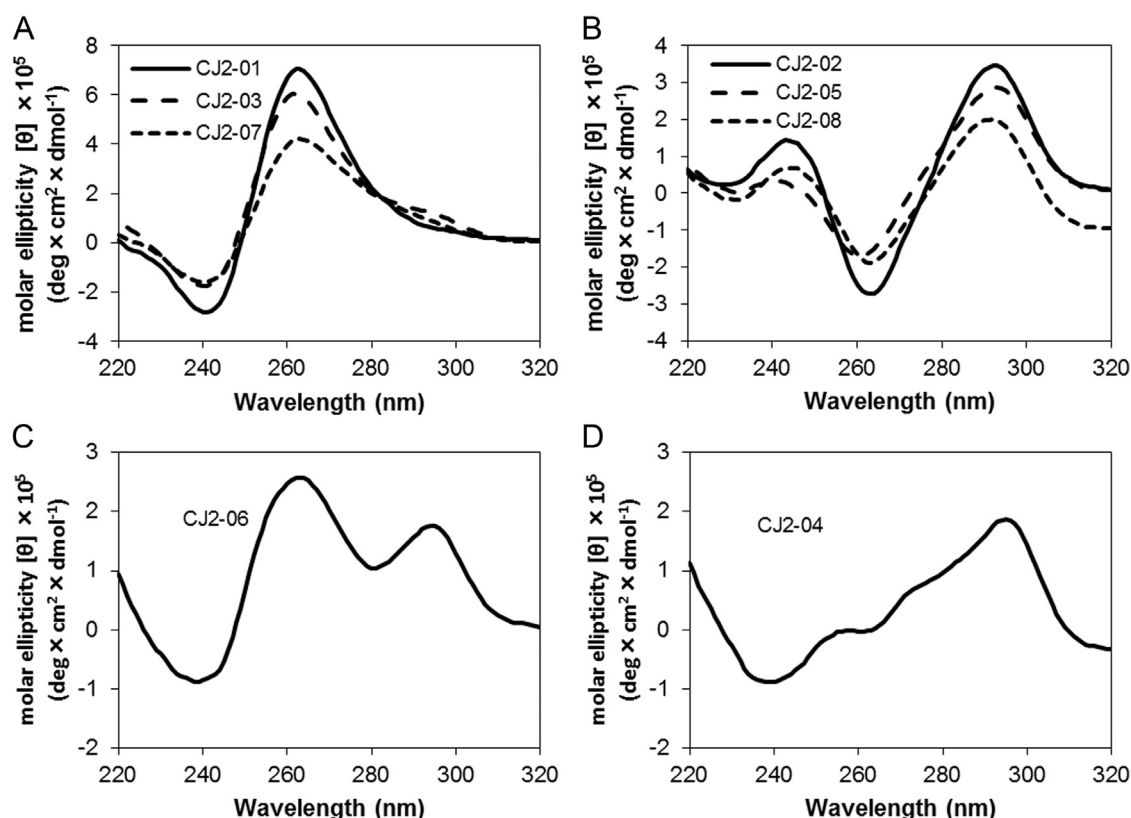


Fig. 2. CD spectra of aptamers against Cry j 2. (A) CJ2-01, -03, and -07 in group I. (B) CJ2-02, -05, and -08 in group II. (C) CJ2-06 in group II. (D) CJ2-04 in group II.

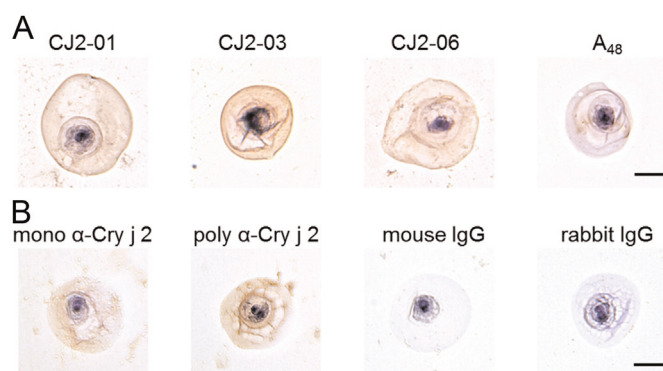


Fig. 3. Histochemical staining of ruptured Japanese cedar pollen. (A) Aptamers (CJ2-X<sub>A24</sub>) were used as a molecular recognition element of Cry j 2. (B) Anti-Cry j 2 antibodies were used for immunostaining to detect Cry j 2 in Japanese cedar pollen. Scale bar, 20  $\mu$ m.

### 3.4. Detection of Cry j 2 contained in house dust

In an aptamer blotting assay using BioCJ2-06, the signal dose-dependently increased against Cry j 2. We clearly observed a spot where 0.7 pmol of Cry j 2 was immobilized on a nitrocellulose membrane, which is equivalent to 25 ng of Cry j 2 (Fig. 5A). This is equivalent to the amount of Cry j 2 included in several tens of micrograms of pollen, as estimated from a previous report (Goto-Fukuda et al., 2007). The amount of Japanese cedar pollen typically carried indoors has been estimated as 9.6 counts/cm<sup>2</sup>/day on floors (Takahashi et al., 2008). Considering that the weight of a pollen grain is nearly 10 ng (Saito and Teranishi, 2002), this estimate suggests that in several days pollen would reach an amount measurable by the aptamer blotting assay in a floor area

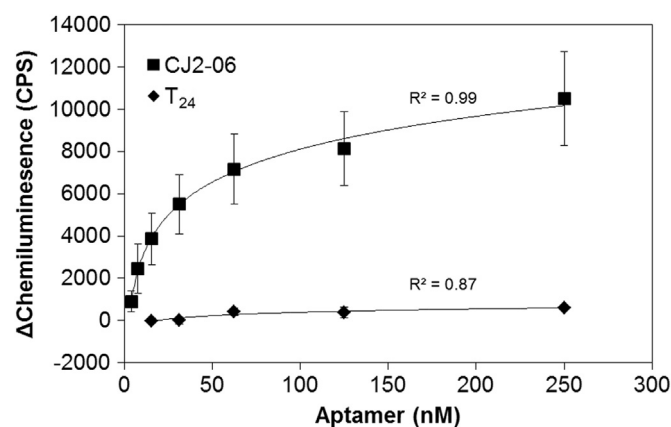


Fig. 4. Binding saturation curves of CJ2-06 to Cry j 2. ELONA was performed using BioCJ2-06 (0–250 nM) against Cry j 2 immobilized on a microtiter plate. The values are presented as mean  $\pm$  SE.

of < 10 cm  $\times$  10 cm. In addition, pollen was attached to textile products outdoors at 9–19 counts/cm<sup>2</sup>/h (Takahashi et al., 2008) and could be brought indoors. Sawatani et al. (1994) reported that Cry j 2 ELISA showed sensitivity at 0.6 ng/mL using 100  $\mu$ L of Cry j 2 solution. Commercially available Cry j 2 ELISA kit (TAC<sup>®</sup> Cry j 2 ELISA Kit, Shibayagi, Gunma, Japan) shows sensitivity at 0.39 ng/mL using 100  $\mu$ L of the solution. With respect to sample preparation, the same sample as that for ELISA would be applicable to aptamer blotting assay if a dot blotter was used for immobilization of the sample on a nitrocellulose membrane. In a spike test using house dust (Fig. S7A), we obtained signals dependent on the concentration of added Cry j 2 in an aptamer blotting assay with BioCJ2-06 (Fig. 5B). In addition, the recovered Cry j 2 was detected by an immunoblotting and an aptamer

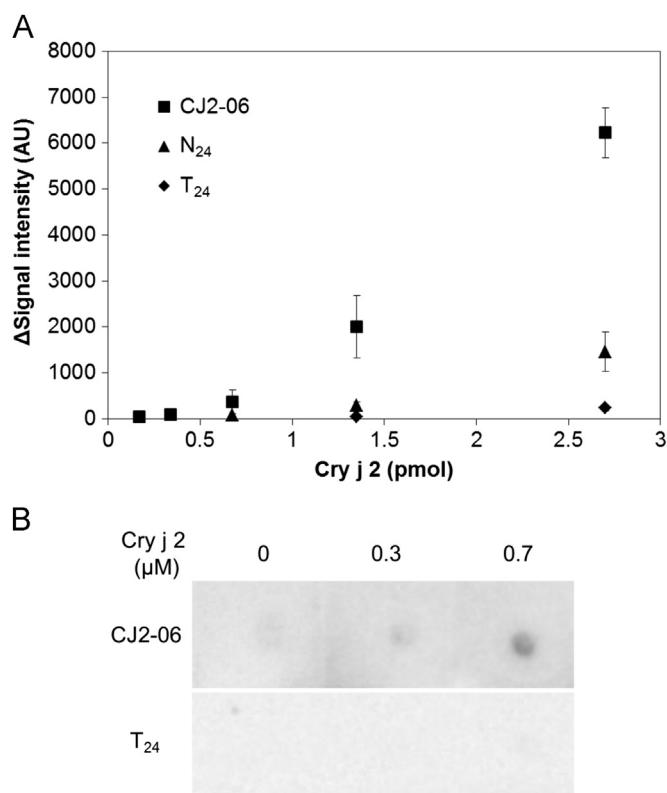


**Fig. 5.** Detection of Cry j 2 by aptamer blotting assay. (A) Each amount of Cry j 2 spotted on a nitrocellulose membrane was detected using BioCJ2-06. Spot intensity was assessed using ImageJ software and plotted against the amount of Cry j 2 ( $n=5$ ). The values presented are mean  $\pm$  SD. (B) Extract from house dust including Cry j 2 was spotted on a nitrocellulose membrane at 2  $\mu$ L and signal was detected using BioCJ2-06.

blotting assay with the recovered quantity slightly lower than that of Cry j 2 prior to addition to house dust (Fig. S7B and C). We infer that BioCJ2-06 has sufficient binding ability to detect Cry j 2 contained in house dust, although the conditions of extraction need improvement.

At present, people have no measures except for wearing eyeglasses and facemask to avoid exposure to allergens or removing the allergens randomly by cleaning the living environment. Given that large-scale production of aptamers is more time- and cost-effective than that of antibodies (Kedzierski et al., 2013), they could be applied as new measures against allergic disorders caused by Japanese cedar pollen. The chemically synthesized aptamers have potential applications, such as in immunochromatography, for detection of Cry j 2 to bring about the efficient avoidance and elimination of antigens. Anti-Cry j 2 aptamers reported in this study are allergen recognition elements that can be used daily for detection.

#### 4. Conclusions

In this study, we developed aptamers against Cry j 2, which is one of the major allergens of Japanese cedar pollen. This is the first report of anti-Cry j 2 DNA aptamers or of a sensing method for Cry j 2 in the ruptured Japanese cedar pollen using histochemical staining. Anti-Cry j 2 aptamers selected by SELEX are considered to form G-quadruplex structures. In histochemical staining of the ruptured pollen, these aptamers generated staining comparable to that of anti-Cry j 2 antibodies. Detection of Cry j 2 in the ruptured pollen by sample preparation is more rapid than by conventional methods such as ELISA. Staining using the ruptured pollen is thus

convenient for the selection of Japanese cedar with low Cry j 2 pollen in forest tree breeding. The aptamer CJ2-06 displayed a dissociation constant equivalent to that of typical antibodies. In an aptamer blotting assay, CJ2-06 detected an amount of Cry j 2 corresponding approximately to several tens of micrograms of pollen grains. Furthermore, a dose-dependent signal was detected using purified Cry j 2 and a mixture of Cry j 2 and house dust. These results indicate that CJ2-06 would be applicable to the detection of Cry j 2 indoors during the pollen dispersal season. This work facilitates the detection of Cry j 2 and via daily detection of Cry j 2, will lead to new preventive measures against allergic disorders caused by Japanese cedar pollen.

#### Acknowledgments

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#### Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bios.2014.07.030>.

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## Supporting Information

### **DNA aptamers against the Cry j 2 allergen of Japanese cedar pollen for biosensing applications**

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Table S1. Putative quadruplex-forming G-rich sequences in aptamers

| Clone  | Position | Length | QGRS                                           | G-Score |
|--------|----------|--------|------------------------------------------------|---------|
| CJ2-01 | 2        | 19     | <u>GGG</u> CTGTC <u>GGGGGGGGTGGG</u> *         | 38      |
| CJ2-02 | 1        | 19     | <u>GG</u> CAG <u>GG</u> AAAG <u>GGGTGTGG</u>   | 20      |
| CJ2-03 | 1        | 24     | <u>GGGTGGTGGCCGGGGAGGGGGGGG</u>                | 36      |
| CJ2-04 | 1        | 20     | <u>GG</u> CGAG <u>GG</u> AAAG <u>GGGGGGCGG</u> | 21      |
| CJ2-05 | 1        | 20     | <u>GG</u> CGAG <u>GG</u> AAAG <u>GGGGGGCGG</u> | 21      |
| CJ2-06 | 1        | 20     | <u>GGTGCAGGG</u> AAAG <u>GGGGGGGG</u>          | 21      |
| CJ2-07 | 2        | 22     | <u>GGGTGGGT</u> CGTGTCT <u>GGGGGGG</u>         | 35      |
| CJ2-08 | 1        | 19     | <u>GGTAGGG</u> AAAG <u>GGGTGTGG</u>            | 20      |

\* Underlined guanines were predicted to form G-quartet by QGRS mapper.

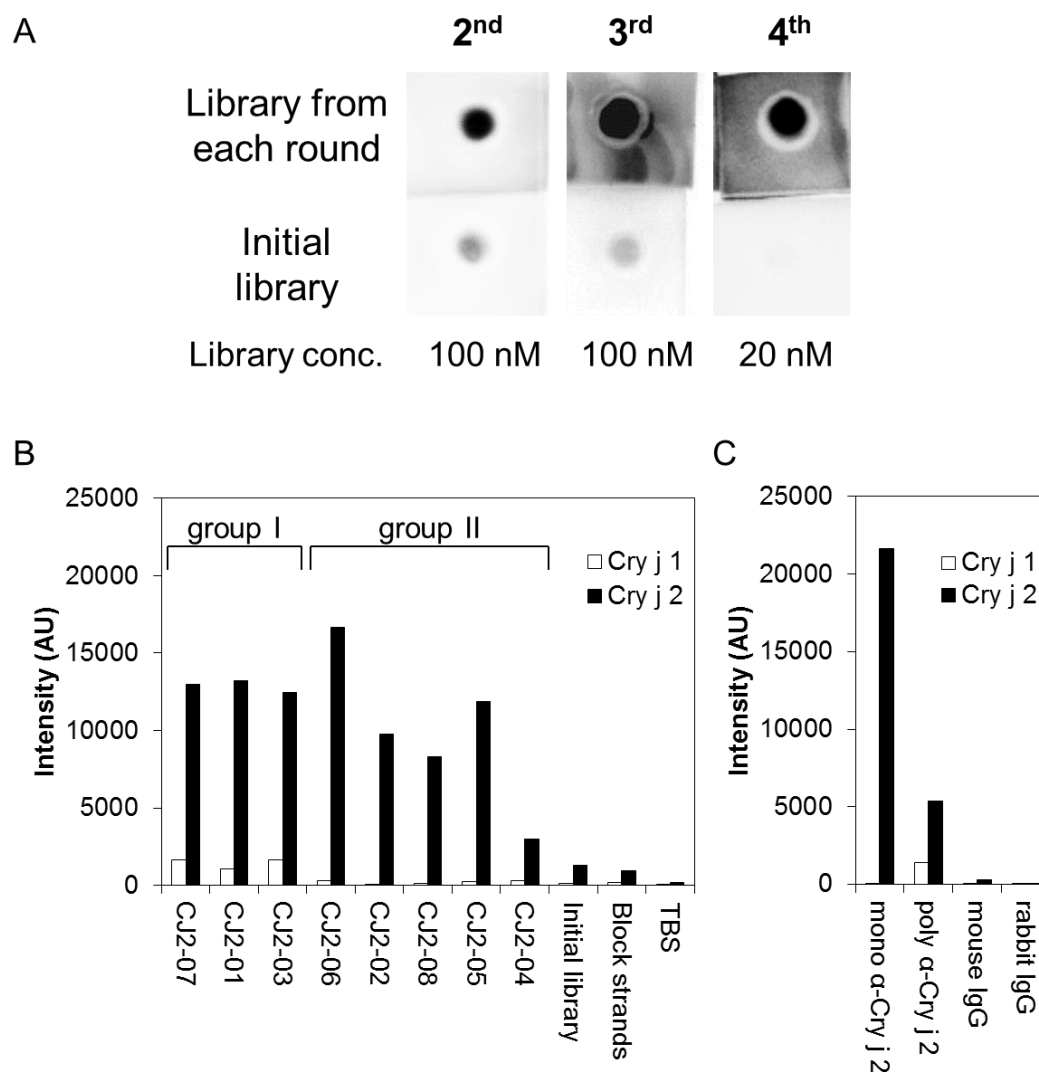


**Fig. S1. Dot blotting assay for Cry j 2 on nitrocellulose membrane.** (A) Cry j 2 (27 pmol) was spotted in the center of a membrane and detected using the biotin-labeled library of each round. (B) The aptamer blotting assay shown in Fig. 1A and (C) the dot blotting assay using anti-Cry j 2 antibody shown in Fig. 1B were quantified using ImageJ software.

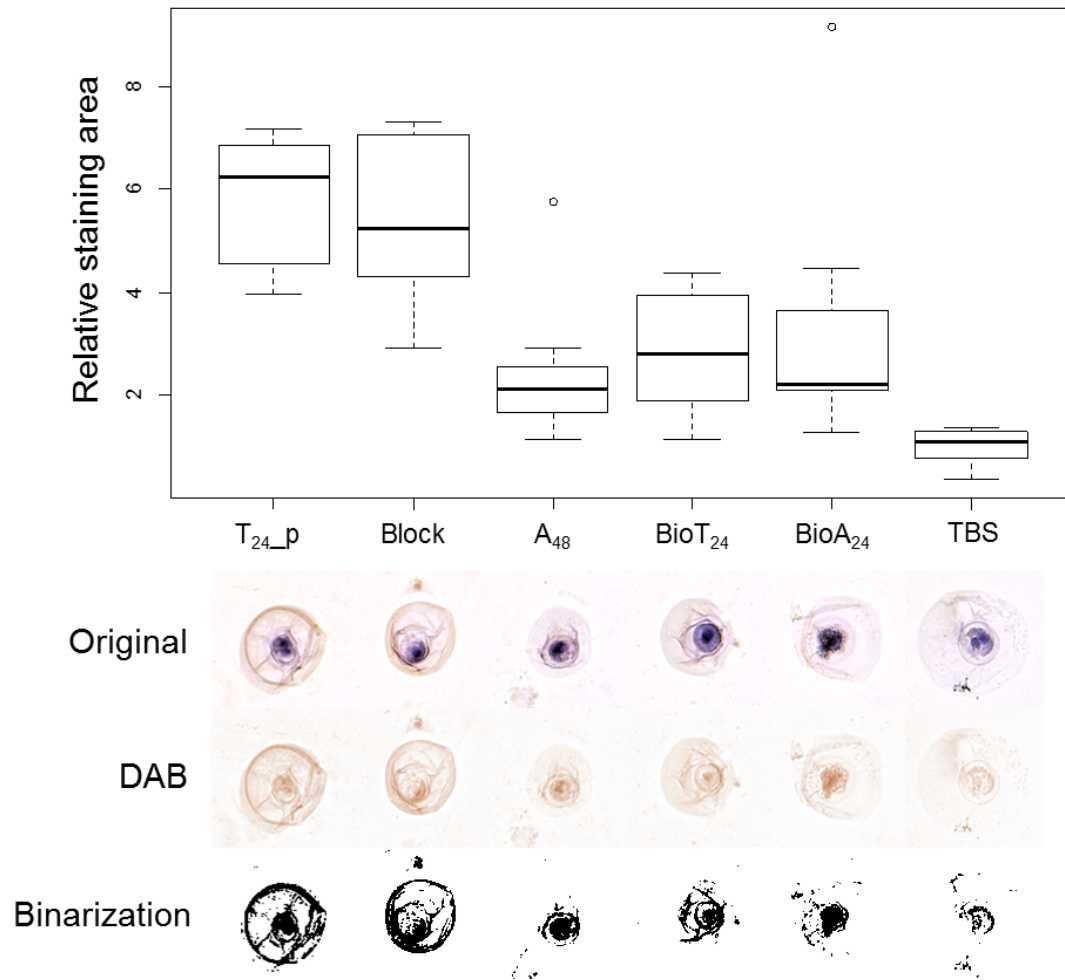


**Fig. S2. Nonspecific staining caused by differences between methods of labeling with biotin.** Oligonucleotides were indirectly or directly labeled with biotin. Stainability was evaluated by comparison of the relative staining area in ruptured cedar pollen normalized with the average of TBS without oligonucleotides. The DAB staining area was separated using the color deconvolution option of the ImageJ software. The area of staining was binarized before measuring and the values are shown in a boxplot ( $n = 8$ ). The boxes show the median and the upper and lower quartiles of each distribution. Box whiskers show the maximum and minimum range excluding outliers

(more than 1.5 interquartile ranges below the first quartile or above the third quartile).

Outliers are shown as circles. T<sub>24\_p</sub>: T<sub>24</sub> labeled on the complementary strand with a biotinylated Rev primer and blocked with anti-Fw primer; Block: a biotinylated Rev primer and complementary strand of Fw primer; A<sub>48</sub>: A<sub>48</sub> labeled with biotinylated T<sub>24</sub>; BioT<sub>24</sub>: biotinylated T<sub>24</sub>; BioA<sub>24</sub>: biotinylated A<sub>24</sub>; TBS: TBS buffer.

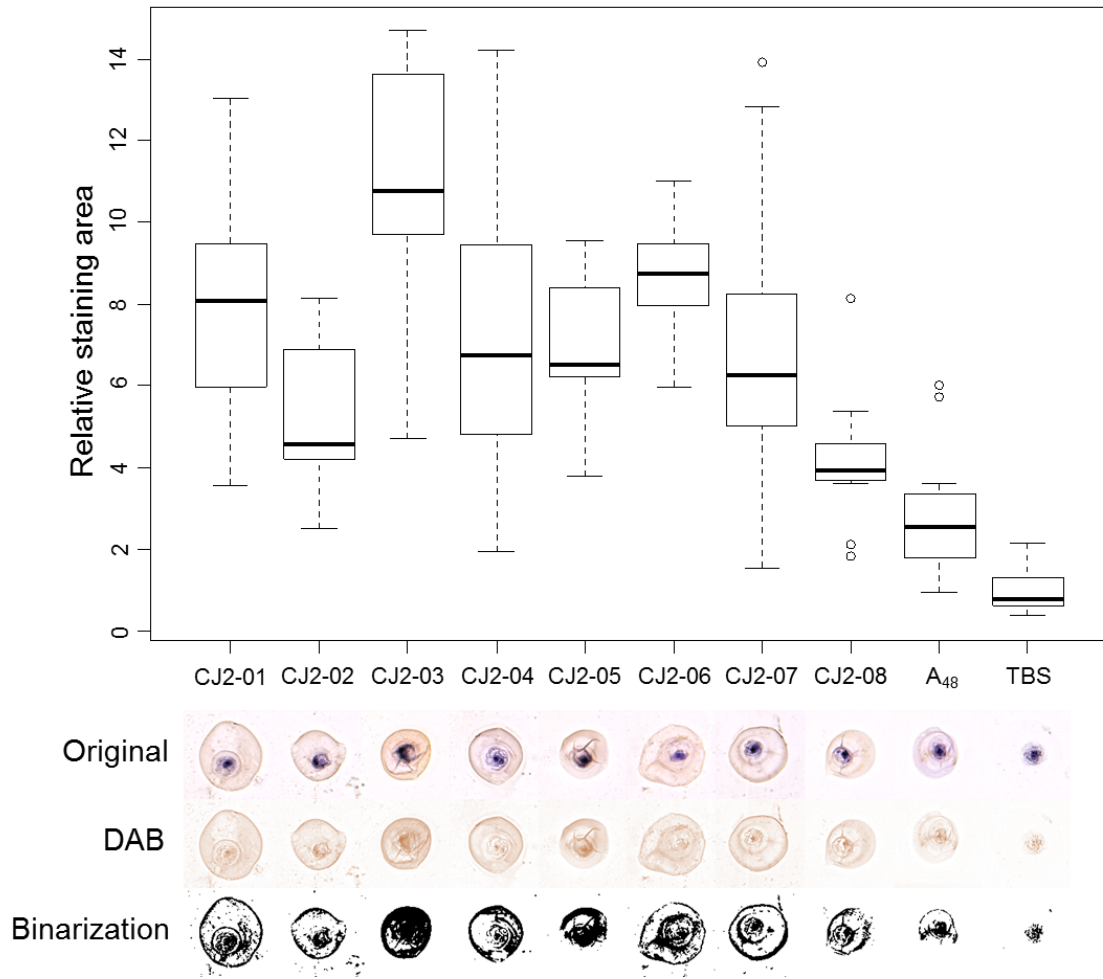


**Fig. S3. Stainability of aptamers against Cry j 2 of the ruptured pollen.** Each aptamer was indirectly labeled with biotinylated T<sub>24</sub> through A<sub>24</sub> (CJ2-X\_A<sub>24</sub>). Stainability was evaluated by comparison of the relative staining area in the ruptured cedar pollen normalized to the average of TBS. The DAB staining area was separated using the color deconvolution by the ImageJ software. The area of staining was binarized before measuring, and the values were shown in boxplot (n = 13). The boxes show the median and the upper and lower quartiles of each distribution. Box whiskers exhibit the maximum and minimum range excluding outliers (more than 1.5

interquartile ranges below the first quartile or above the third quartile). Outliers are shown as circles. The original images of CJ2-01, -03, and -06 and A<sub>48</sub> are shown in Fig. 3A.

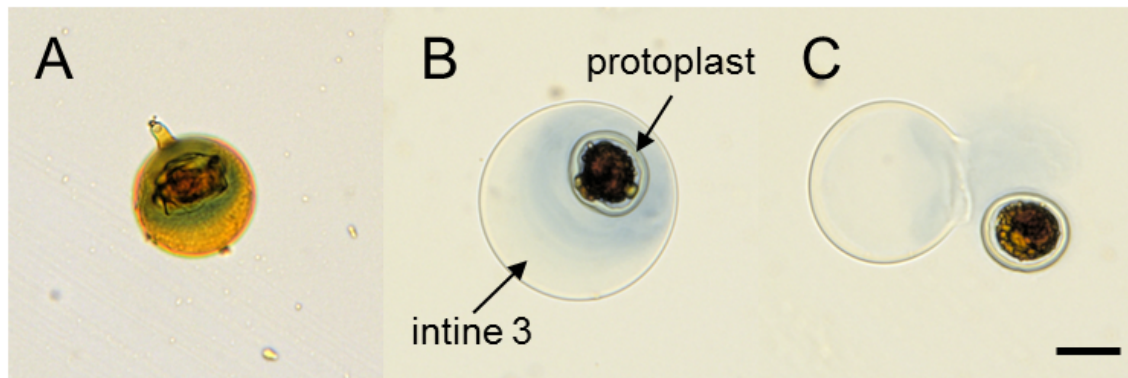


**Fig. S4. Localization of starch in Japanese cedar pollen.** The ruptured Japanese cedar pollen grains were prepared on glass slides as described. After fixation in the same manner as the staining with aptamers, the pollen was incubated with KI-I<sub>2</sub> solution (1 g of I<sub>2</sub> dissolved in 100 mL of 2% KI solution) and covered with a cover slip. Staining was observed under a microscope VHX-1000 (Keyence, Osaka, Japan). Scale bar, 20  $\mu$ m. (A) In pollen grains, starch was observed in the cytosol. (B) The staining of starch in the ruptured pollen was observed inside intine 3, similar to the staining using anti-Cry j 2 aptamers and antibodies. In addition, (C) starch was observed to flow out when the protoplast was released from intine 3. These results suggest that Cry j 2 diffuses from the protoplast to the expanded intine 3 together with starch when the pollen is ruptured and may be released outside with starch efflux.

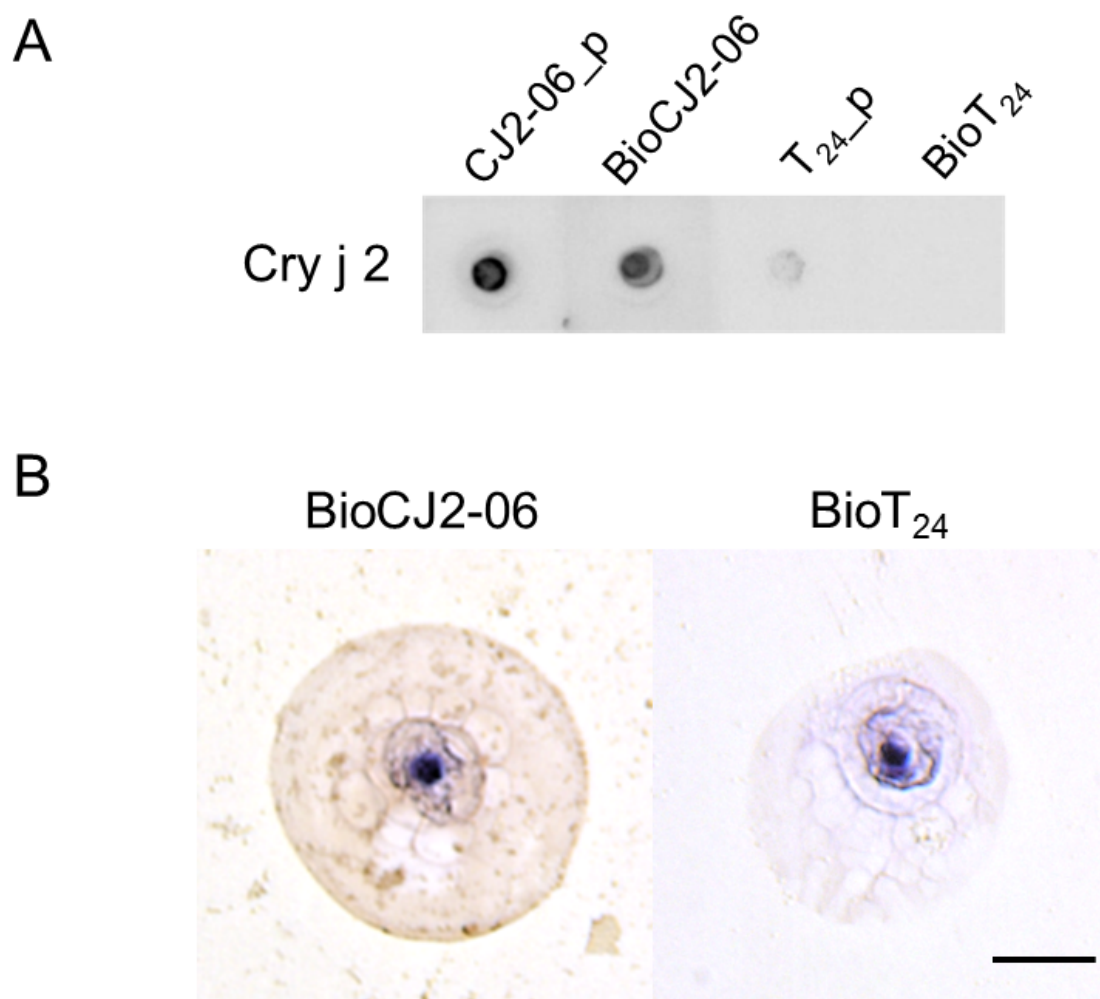


**Fig. S5. Characteristics of directly labeled aptamer with biotin.** (A) Aptamer blotting using CJ2-06\_p or BioCJ2-06 was performed against Cry j 2 spotted at 2.7 pmol on a nitrocellulose membrane. (B) Histochemical staining was performed using BioCJ2-06. Scale bar, 20  $\mu$ m.

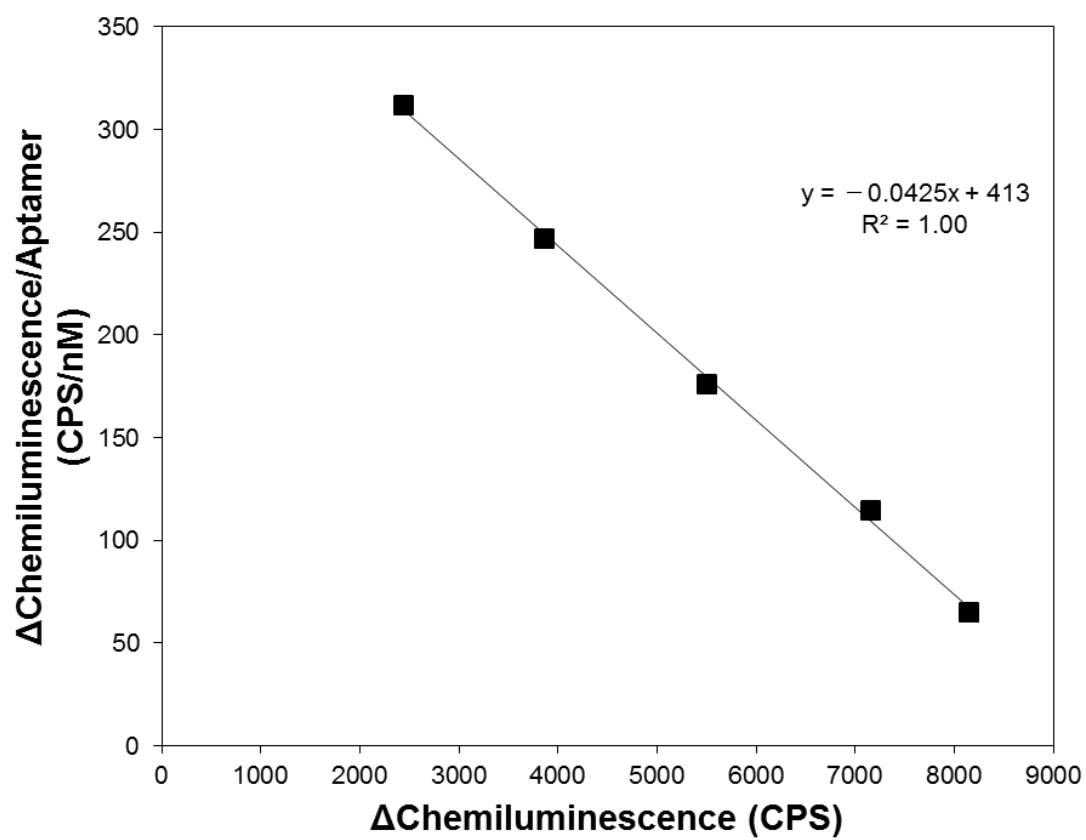


**Fig. S6. Scatchard analysis based on ELONA.** The  $K_d$  value was estimated by the Scatchard plot analysis following a previous report (Tsukakoshi et al., 2012).

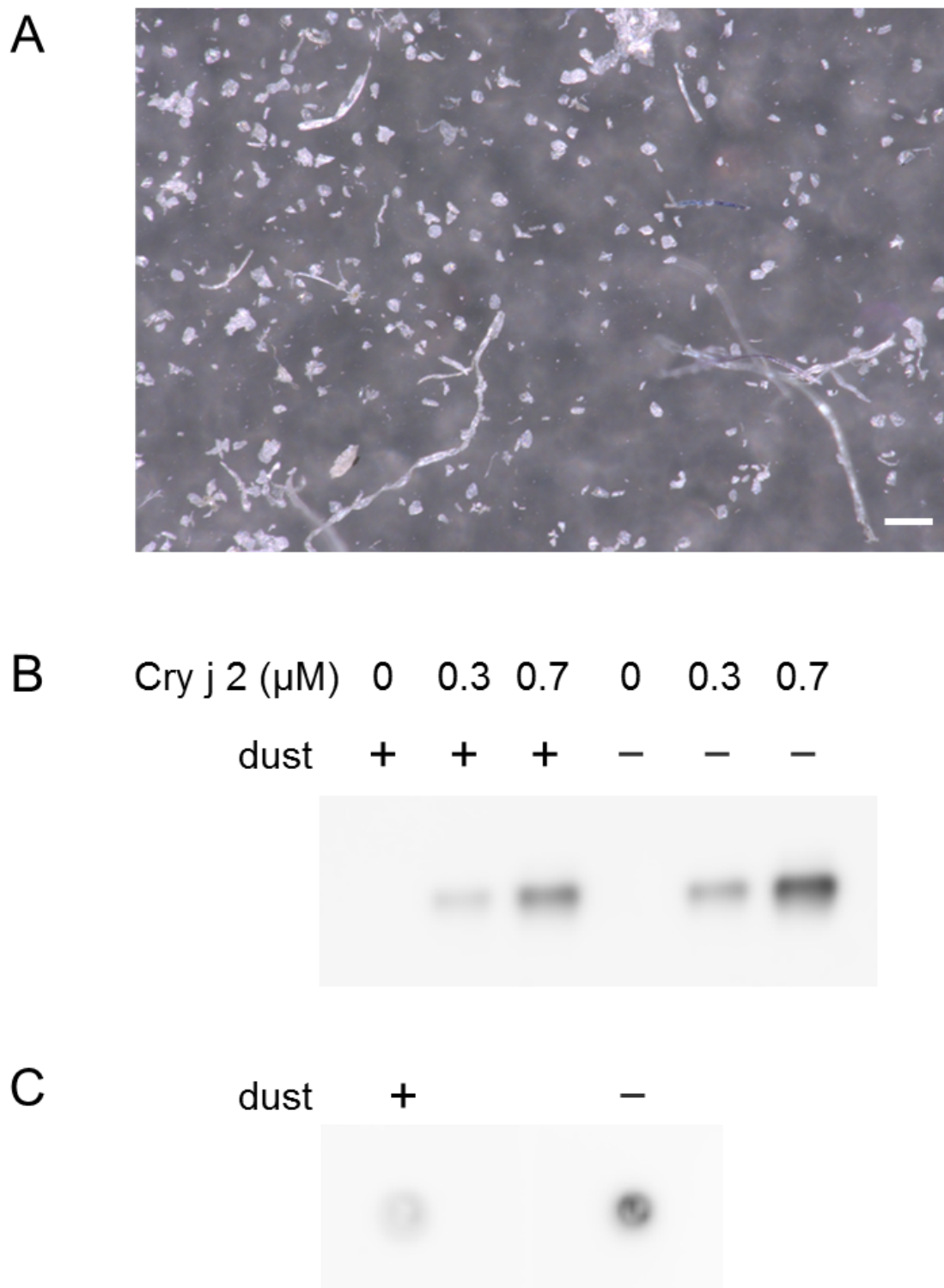


**Fig. S7. Recovery of Cry j 2 contained in house dust by a spike test.** (A) House dust was collected from an air conditioner filter. Scale bar, 100  $\mu$ m. Recovery of Cry j 2 in a

spike test was evaluated by (B) an immunoblotting assay and (C) an aptamer blotting assay. (B) The immunoblotting assay was performed using anti-Cry j 2 antibody (T27) to detect Cry j 2 added to house dust. Extracts of house dust containing Cry j 2 (2  $\mu$ L) was dissolved in SDS-sample buffer, separated by SDS-PAGE using Any kD Mini-PROTEAN TGX Gel (Bio-Rad, CA, USA), and transferred to a polyvinylidene difluoride membrane (Bio-Rad, CA, USA). The membrane was blocked with 2% (w/v) bovine serum albumin in TBST and incubated with anti-Cry j 2 monoclonal antibody T27 (250 ng/mL) as an initial antibody at RT for 1 h. After washing in TBST, the membrane was incubated with HRP-conjugated anti-mouse Igs (Dako A/S, Glostrup, Denmark) (1:1000) as a secondary antibody at RT for 1 h. Detection was performed using ECL (GE Healthcare, Chalfont St Giles, UK). Initial and secondary antibodies were diluted in Can Get Signal (TOYOBO, Osaka, Japan). (C) The aptamer blotting assay was performed to detect Cry j 2 added to house dust. Two micro-litter aliquots of the extract from house dust including 0.7  $\mu$ M Cry j 2 and the Cry j 2 solution before added to the house dust were spotted on a nitrocellulose membrane, and signal was detected using BioCJ2-06 in the same manner as described in 2.3 (Aptamer blotting assay).

**Reference**

Tsukakoshi, K., Abe, K., Sode, K., Ikebukuro, K., 2012. *Anal. Chem.* 84, 5542–5547.