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Homology-dependent Suppression of Stigma Phenotype by an Antisense *S*-locus Glycoprotein (*SLG*) Gene in *Brassica rapa* L.

Takeshi Takasaki*[1),2)], Katsunori Hatakeyama[1)], Masao Watanabe[3)], Kinya Toriyama[4)] and Kokichi Hinata[1)]

[1)] *Research Institute of Seed Production Co., Ltd.* 6-6-3 Minamiyoshinari, Aoba, Sendai, Miyagi 989-3204, Japan
[2)] *Faculty of Agriculture, Kobe University,* 1-1 Rokkodai, Nada, Kobe, Hyogo 657-8501, Japan
[3)] *Faculty of Agriculture, Iwate University,* 3-18-8 Ueda, Morioka, Iwate 020-8550, Japan
[4)] *Graduate School of Agricultural Sciences, Tohoku University,* 1-1 Tsutsumidori Amamiya-machi, Aoba, Sendai, Miyagi 981-8555, Japan

Self-incompatibility (SI) in Brassicaceae is sporophytically controlled by haplotypes of the polymorphic *S* locus complex. Two tightly linked polymorphic genes at the *S* locus, *S*-locus glycoprotein (*SLG*) and *S*-receptor kinase (*SRK*) genes, are specifically expressed in the stigma. *S*-haplotypes have been classified into class I and class II types based on the sequence similarity of their SLGs, and their SRKs. To investigate the effect of an antisense *SLG* gene on the class divergency of the endogenous *SLG* and *SRK* genes, we introduced an antisense class I SLG^{43} cDNA into a cultivar Osome in *Brassica rapa* which was heterozygous for class I S^{52} haplotype and class II S^{60}. SLG^{43} is more similar to the endogenous SLG^{52} (87.8 % identity) than to SLG^{60} (74.8 % identity). Out of ten primary transformants analyzed, two were completely self-compatible; the SI phenotype of stigma was altered from $S^{52}S^{60}$ to S^{60}, but that of pollen was not. In these two plants, the expression levels of mRNA and protein of SLG^{52} were reduced, whereas those of SLG^{60} were not. We suggest that an antisense class I *SLG*-transgene causes homology-dependent suppression, which leads to breakdown of the class I *S*-haplotype specificity in stigma but not the class II *S*-haplotype.

Key Words: ***Brassica*, antisense RNA, self-incompatibility, *S*-locus glycoprotein, transgenic plant.**

Introduction

Self-incompatibility (SI) in the Brassicaceae is sporophytically controlled by haplotypes of the *S* locus complex (Bateman 1955). Self pollen fails to germinate or pollen tubes are arrested on the stigma surface. The *S*-locus contains at least two highly polymorphic genes that are expressed in the stigma: the *S*-locus glycoprotein (*SLG*) gene and the *S*-receptor kinase (*SRK*) gene (Hinata *et al.* 1993, Nasrallah and Nasrallah 1993). *SLG* encodes a secreted glycoprotein (Nasrallah *et al.* 1987, Takayama *et al.* 1987) and *SRK* encodes a transmembrane protein kinase that is composed of an extracellular domain (S domain) similar to SLG, a transmembrane domain and a serine/threonine protein kinase domain (Stein *et al.* 1991, Goring *et al.* 1992). SLG and the S domain of SRK of the same haplotype share a high degree of sequence similarity (Stein *et al.* 1991, Watanabe *et al.* 1994). We have recently elucidated that SRK alone determines the *S* haplotype specificity of the stigma and that SLG acts to promote a full manifestation of SI response (Takasaki *et al.* 2000).

Shiba *et al.* (1995, 2000) introduced an antisense class I SLG^{43} cDNA driven by an SLG^{43} promoter with an enhancer of CaMV 35S promoter into *B. rapa* ssp. *oleifera* cv. Candle and ssp. *chinensis* cv. Kohsaitai and obtained two transgenic plants. These plants were self-compatible and the amount of transcripts of endogenous *SLG*/*SRK* genes was reduced. However, the *S*-haplotypes and the endogenous *SLG*/*SRK* genes in the recipient plants have not been characterized fully.

Both *SLG* and *SRK* genes have been classified into class I and class II types based on sequence divergence (Nasrallah and Nasrallah 1993). Class I *SLG* genes exhibit about 65 % identity in amino acid sequence to class II *SLG* genes. Besides, the class II *S* haplotypes are, in most cases, recessive to class I *S* haplotypes in pollen (Hatakeyama *et al.* 1998). An important factor in the breakdown of SI using an antisense RNA strategy is the nucleotide sequence similarities between the antisense *SLG* transgene and the endogenous *SLG*/*SRK* genes. Therefore, to investigate the breakdown of SI phenotypes by the antisense transgene in relation to the class divergency of the endogenous genes, we introduced an antisense class I SLG^{43} cDNA fused with the SLG^{43} promoter into a cultivar Osome which was heterozygous for class I S^{52} haplotype and class II S^{60}. The expression of the endogenous *SLG* genes and the alteration of the SI phenotypes in ten transformants were studied.

Materials and Methods

Construction of the antisense SLG^{43}*-transgene*

The promoter region of the SLG^{43} genomic clone (Dwyer *et al.* 1991) was amplified by PCR with the M13RV univer-

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*Corresponding author (e-mail: taka@kobe-u.ac.jp)

sal primer and SLG43P primer (-19-+ 1: 5′-ATTTGGATCC CCACCTTG-3′, the underline shows the *Bam*HI site designed). An 1.9kb amplified promoter fragment was cloned into pPCRII (Invitrogen) and then was sequenced. The nucleotide sequence of this promoter fragment coincided with that of the original promoter. The 1.9kb *Eco*RI/*Bam*HI SLG^{43} promoter fragment was inserted into a binary vector pROK1 (Baulcombe *et al.* 1986) from which the CaMV 35S promoter had been removed. Then, an 1.3 kb *Bam*HI SLG^{43} cDNA fragment (Yamakawa *et al.* 1994) was fused downstream of the SLG^{43} promoter in antisense orientation. A hygromycin-resistant cassette containing a gene coding for hygromycin phosphotransferase (HPT; Cullen *et al.* 1987) was inserted at the *Hin*dIII site in this vector to yield pAntiSLG43 (Fig. 1A). This construct was introduced into *Agrobacterium tumefaciens* strain EHA101 (Hood *et al.* 1986) using the procedure described by An *et al.* (1988).

Plant transformation

The cultivar Osome, a commercial F_1 hybrid variety of *B. rapa* ssp. *chinensis* (Takii Seed, Co., Kyoto, Japan), was used as a recipient plant, because of its high transformation efficiency (Takasaki *et al.* 1997). The *S*-genotype of Osome was $S^{52}S^{60}$-heterozygote. S^{52} and S^{60} were co-dominant in the stigma. S^{60} was recessive to S^{52} in the pollen (Takasaki *et al.* 1999). Hypocotyl explants were transformed with *A. tumefaciens* strain EHA101 harboring the pAntiSLG43 according to the procedure described by Takasaki *et al.* (1997).

Pollination tests

Open flowers on the day of pollination were collected from each plant and were used for self- or cross-pollination. The pollinated flowers were cut at the peduncle and stood on 1% solid agar overnight under room conditions. The stigmas were softened in 1 N NaOH at 60°C for 1.5 h and stained with decolorized aniline blue at 60°C for 1.5 h. Pollen tube behavior was observed under a UV fluorescent microscope (Kho and Baer 1968). Five flowers each were used for self-pollination and cross combination on each

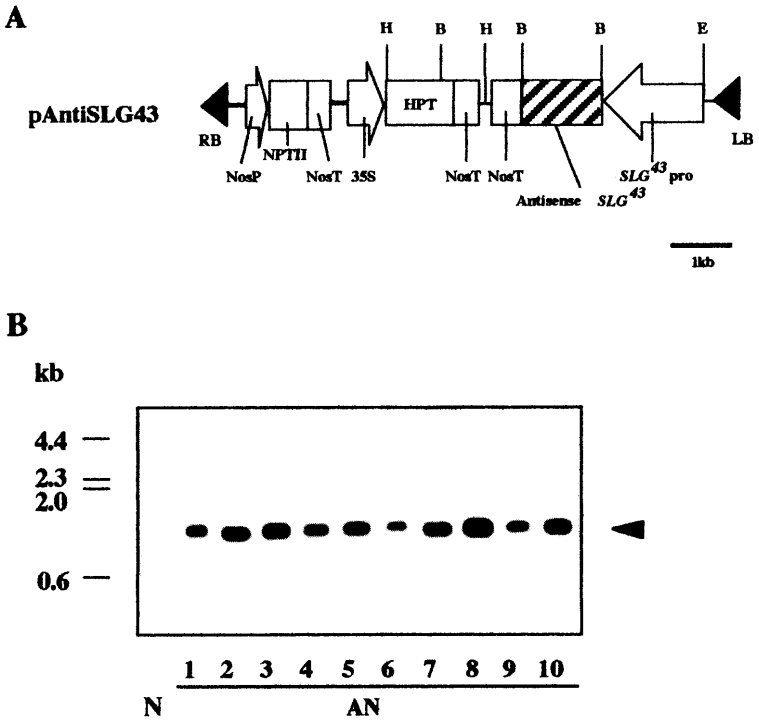

Fig. 1. Vector construction and detection of transgene. **A**, Representation of the T-DNA region of transformation vector pAntiSLG43. Antisense SLG^{43}: the SLG^{43} cDNA in the antisense orientation is indicated by the hatched area, NPTII: neomycin phosphotransferase, SLG^{43}pro: SLG^{43} promoter region, HPT: hygromycin phosphotransferase, NosP: nopaline synthase promoter, NosT: nopaline synthase terminator, 35S: 35S promoter, RB: right border, LB: left border, E: *Eco*RI, H: *Hin*dIII, B: *Bam*HI. **B**, DNA gel blot analysis of plants transformed with the antisense SLG^{43}-transgene. Two μg of DNA digested with *Bam*HI was loaded in each lane and hybridized with the SLG^{43} cDNA probe. N: a non-transformant of Osome ($S^{52}S^{60}$-heterozygote), AN-1 to -10: transformants. The arrowhead points to *Bam*HI fragment of the antisense SLG^{43}-transgenes.

occasion and the observation of pollen tube behavior was generally replicated 6 or 7 times in respective pollination on different dates. At first, each flower was classified into incompatible or compatible when the number of penetrated pollen tubes was less than 5 or more than 150, respectively. The phenotype of a plant was indicated to be incompatible (−) when all observed flowers were incompatible, and to be compatible (+) when all observed flowers were compatible.

DNA gel blot analysis

Total DNA was extracted from young leaves by the cetyldimethylammonium bromide (CTAB) method (Murray and Thompson 1980). The DNA was digested with *Bam*HI or *Hin*dIII, and electrophoresed on 0.8% agarose gels. DNA fragments were transferred to nylon membranes and allowed to hybridize with a digoxigenin (DIG)-labeled SLG^{43} cDNA probe (Yamakawa *et al.* 1994) and SLG^{52}-PCR fragment probe (Takasaki *et al.* 1999). Washing and detection were performed according to the instruction manual accompanying the DIG Nucleic Acid Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany). After hybridization, the membranes were washed twice in 0.1% SSC and 0.1% SDS at 65°C for 20 min.

Immunoblot analysis

Total protein was extracted from the stigma of open flowers in 50 mM Tris-HCl (pH 7.5). The extract was subjected to thin-layer polyacrylamide gel isoelectric focusing (Ampholine PAG Plate, pH 3.5-9.5, Amersham-Pharmacia Uppsala, Sweden) and then transferred to a PVDF membrane by electroblotting. SLG^{52} was detected with an anti-SLG^{43} (class I SLG) monoclonal antibody (Shiba *et al.* 1995).

RNA gel blot analysis

Poly(A)$^+$RNA was isolated from stigmas at one day before anthesis using the Micro FastTrack mRNA isolation kit (Invitrogen, San Diego, CA, USA) according to the manufacturer's instruction manual. After denaturation in glyoxal, mRNA was loaded on 1% agarose gels in 10 mM sodium phosphate buffer (pH 7.0). After electrophoresis, the mRNA was transferred to nylon membranes and allowed to hybridize with a DIG-labeled SLG^{52}- or SLG^{60}-PCR fragment probe (Takasaki *et al.* 1999). Washing and detection were performed as described for DNA gel blot analysis. The intensity of the bands was determined using a the Bio-Rad Model GS-670 densitometer.

Results and Discussion

The cultivar Osome of *B. rapa* used as the recipient plant in the present study, carried S^{52} (class I)- and S^{60} (class II)-haplotypes (Takasaki *et al.* 1999). We introduced an antisense class I SLG^{43} cDNA fused with the SLG^{43} promoter into this cultivar. Sixteen independent kanamycin-resistant plants were obtained from *Agrobacterium*-mediated transformation of Osome with the antisense SLG^{43}-transgene. Ten plants were chosen for further analysis. No visible morphological differences were observed between these plants and the non-transformant of Osome. Gel blot analysis of *Bam*HI-digested genomic DNA using the SLG^{43} cDNA as a probe showed that all ten transformants carried the 1.3 kb antisense SLG^{43}-transgene (Fig. 1B). We estimated one copy for plants AN-1, 6 and 9; two copies for plants AN-5, 7 and 10; three copies for plants AN-2 and -3; four copies for plants AN-4 and AN-8 according to *Hin*dIII digestion. On the other hand, two intense bands at 10.5 kb and 6.2 kb corresponding to endogenous SLG^{52} and SRK^{52} were detected in Osome and all ten transformants by gel blot analysis of *Hin*dIII-digested DNA using SLG^{52}-PCR fragment probe (data not shown).

Pollination tests were carried out to investigate the SI phenotypes of the primary 10 transformants carrying the antisense SLG^{43}-transgene. In the self-pollination test, two transformants (AN-1 and AN-4) were found to have become completely self-compatible, though the non-transformant, Osome plant is self-incompatible. These two transformants were compatible with the pollen from Osome plant ($S^{52}S^{60}$-heterozygote with the pollen *S* phenotype being S^{52}) and $S^{52}S^{52}$-homozygotes, but incompatible with the pollen from $S^{60}S^{60}$-homozygotes (Table 1). We concluded that the two transformants lost the ability completely to reject the pollen of S^{52}-haplotype, but retained the ability to reject the pollen of S^{60}-haplotype. This showed that the SI phenotype of their stigmas had been changed from $S^{52}S^{60}$ to S^{60}. The other eight transformants did not show any changes in the SI phenotype of the stigma. The SI phenotype of the pollen was not changed in any of the ten transformants.

Table 1. Pollination tests of the transformed plants carrying an antisense SLG^{43}-transgene

Transform	Self	× N	N ×	× S^{52}	S^{52} ×	× S^{60}	S^{60} ×
N	−	−	−	−	−	−	+
AN-1	+	+	−	+	−	−	+
-2	−	−	−	−	−	−	+
-3	−	−	−	−	−	−	+
-4	+	+	−	+	−	−	+
-5	−	−	−	−	−	−	+
-6	−	−	−	−	−	−	+
-7	−	−	−	−	−	−	+
-8	−	−	−	−	−	−	+
-9	−	−	−	−	−	−	+
-10	−	−	−	−	−	−	+

N: non-transformant of Osome ($S^{52}S^{60}$), S^{52}: S^{52}-haplotype, S^{60}: S^{60}-haplotype.

S^{52}- and S^{60}-haplotypes were isolated from Osome ($S^{52}S^{60}$-heterozygote).

Self: self-pollination of transformants.

× N, × S^{52}, × S^{60}: the pollen of N, S^{52} and S^{60} were pollinated to the transformed stigma.

N ×, S^{52} ×, S^{60} ×: the pollen of the transformants were pollinated to N, S^{52} and S^{60} stigma.

+: compatible, −: incompatible.

SLGs in the stigmas of the transformants and an Osome plant ($S^{52}S^{60}$) were examined by IEF-immunoblot analysis using an anti-SLG^{43} (class I SLG) monoclonal antibody. The anti-SLG^{43} monoclonal antibody crossreacted only with the SLG^{52} protein, but not with the SLG^{60} protein (Takasaki *et al.* 1999). As shown in Fig. 2, in the two self-compatible transformants (AN-1 and AN-4), no bands corresponding to the SLG^{52} protein were detected, whereas the three bands, all of which corresponded to the SLG^{52} protein, were detected at a normal level in the other transformants. The level of the endogenous SLG^{52} protein was confirmed by independent analyses replicated three times. These results showed that the endogenous SLG^{52} protein was reduced in two self-compatible plants (AN-1 and AN-4).

The levels of *SLG*^{52} and *SLG*^{60} RNA transcripts produced in the transformants were examined by gel blot analysis of poly(A)$^+$ RNA from the stigmas using the *SLG*^{52}- or *SLG*^{60}-PCR fragment as a probe. The *SLG*^{52} probe hybridized to both *SLG*^{43} and *SLG*^{52} RNA transcripts, but not to *SLG*^{60} RNA transcripts (Fig. 3A); this was due to the sequence divergence between class I and class II *SLG*s. Using the *SLG*^{52} probe, we detected some *SLG*^{52} RNA transcripts in AN-4, but not in AN-1. The amount of *SLG*^{52} RNA transcripts produced by AN-4 was estimated to be ca. 25% (average of two independent experiments) of that produced by the Osome plant (Fig. 3A). No reduction of *SLG*^{52} RNA transcripts was observed in the other transformants. Because *SRK*^{52} transcripts in Osome plants could only be detected as a very faint band even after a very long exposure to X-ray film, it is difficult to determine accurately whether the expression level of *SRK*^{52} was suppressed in the transformants analyzed here. However, *SLG* and the *S*-domain of *SRK* isolated from the same haplotype share a high level (> 87%) of nucleotide sequence identity (Watanabe *et al.* 1994, Delorme *et al.* 1995, Hatakeyama *et al.* 1998). The

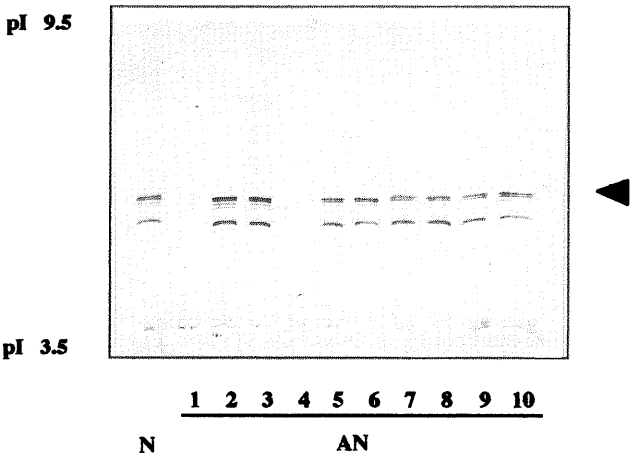

Fig. 2. IEF-immunoblot analysis of levels of SLG^{52} protein using anti-SLG^{43} monoclonal antibody. Stigma proteins of Osome plant (lane N, non-transformant) and transformants (AN-1 to -10). Total protein was extracted from 10 stigmas for each plant. The arrowhead indicates SLG^{52} protein.

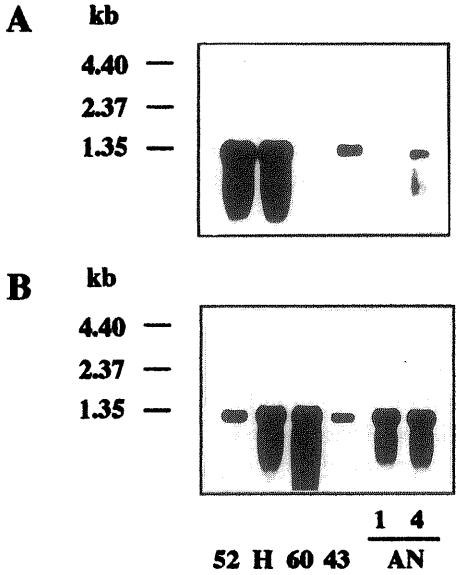

Fig. 3. RNA gel blot analysis of the expression of *SLG*s using PCR fragments of *SLG*^{52} **(A)** and *SLG*^{60} **(B)** as probes. Poly(A)$^+$ RNA was isolated from stigmas of $S^{52}S^{52}$-homozygote (lane 52), $S^{52}S^{60}$-heterozygote (non-transformant; lane H), $S^{60}S^{60}$-homozygote (lane 60), $S^{43}S^{43}$-homozygote (lane 43) and from the transformants AN-1 and AN-4. In lanes 52, 60 and 43, 0.5 μg of poly (A)$^+$ RNA was loaded, and in the other lanes 1 μg of poly (A)$^+$ RNA was loaded.

expression of endogenous *SLG*/*SRK* was suppressed in transgenic *B. rapa* plants carrying an antisense *SLG*^{43} gene (Shiba *et al.* 2000). We have shown by gain-of-function experiments that SRK alone determines *S*-haplotype specificity in the stigma but SLG does not (Takasaki *et al.* 2000). Therefore, the expression of endogenous *SRK*^{52} in these two plants (AN-1 and AN-4) had to be suppressed by the antisense *SLG*^{43}-transgene, in the same way as for *SLG*^{52}. The lower amount of *SLG*^{52} RNA transcripts produced by AN-4 compared with the Osome plant was compatible with the pollen of S^{52}. In AN-4, the antisense *SLG*^{43} RNA would be successful to suppress the expression of *SRK*^{52} perfectly but not to do that of *SLG*^{52}.

It is known that probes derived from class II *SLG* genes hybridize not only to the RNA transcripts of class II *SLG* genes, but also to those of *SLR*2, which shares a high degree of sequence similarity with class II *SLG*s but is not linked to the *S*-locus (Boyes *et al.* 1991). Indeed, the *SLG*^{60} probe detected a very intense band in all transformants, Osome plant ($S^{52}S^{60}$) and $S^{60}S^{60}$-homozygote. In contrast, a much weaker hybridizing band was observed in the $S^{43}S^{43}$-homozygote and $S^{52}S^{52}$-homozygote. This band likely represented *SLR*2 RNA transcripts (Fig. 3B). We concluded that in the two self-compatible transformants (AN-1 and AN-4) the expression of *SLG*^{52}, but not *SLG*^{60}, was suppressed.

Two transformants (AN-1 and AN-4) that had become

self-compatible lost the ability to reject the S^{52} pollen and only the expression levels of the endogenous SLG^{52} gene was reduced at the mRNA and protein levels. The antisense SLG^{43} transgenes in AN-1 and AN-4 did not appear to be integrated into SLG^{52} and SRK^{52} because two bands (10.5 kb and 6.2 kb) corresponding to SLG^{52} and SRK^{52} were detected both in the Osome plant and in these two plants by gel blot analysis using the SLG^{52}-PCR fragment probe. AN-1 and AN-4 plants carried a single copy and 4 copies of SLG^{43} transgene, respectively, while the other transformants that showed no change in stigma phenotype had a variable copy number. The expression of the antisense SLG^{43} transgenes was likely to be independent of the copy number. SLG^{43} (class I *SLG*) shares a higher degree of sequence similarity (87.8%) to SLG^{52} (a class I *SLG*) than to SLG^{60} (74.8%, a class II *SLG*) (Takasaki *et al.* 1999), and the SLG^{52} probe hybridized to SLG^{43} RNA transcripts, but not to SLG^{60} RNA transcripts. The mechanism of the antisense suppression presumably occurs by the formation of a duplex between complementary RNAs and the rapid selective degradation of double-stranded molecules by cellular RNase (Zabaleta *et al.* 1994). In the two transformants (AN-1 and AN-4), the antisense SLG^{43} RNA may form a duplex with the endogenous SLG^{52}/SRK^{52} RNA transcripts but not with the endogenous SLG^{60}/SRK^{60} RNA transcripts.

We have shown that an antisense class I *SLG* gene led to loss of the class I haplotype specificity in stigma but not class II haplotype. This means that the homology-dependent suppression was caused by the antisense SLG^{43}-transgene. The sequence similarity between the antisense *SLG*-transgene and endogenous *SLG*/*SRK* genes of recipient plants is an important point to establish the self-compatible *Brassica* plants using an antisense RNA technique.

In order to breakdown the SI in the cases of selfing and crossing between lines with the same *S*-allele, several physiological methods have been utilized such as bud pollination, CO_2 gas treatment (Nakanishi *et al.* 1969) and NaCl treatment (Monteiro *et al.* 1988). On the other hand, the breakdown of SI by the introduction of an antisense *SLG* is based on genetics. Therefore, it could be expected to utilize for mass propagation in pure-bred varieties and to provide higher seed production.

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