

Possible surface carbohydrates involved in signaling during conjugation process in *Zygnema cruciatum* monitored with fluorescein isothiocyanate-lectins (Zygnemataceae, Chlorophyta)

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SUMMARY

The changes of cell surface carbohydrates were examined with FITC (fluorescein isothiocyanate)-labeled lectins during the conjugation process of the green alga *Zygnema cruciatum*. The *Ulex europaeus* agglutinin (UEA)-specific materials were detected consistently on the surface of vegetative cells, but were absent on the surface of protruding papillae or conjugation tube. The tips of male and female papillae were labeled with soybean agglutinin (SBA) and peanut agglutinin (PNA) during conjugation. The SBA- and PNA-specific materials appeared first at the tip of male papillae and began to accumulate on the surface of female papillae. No labeling of these lectins was detected on the surface of vegetative filaments throughout the conjugation process. FITC-ConA (Concanavalin A) and FITC-RCA (*Ricinus communis* agglutinin) did not label the vegetative filaments of *Z. cruciatum*, but a trace labeling of these lectins was observed on the surface of some swollen papillae occasionally. Blocking experiments with various lectins showed that these SBA- and PNA-specific glycoconjugates might be involved in the signaling between male and female papillae.

Key words: cell–cell recognition, conjugation, fluorescein isothiocyanate-lectin, secretion of glycoconjugates, *Zygnema*.

INTRODUCTION

In zygnematacean algae, sexual reproduction is achieved by direct conjugation between filaments. Two compatible filaments aligned parallel to one another make numerous papillae, which connect opposite cells of two filaments and serve as a passage for a male protoplasm to move into the female cell (van den Hoek *et al.* 1995; Sze 1998; Kim & Kim 2002). Although complete conjugation involves a complex series of events, it always begins with the contact between two

papillae protruding from each opposite cell. Therefore, recognition factors are expected along their surfaces.

Considering that the conjugation process of *Spirogyra* and *Zygnema* has been introduced even in high school textbooks in many countries, it is surprising that still little is known about its chemical process (Kim *et al.* 2005). There are a few studies on the environmental factors inducing conjugation in the zygnematacean species (Grote 1977; Simons *et al.* 1984; Stabenau & Saeftel 1989; Kato & Ooshima 1997), but the key factors, which trigger the conjugation in these species, are still unknown.

Cell surface carbohydrates are highly suitable for encoding biological information because of their complexity and structural diversity, and hence they have been reported as primary markers for cell–cell recognition events in many organisms (Sharon & Lis 1993). Such recognition systems depend on the complementary binding between carbohydrate moieties of a glycoconjugate on one cell with a lectin protein on another cell (Sharon & Lis 1989; Chrispeels & Raikhel 1991; Ridge *et al.* 1998; Kim & Kim 1999; Kim *et al.* 2006). Because of their sugar-binding properties, fluorescein-labeled lectins have been used as a powerful tool to analyze the characteristics and the distribution of cell surface carbohydrates in many organisms (e.g. Wassarman 1987; Sharon & Lis 1989; Karlsson 1991; Kim & Fritz 1993a,b; Kim *et al.* 1996; Kim & Kim 1999).

In an elaborate ultrastructural study, Cheli and De Vecchi (1989) showed that the extracellular mucilage of *Spirogyra* filaments disappeared during conjugation and some glycoproteinaceous materials were accumulated in the cell wall of the developing conjugation

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Table 1. Lectin-binding pattern of the vegetative and reproductive cells of *Zygnema cruciatum* during the conjugation

Lectin	Specificity	Vegetative filament	Papillae	Contact area between male and female papillae
<i>Ulex europaeus</i> agglutinin (UEA)	L-fucose	+	–	–
Soybean agglutinin (SBA)	N-acetyl-D-galactosamine	–	+	+
Peanut agglutinin (PNA)	D-galactose (1→3), N-acetyl-D-galactosamine	–	+	+
Concanavalin A (ConA)	D-mannose, D-glucose	–	*	–
<i>Ricinus communis</i> agglutinin (RCA)	D-galactose	–	*	–
<i>Dolichos biflorus</i> agglutinin (DBA)	N-acetyl-D-galactosamine	–	–	–
Wheat germ agglutinin (WGA)	N-acetyl-D-glucosamine, Neuramic acid	–	–	–

+, positive labeling; –, negative labeling; *occasional and weak labeling.

papilla, suggesting that the chemical composition in the cell surface changed during the process. Surface carbohydrates on some vegetative cells and differentiating rhizoid cells were observed in several species of *Zygnema* and *Spirogyra* by the use of fluorescein isothiocyanate (FITC)-lectins (Sengbusch *et al.* 1982; Inoue *et al.* 1999), but there is no analytical study on surface carbohydrates during conjugation in the zygnematacean species.

In this study, we examined the changes of cell surface carbohydrates during the conjugation process of *Zygnema cruciatum* (Vaucher) Agardh using various FITC-lectins. Results show that there are significant changes of surface carbohydrates during the conjugation process and some signaling glycoconjugates are secreted from the apical portion of the developing papilla.

MATERIALS AND METHODS

Plant material and laboratory culture

Algal materials were collected from ponds in Kongju, Korea, from February to March 2004. The plants were washed three times with BBM medium (Bolds basal medium; Bischoff & Bold 1963) and kept in the same medium at 20°C, 16:8 h light/dark (LD) cycle under more than 20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ provided by cool-white fluorescent lamps. The spores and fragments of sexual filaments were isolated from conjugating plants and kept separately. GeO_2 was added to the medium (final concentration 1 mg/L) for 2 weeks to eliminate diatoms. Three months later, the zygospores germinated and a unialgal culture was established in the same condition. The cultured materials were exposed to the various combinations of temperature (10, 15, 20, 25, 30°C), irradiance (10, 20, 50, 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$), and LD regime (10:14, 12:12, 14:10 h LD) to induce conjugation. Nitrogen- (N) depleted BBM medium was used in combination with the above environmental factors.

Application of fluorescein-labeled lectins

Fluorescein isothiocyanate-labeled lectins (Table 1; Fluorescent Lectin Kit-2100, Vector laboratories, Burlingame, CA, USA) were diluted in phosphate buffer of pH 7.4, consisting of KCl 0.2 g, Na_2HPO_4 1.44 g, KH_2PO_4 0.24 g, double-distilled water 1 litre. Lectins were added to plants in BBM medium to give a final concentration of approximately 10 $\mu\text{g/mL}$ and the container was rotated mildly during incubation for 1 h. Unbound lectin was removed by three washes in BBM medium for 5 min each, and materials were examined with a confocal laser scanning microscope (Fluoview, v. 2.0.28, Olympus). Fluorescence was detected with the BA510IF and BA510-540 filter set (Ex. $\lambda = 490 \text{ nm}$, Em. $\lambda = 525 \text{ nm}$). No auto-fluorescence of the material was observed in this setting. Sugar specificity of the lectin–ligand interactions was assayed as described by Kim and Fritz (1993c).

Blocking of conjugation with lectins

Each lectin (Lectin Kit-2000, Vector Laboratories, Burlingame) was dissolved in BBM medium of pH 7.4 to a final concentration of 25 $\mu\text{g/mL}$. About 20 fragments of the male and the female filaments were placed together in a glass tube containing 5 mL of each lectin solution and kept at the culture condition as described above. The number of conjugating filaments (those with conjugating tubes and zygospores) was counted after 3 days. The filaments with conjugating tubes and spores were considered as conjugating filaments. Each experiment was repeated 10 times and the percentage in control (without lectin), the means and standard deviations were calculated.

Microscopy

For time-lapse video-microscopy, the conjugating filaments were placed on a glass slide and a coverslip was lowered and sealed with VALAP (1:1:1; Vaseline: Lanolin: Paraffin) melted on a hot plate at 70°C. The

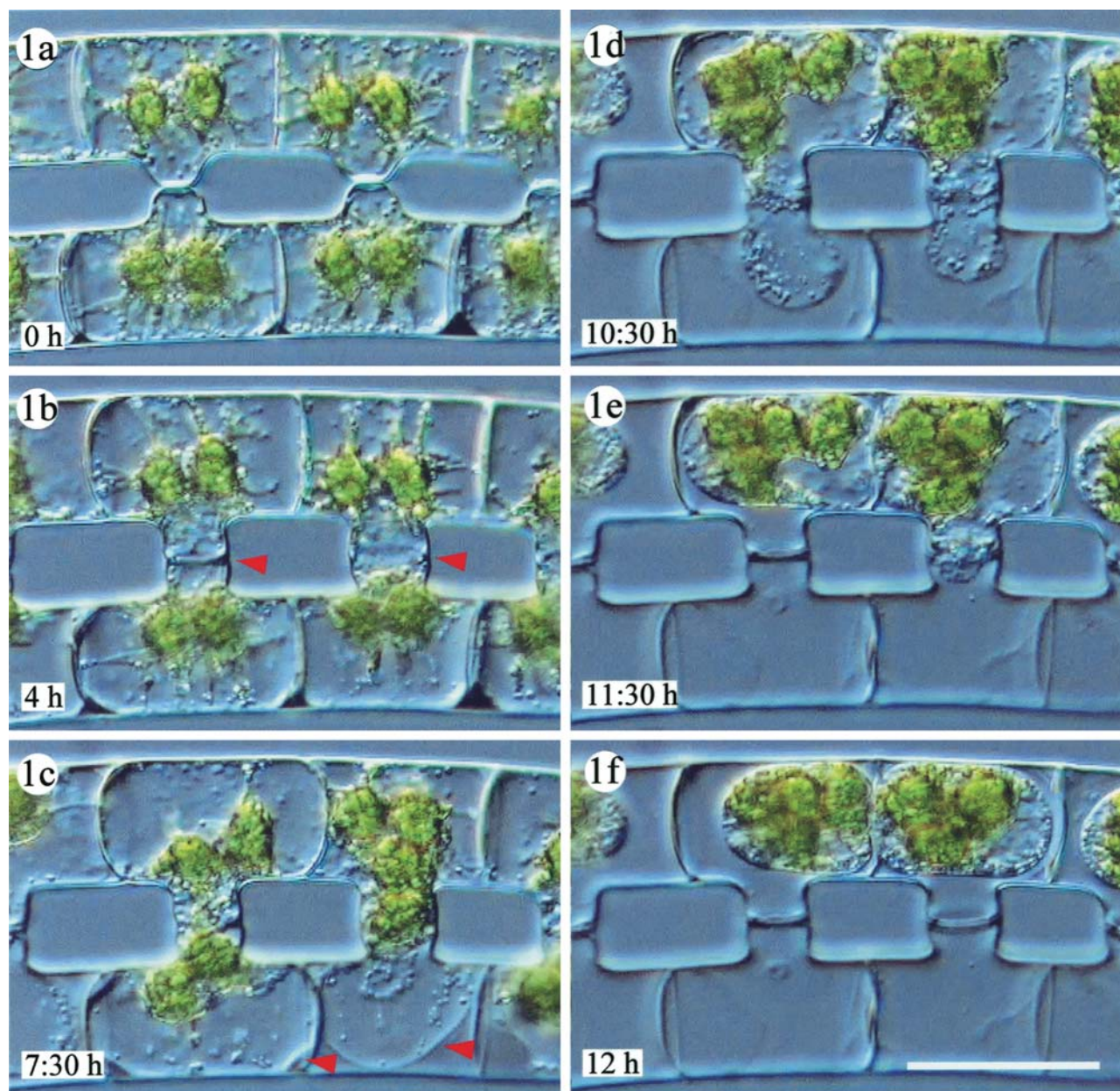


Fig. 1. The conjugation events leading up to zygote formation in *Zygnema cruciatum*. The times indicated on each figure represent the period, which had past since the beginning of filming. (a) Male and female papillae contacted each other. (b) The conjugation tube developed. Note that the contacting area is opening (arrowheads). (c–e) Movement of male protoplasm into the female filament (arrowheads). (f) Zygote formation. Scale bar, 50 μm .

slide preparations were examined on an Olympus BX-51 microscope under the oil immersion $\times 20$ objective lens and recorded on a Digital Imaging Time-Lapse Recorder (Seoulin Bioscience, Seoul, Korea).

RESULTS

Environmental factors for conjugation

Zygnema cruciatum bloomed in several ponds in Kongju area from February to March 2004. The water

temperature of the ponds ranged from 5 to 12°C and the light/dark regime was about 12:12 h LD. Some (<10%) of the field materials were already undergoing conjugation. Almost all plants began conjugation in 2 days when they were moved to the laboratory and kept in BBM medium at 20°C in 16:8 h LD cycle at more than 20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ provided by cool-white fluorescent lamps (Fig. 1).

Conjugation of *Z. cruciatum* started with protrusion of papilla on the male plants, followed by the development of female papilla on the opposite filament

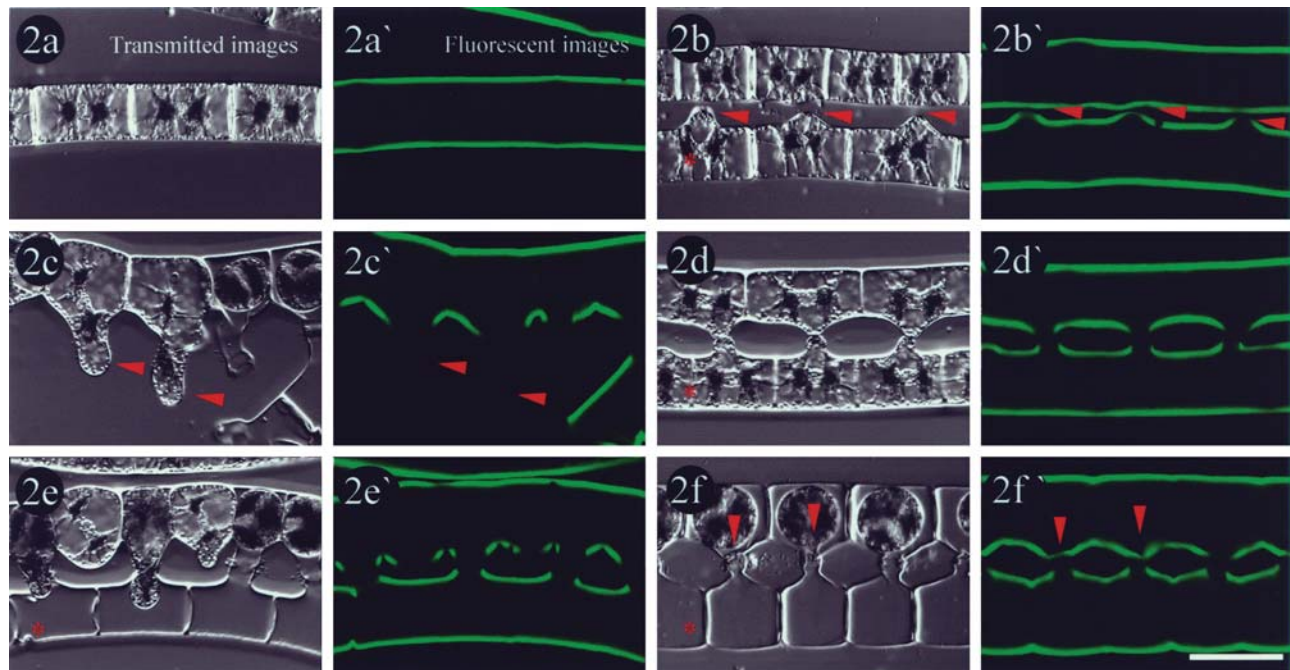


Fig. 2. *Ulex europaeus* agglutinin (UEA) labeling patterns during conjugation in *Zygnema cruciatum*. Differential interference micrographs of the filaments (a–f) and fluorescence micrographs of fluorescein isothiocyanate (FITC)-lectin labeling of the same filaments (a'–f'). (a–a') Vegetative filaments showing dense labeling of UEA on the surface were placed side by side. (b–b') Initial stage of conjugation. Arrowheads point to papilla protruding from the male filament. (c–c') Protruding papillae are not labeled (arrowheads). (d–d') No labeling appeared on the surface of conjugation tube during fusion stage. (e–e') Male protoplasm moved to the female filament. (f–f') Zygote formation. Arrowheads point to sealed labeling gap in the female filament bearing zygotes. Asterisks show male filaments. Scale bar, 50 μ m.

(Fig. 1a). Two papillae grew towards each other and fused to form a conjugation tube (Fig. 1b). The male protoplasm migrated into the female cell through the conjugation tube (Fig. 1c,d) and the zygote began to develop in the female plant (Fig. 1e,f). This phenomenon was observed more than 10 times in all the materials collected during February to March 2004.

The cultures from germinating zygospores grew well in the laboratory conditions, but conjugation was not induced when we repeated the same environmental conditions described above. The cultured plants were subjected to various environmental conditions, including N depletion, changing temperature, light and dark cycle, and light intensity, but conjugation was not induced in any of these conditions.

Lectin labeling during conjugation

Most of FITC-lectin labeling experiments were carried out with field material washed three times with BBM medium. The labeling pattern of seven FITC-lectins during conjugation of *Z. cruciatum* is listed in Table 1. Three lectins, peanut agglutinin (PNA), soybean agglutinin (SBA), and *Ulex europaeus* agglutinin (UEA), bound to either vegetative or reproductive cells

(Figs 2,3). Other lectins had no labeling during conjugation, except for some occasional and weak labeling of Concanavalin A (ConA) and *Ricinus communis* agglutinin (RCA) (Table 1).

The cell surface of vegetative plants was strongly labeled with FITC-UEA (Fig. 2a'). The labeling continued throughout the conjugation process except on the protruding papillae (Fig. 2b',c'). No UEA-labeling was observed on the developing papilla and the conjugation tube (Fig. 2c'–e'). When conjugation was completed, the labeling gap in the female filament bearing zygotes began to seal up again, but it remained open in the male plant (Fig. 2f', arrowheads).

No vegetative filament was labeled with FITC-SBA (Fig. 3a'). The labeling began to appear at the tips of male papillae first (Fig. 3b',c', arrowheads). The labeling on female papillae appeared later and was always weaker than that on the male papillae (Fig. 3c',d'). Only the tip of both papillae was labeled with FITC-SBA (Fig. 3b'–d'). When the conjugation tube was formed the labeling remained at the site where the two papillae met (Fig. 3e'). The labeling began to disappear when the zygospore was completely formed.

The SBA-binding material first appeared at the tip of male papillae and began to appear on the surface of the

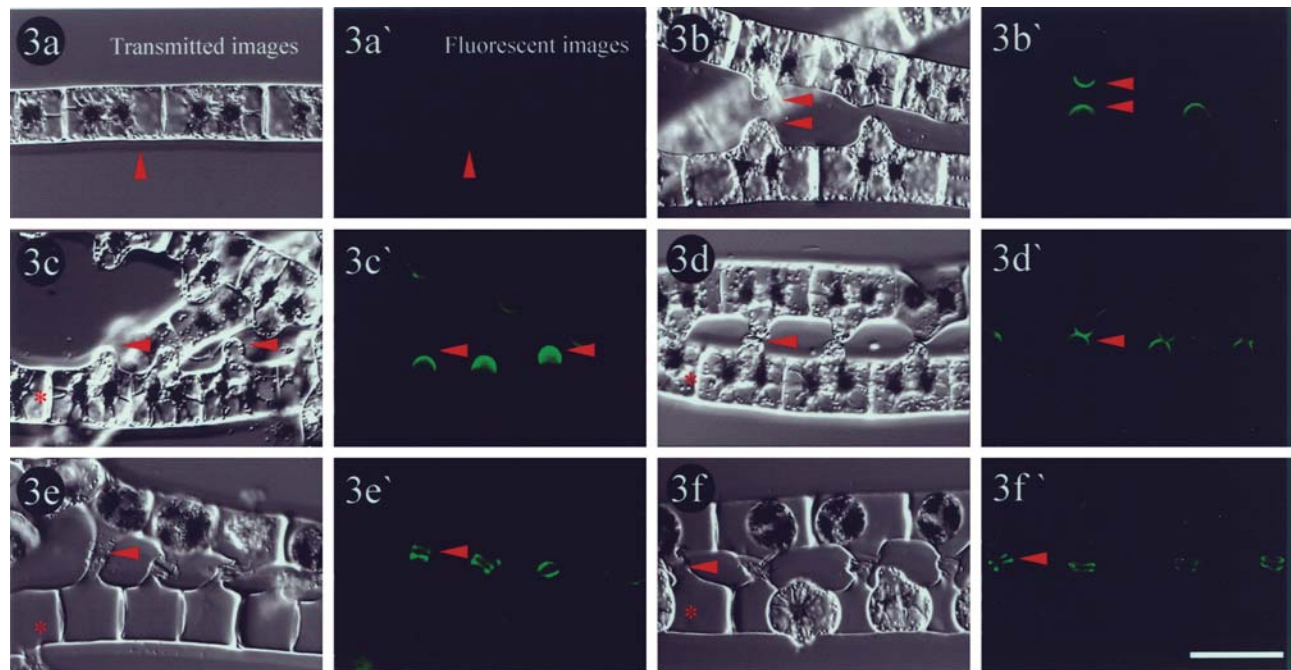


Fig. 3. Soybean agglutinin (SBA) (a–e) and peanut agglutinin (PNA) (f) labeling patterns during conjugation in *Zygnema cruciatum*. Differential interference micrographs of the filaments (a–f) and fluorescence micrographs of fluorescein isothiocyanate (FITC)-lectin labeling of the same filaments (a’–f’). (a–a’) Single filament showing no SBA labeling on the surface. (b–b’) SBA labeling appeared on the papilla formed from male and female filaments (arrowheads). (c–c’) Dense SBA labeling on the papilla in male filament (arrowheads). (d–d’) Papilla contact stage (arrowheads). (e–e’) and (f–f’) Two layers of SBA (e–e’) and PNA (f–f’) labeling appeared on the contact area between the male and female papilla during zygote formation stage (arrowheads). Asterisks show male filaments. Scale bar, 50 μ m.

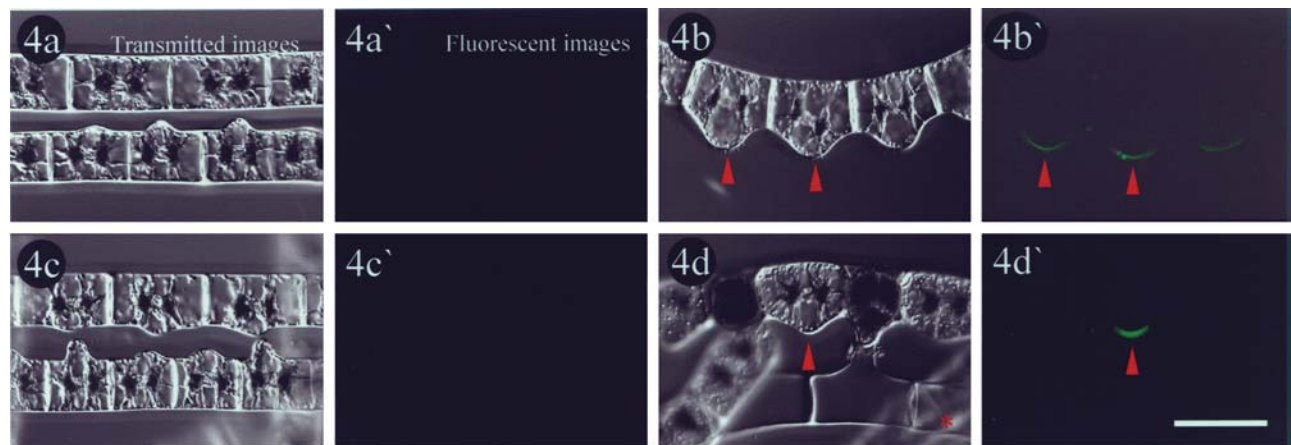


Fig. 4. Concanavalin A (ConA) (a–b’) and *Ricinus communis* agglutinin (RCA) (c–d’) labeling patterns during conjugation in *Zygnema cruciatum*. Differential interference micrographs of the filaments (a–d) and fluorescence micrographs of fluorescein isothiocyanate (FITC)-lectin labeling of the same filaments (a’–d’). (a–a’) Papilla formation stage showing no labeling of ConA on the surface. (b–b’) ConA labeling was rarely found on the papilla in some filaments (arrowheads). (c–c’) No RCA labeling was detected in the stage of papilla formation. (d–d’) Rarely, RCA labeling appeared on the papilla. Asterisk shows male filament. Scale bar, 50 μ m.

female papillae later (Fig. 3c’). There was a trace of SBA-specific materials in the gap between them. The number of male and female papillae labeled with FITC-SBA was counted over the time course during the conjugation process (Table 2). At 6 h after mixing of male and female filaments, 67% of male papillae were labeled

with FITC-SBA, but only 23.5% of female papillae were labeled. Most (92.6%) male papillae were labeled at 12 h after mixing and the percentage of labeled female papillae also increased to 61.1% (Table 2).

The FITC-PNA labeling pattern (Fig. 3f’) was similar to that of FITC-SBA (Fig. 3e’). No vegetative filament

Table 2. Comparison of labeling ratio on the papilla between male and female filaments

Time after mixing	Filament	SBA Labeled papillae/ total papillae	Percentage of labeled papillae (%)	PNA Labeled papillae/ total papillae	Percentage of labeled papillae (%)
6 h	Male	61/91	67.0	47/80	58.7
	Female	16/68	23.5	17/59	28.8
12 h	Male	88/95	92.6	66/74	89.2
	Female	44/72	61.1	35/67	52.2

PNA, peanut agglutinin; SBA, soybean agglutinin.

Table 3. Inhibition of conjugation in *Zygnema cruciatum* by lectins (25 µg/mL)

Treatment	Control*	ConA	RCA	WGA	DBA	UEA	PNA	SBA	PNA + SBA
Percentage (%)	71 ± 4.7	65.0 ± 7.0	58.9 ± 4.7	64.5 ± 5.8	63.4 ± 4.7	60.1 ± 3.9	24.0 ± 3.8	21.0 ± 4.6	20.2 ± 5.7

*BBM medium without lectins. Data are means ± standard deviation. ConA, Concanavalin A; DBA, *Dolichos biflorus* agglutinin; PNA, peanut agglutinin; RCA, *Ricinus communis* agglutinin; SBA, soybean agglutinin; UEA, *Ulex europaeus* agglutinin; WGA, wheat germ agglutinin.

was labeled with the lectin and the labeling was observed only on the tip of male and female papillae (data not shown). When the number of male and female papillae labeled with FITC-PNA was counted, it also showed the increase of labeled female papillae over the time course (Table 2).

Fluorescein isothiocyanate-ConA and FITC-RCA did not label the vegetative filaments of *Z. cruciatum* (Fig. 4a',c'). However, we observed a weak labeling by these lectins on the surface of some swollen papillae occasionally (Fig. 4b',d').

Blocking of conjugation with lectins

To determine if the PNA-, SBA-, and UEA-specific materials were involved in signaling or cell-cell recognition during conjugation of *Z. cruciatum*, an inhibition experiment using various lectins was carried out (Table 3). When the filaments were placed in the solution containing 25 µg/mL of PNA or SBA, only 24 and 21% of filaments, respectively, underwent conjugation (Table 3). The male and female papillae in the solution containing the above lectins showed slower growth, and the cell fusion did not occur even after the papillae contacted each other (data not shown). The inhibition effect did not increase significantly (20.2%) even when two lectins were combined. The other lectin treatment showed 58.9–65.0% of conjugating filaments (Table 3).

DISCUSSION

Our results indicate that there are significant changes in the composition of cell surface carbohydrates during

the conjugation process of *Zygnema cruciatum*. Some glycoconjugates with N-acetyl-D-galactosamine residues appeared at the tip of the male papillae and accumulated later on the tip of female papillae during the process. The blocking experiment with its complementary lectins, SBA and/or PNA, showed that these secreted materials might be involved in signaling or recognition between male and female papillae.

A detailed description of the morphological changes during the conjugation process in *Spirogyra* and *Zygnema* can be found in most algal textbooks. Thus, it is surprising that there are very few biochemical or cytochemical studies on this process. It may be due to the difficulty in controlling the conjugation process of laboratory-cultured materials. The filamentous zygnematacean species are notoriously difficult to establish unialgal cultures directly from the field-collected vegetative plants. Even when the cultures are established from field-collected vegetative plants or their germinating spores, it is again difficult to initiate the conjugation in a controlled manner. In most cases, the cultured plants appear to lose the ability of conjugation soon after they adapt to the culture condition (Kim & Kim 2002). The *Z. cruciatum* isolate used in this study started sexual reproduction as soon as it was transferred to the laboratory, making it easy to carry out the experiment in a short time. However, it would be essential to know the key environmental factors, which initiate conjugation of this species for further biochemical studies.

There are a few studies on the environmental factors inducing conjugation of filamentous zygnematacean species (Grote 1977; Simons *et al.* 1984; Stabenau & Saefel 1989; Kato & Ooshima 1997; Kim & Kim 2002). N depletion and light intensity have been

regarded as key factors for the induction of zygotes in *Spirogyra* spp. (Grote 1977; Simons *et al.* 1984). A high C/N ratio produced by depletion of N compounds was also found to be effective for induction of sexual reproduction in some *Spirogyra* (Yamashita & Sasaki 1979). In *Mougeotia*, aeration of the cultures was an essential factor together with N depletion and high light intensity (Stabenau & Saefel 1989). On the contrary, high temperature was reported as the only important factor to induce conjugation in *Zygnema extenuae* Jao (Kato & Ooshima 1997). Although there are numerous studies showing positive induction of conjugation by various environmental factors, the key factors that trigger this process in Zygnemataceae seem still inconclusive. Our cultures of *Z. cruciatum* were healthy and grew well but sexual reproduction was not induced with any of the above factors for 2 years.

Healthy filaments of the zygnematacean species secrete copious mucilage apparently to keep the external cell surfaces free of epiphytes. The mucilage on the surface of filaments may also be involved in the conjugation processes (e.g. Sze 1998). However, our results show that the composition of surface carbohydrates on conjugation papilla is different from that of external mucilage and some new glycoconjugates were produced and secreted from the tip of protruding papilla, indicating that the external mucilage is not directly involved in the signaling or cell-cell recognition during the conjugation process. This suggestion is supported by an ultrastructural study by Cheli and De Vecchi (1989), which showed that the extracellular mucilaginous sheath disappeared, and a glycoproteinaceous material was accumulated in the cell wall of the developing papillae during conjugation.

Blocking experiments show that two lectins, SBA and PNA, inhibited fusion between the male and female papillae. As the lectins are specific to the different derivatives of N-acetyl-D-galactosamine, we may conclude that glycoconjugates (probably glycoproteins) with the complement sugar residues are involved in signaling and adhesion between the male and female papillae. The lectins could block the signaling either by competing with the sugar-binding receptors on female papillae or by binding with the secreted glycoconjugates making it structurally difficult to bind with female receptors. Signaling glycoproteins involved in conjugation of unicellular algae, *Closterium* spp., have been studied extensively (e.g. Sekimoto *et al.* 1998). Recently, the cDNA encoding one of the signal glycoproteins have been cloned and characterized in *Closterium ehrenbergii* Meneghini ex Ralfs (Fukumoto *et al.* 2003). A signaling glycoprotein, rhodomorphin, was reported in a marine red alga *Griffithsia pacifica* Kylin, which induces elongation of repair cells and mediates a somatic cell fusion between repair cells during the wound-healing response of the species (Watson &

Waaland 1983, 1986). Similar glycoprotein has been found in other red algal species, *Antithamnion* spp., and its secretion process from the tips of repair cells has been visualized by the use of FITC-lectins (Kim & Fritz 1993c; Kim *et al.* 1995, 1996). The conjugation process in *Z. cruciatum* shares some common features with red algal somatic cell fusion in having a glycoconjugate as a signal molecule and showing the directional elongation of a cell protrusion towards its compatible partner. Further biochemical and cytochemical studies are necessary to elucidate the function and properties of the signaling glycoconjugates in *Z. cruciatum*.

With extensive further investigation on the inducing factor for conjugation and the establishment of culture methods to maintain sexually active plants in a controlled manner, the conjugation process of zygnematacean species might serve as a good model system for studies on the sexual differentiation, cell signaling and recognition of self from non-self.

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