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Note

The shoot of *Ranunculus nipponicus* var. *submersus*, a submerged vascular plant, can actively take up nitrate from cool water

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Abstract Nitrate uptake characteristics in the shoots of *Ranunculus nipponicus* var. *submersus* (Japanese name: Baikamo), a submerged eudicot adapted to groundwater temperature (approximately 15°C), were investigated for the phytoremediation of nitrate-polluted groundwater. For the experiments, we developed a culture system that allows *R. nipponicus* growth under laboratory conditions. ^{15}N-nitrate feeding experiments showed that apparent nitrate uptake by the shoots of *R. nipponicus* was approximately three times of that by the shoots of *Egeria densa*, a model submerged monocot, at 15°C. A DNA fragment of the *R. nipponicus* high-affinity nitrate transporter (NRT2) was isolated and the deduced amino acid sequence of the partial RnNRT2 protein was similar to that of NRT2 in monocots rather than in eudicots. Real-time reverse transcription-PCR analysis revealed that after the shoots were fed 0.2 mM nitrate, *RnNRT2* transcripts in the shoots of *R. nipponicus* were induced within 1 h, reached a maximum by 6 h and then decreased. At 15°C, *RnNRT2* transcripts in the shoots of *R. nipponicus*, in contrast to *EdNRT2* transcripts in shoots of *E. densa*, were rapidly and strongly induced by nitrate. We concluded that the shoots of *R. nipponicus* have a system of high-affinity nitrate uptake actively functioning under cool conditions (15°C) and may be useful for the clean-up of nitrate-contaminated groundwater.

Key words: High-affinity nitrate transporter, nitrate uptake, NRT2, *Ranunculus nipponicus* var. *submersus*, submerged vascular plant.

Submerged vascular plants cause a reduction in the inorganic nitrogen concentration of the water column and contribute to the improvement of water quality in aquatic ecosystems (van Donk et al. 1993). Our previous results showed that in *Egeria densa*, a model submerged monocot, nitrate uptake ability of shoots was higher than that of roots at a relatively high water temperature (25°C) (Takayanagi et al. 2012). This finding indicates that the shoots of submerged vascular plants are useful for water quality management not only for aquatic ecosystems but also for drinking water. In Japan, groundwater quality monitoring results in the past decade revealed that approximately 4% of surveyed wells exceeded the environmental quality standard for nitrate of 44.3 mg l^{-1} (0.7 mM) (Ministry of the Environment, Japan 2014). It is thought that the shoots of *E. densa* could contribute in converting these nitrate-polluted groundwaters into drinkable water. However, the average temperature of the nitrate-polluted groundwater is 14±2°C [calculated using data from Miyashita (2004)]. The elongation and relative growth rates of *E. densa* at 17°C were severely reduced compared with that at 25°C (Motonaga et al. 2011). We further expect that nitrate uptake by this plant would be drastically decreased at 15°C because nitrate uptake in the roots of coffee (*Coffea arabica*) and rice (*Oryza sativa*) is depressed at this temperature (Hasegawa 1990; Sasakawa and Yamamoto 1978; Vaast et al. 1998). For removing nitrogen from groundwater using the shoots of submerged vascular plants, it is necessary to identify plants that actively grow and take up nitrate in cool (15°C) groundwater.

Ranunculus nipponicus var. *submersus* (Japanese name: Baikamo), a submerged eudicot of the Ranunculaceae, is widely distributed in the Japanese archipelago from the Hokkaido Island to the Honshu Island (Koga et al. 2007, 2008). The plant grows in cool water (<20°C) and its growth is maximal at water temperatures of 10–15°C (Kimura and Kunii 1998; Kunii and Inoue 1997). Therefore, we consider *R. nipponicus* a candidate plant for removing nitrogen from cool eutrophic groundwater.

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In the present study, as a first step in a comprehensive study of nitrate uptake in the shoots of *R. nipponicus* for the phytoremediation of nitrate-polluted groundwater, we evaluated, in the shoots of *R. nipponicus*, the nitrate uptake ability and expression pattern of a high-affinity nitrate transporter (*NRT2*) that is known to play an important role in nitrate uptake at nitrate concentrations below 1 mM and may still contribute to nitrate uptake above 1 mM (Forde 2000; Miller et al. 2007).

A *R. nipponicus* clone, S-c (Figure 1A), was isolated and vegetatively propagated from a plant growing naturally in the Jizo-gawa River in Maibara City (35.33°N; 136.35°E), Shiga Prefecture, Japan. The annual water temperature of the river was between 13°C and 16°C (Kandori 2008). Nitrate concentrations in the river water were between 0.075 mM and 0.093 mM through the year (data not shown). For the experiments, we developed a culture system for *R. nipponicus* growth, as shown in Figure 1B. Plants were maintained in a 3-l capacity plastic beaker containing 3 l of medium in a plant growth chamber (LH-200-RDCT, Nippon Medical Chemical Instruments, Osaka, Japan) supplied continuously with CO_2 at 15±2°C under continuous illumination from fluorescent lamps (Sun White 5 FL15N, NEC Lighting, Tokyo, Japan; 8.5 μmol m^{-2} s^{-1} at the water surface; Figure 1B). The culture medium was a ten-fold dilution of modified Gaudet's medium (Takayanagi et al. 2012), except for KNO_3 content, which was reduced to 20.22 mg l^{-1} (0.2 mM). The shoots of small *R. nipponicus* plants (average length, ca. 6.0 cm; average fresh weight, 70.4 mg; Figure 1A), newly developed from a node of the mother plant, were excised and rinsed with 0.4 mM $CaSO_4$ solution before use. No apparent periphyton growth was observed. An *E. densa* clone, A-3, was grown and used, according to the method of Takayanagi et al. (2012), as a control. For *E. densa*, the shoot length was ca. 4.0 cm and average shoot fresh weight was 45.0 mg.

For ^{15}N-nitrate feeding experiments, a shoot of a small plant of each species was fixed to the bottom of a beaker with silicone sealant (Bath caulk N Clear, Cemedine, Tokyo, Japan). The shoots were pre-treated with 0.4 mM $CaSO_4$ (N-free) for 24 h for *R. nipponicus* or for 38 h for *E. densa*. Shoots were treated with 0.2 mM ^{15}N-KNO_3 (98.2at% ^{15}N; Isotec, Miamisburg, OH, USA) and 0.4 mM $CaSO_4$. Both pre-treatment and ^{15}N-nitrate treatment were performed in a plant growth chamber under the same conditions described above, except that light intensity for *E. densa* was 30.0 μmol m^{-2} s^{-1} at the water surface. Shoots were collected 0 and 6 h after treatment initiation, washed with 0.4 mM $CaSO_4$ solution for 1 min and dried for 48 h at 70°C and ground. The ^{15}N content was measured using a dry combustion CN analyser coupled with an isotope ratio mass spectrometer (Tracer MAT, Thermo Quest, Tokyo, Japan). When 0.2 mM ^{15}N-labelled nitrate was applied to shoots at 15°C, the ^{15}N content in shoots of *R. nipponicus* and *E. densa* was 73.9 and 28.7 nmol mg^{-1} DW, respectively (Table 1). Nitrate uptake ability by the shoots of *R. nipponicus* was approximately three-fold higher than that by the shoots of *E. densa*. 0.2 mM ^{15}N-nitrate uptake ability in the shoots of *E. densa* at the 25°C condition has been reported to be

Table 1. ^{15}N absorption by the shoots of *Ranunculus nipponicus* and *Egeria densa* at 15°C.

Plant species	Time after ^{15}N-nitrate treatment (h)	At% ^{15}N in dry shoot	^{15}N content in shoot (nmol mg^{-1} DW)
R. nipponicus	0	0.37±0.00	9.5±2.2
	6	3.23±0.78	73.9±9.8
E. densa	0	0.37±0.00	10.5±1.5
	6	1.06±0.38	28.7±7.6

Shoots were exposed to 0.2 mM ^{15}N-enriched KNO_3 (98.2at% ^{15}N) containing 0.4 mM $CaSO_4$ for 0 or 6 h. Values are mean±SD of three to six replicates, each with three shoots of small plants.

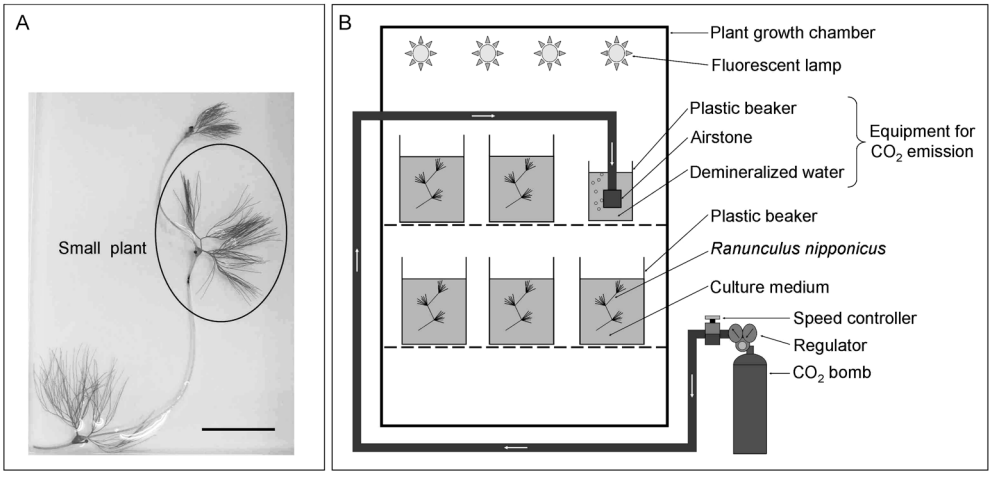

Figure 1. The eudicot submerged plant *Ranunculus nipponicus* and the culture system. (A) The small *R. nipponicus* specimen shown in the ellipse was excised and used for experiments. Scale bar is 50 mm. (B) Schematic diagram of the culture system for *R. nipponicus*.

59.4–71.1 nmol mg^{-1} DW (Takayanagi et al. 2012). Under a 0.2 mM nitrate condition, nitrate uptake ability by the shoots of *R. nipponicus* at 15°C is comparable with that in the shoots of *E. densa* at 25°C. These findings indicate that the shoots of *R. nipponicus* have a high-affinity transport system (HATS) for nitrate that is more active than that of the shoots of *E. densa* at 15°C, because HATS operate to take up nitrate at low external concentrations (below 1 mM) (Forde 2000; Miller et al. 2007).

For *RnNRT2* isolation and cloning, a fragment was amplified by PCR using genomic DNA and a P1-forward primer (5′-TKGAYTCNGARCAC AARGC-3′) (Takayanagi et al. 2012) and a P2-reverse primer (5′-CCC ATDTTNCCC CABCCNGC-3′) derived from the conserved sequences of plant *NRT2s* (Figure 2). Genomic DNA was prepared from the shoots of *R. nipponicus* using a cetyltrimethylammonium bromide method (Doyle 1991). The amplification conditions included initial denaturation at 94°C for 5 min, followed by 35 cycles at 98°C for 10 s, 50°C for 30 s and 72°C for 1 min. The amplified fragments were cloned and sequenced. The 3′ downstream region of fragment was isolated by PCR using the P3-forward primer (5′-CTT TTC TCC TTG GCC AAT CC-3′), whose sequence was derived from the fragment and the P4-reverse primer (5′-CAT GSHDCCC CAYTGNGGRA-3′) also derived from the conserved sequence of plant *NRT2s* (Figure 2). The amplification conditions included initial denaturation at 94°C for 5 min, followed by 35 cycles at 98°C for 10 s, 50°C for 30 s and 72°C for 2 min. The amplified fragments were cloned and sequenced. These two sequences were assembled to yield the final sequence. Multiple sequence alignments were constructed with the DNA Data Bank of Japan (DDBJ) ClustalW version 2.1 program with default parameters (Thompson et al. 1994). A phylogenetic tree was generated using the neighbor-joining method (Saitou and Nei 1987) included in the DDBJ ClustalW version 2.1 program with default parameters and a dendrogram was constructed with TreeView software (Page 1996). A DNA fragment of the putative *R. nipponicus NRT2* was cloned by PCR, and the corresponding gene was designated as partial *RnNRT2* (accession no. AB778032). The deduced amino acid sequence for partial RnNRT2 had 411 amino acid residues and shared 79% identity with OsNRT2.1, 77% with *Arabidopsis thaliana* NRT2.1 and 61% with EdNRT2. Partial RnNRT2 contained the conserved NRT2 motifs A/GGWG/AN/DXG (Okamoto et al. 2003; Pao et al. 1998; Trueman et al. 1996), RP/AXGGXXS/AD and FGMRGRLW (Okamoto et al. 2003), although one substitution (G to A) was found in partial RnNRT2 and OsNRT2.1 in the third motif (Figure 2). Hydropathy profiles obtained using MEMSAT3 program (Jones 2007) predicted that partial RnNRT2 has 12 transmembrane domains (Figure 2). One of the three putative NRT2 phosphorylation sites (S/TXR/K) that are conserved in plant NRT2s (Forde 2000; Okamoto et al. 2003; Tsujimoto et al. 2007) was found in partial RnNRT2 (Figure 2). These results indicate that the partial *RnNRT2* isolated in this study is a large part of a gene encoding a high-affinity nitrate transporter. The phylogenetic analysis of partial plant NRT2s based on their amino acid sequences corresponding to the isolated partial RnNRT2 sequence led to the classification of partial RnNRT2 in a monocot type NRT2 (Figure 3). This finding is curious because *R. nipponicus* is a eudicot. One possibility is that the phylogenetic tree was generated using less-than-full-length NRT2 sequences. Another possibility is that the plant has other NRT2(s) such as a eudicot type NRT2 because in rice and *A. thaliana*, it has been shown that *NRT2* consists of four and seven homologous genes, respectively (Araki and Hasegawa 2006; Okamoto et al.

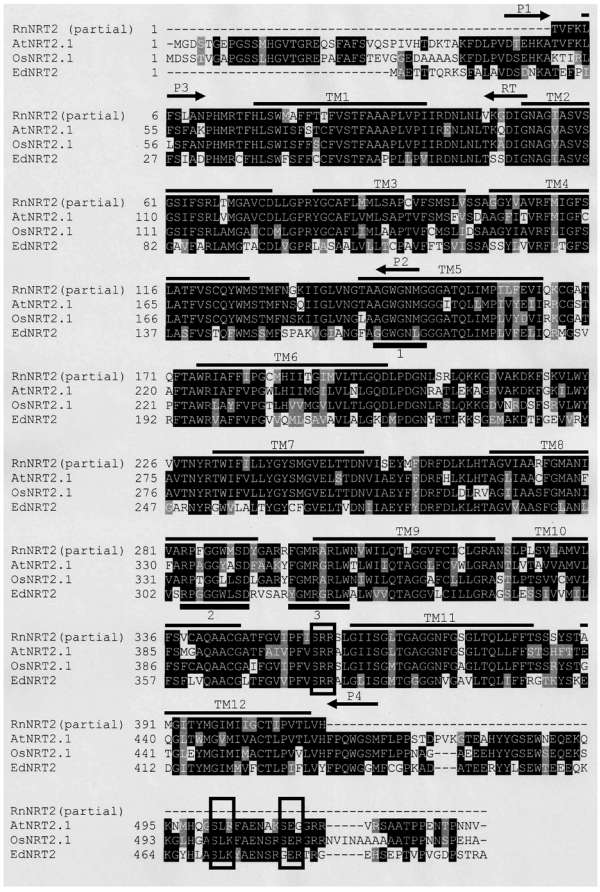

Figure 2. Alignment of partial RnNRT2 deduced amino acid sequences with those of NRT2s from *Arabidopsis thaliana*, *Oryza sativa* and *Egeria densa*. Regions where amino acids were identical within the four sequences are indicated in black. The 12 transmembrane domains of partial RnNRT2 predicted by the MEMSAT3 program are indicated by numbered lines above the sequences. The conserved NRT2 motifs are indicated by the numbered heavy line below the sequences. The conserved putative protein kinase C recognition motifs (S/TXR/K) are boxed. The numbered arrows above the sequences correspond to the amino acid sequences where primers were designed. Protein ID numbers are partial RnNRT2, this study; AtNRT2.1, AAC35883.1; OsNRT2.1, BAA33382.1 and EdNRT2, BAK51923.1.

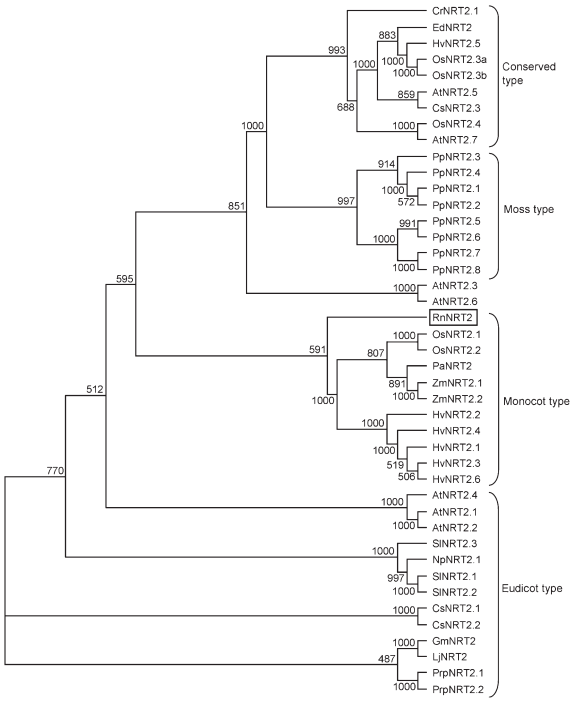

Figure 3. Phylogenetic analysis of deduced partial NRT2 protein sequences that correspond to an isolated partial RnNRT2 sequence. Numbers beside branches represent bootstrap values based on 1,000 replications. RnNRT2 is boxed. Protein ID numbers are *Ranunculus nipponicus* RnNRT2, this study; *Arabidopsis thaliana* AtNRT2.1, AAC35883.1; AtNRT2.2, AAC35884.1; AtNRT2.3, BAB10099.1; AtNRT2.4, BAB10098.1; AtNRT2.5, AAF78499.1; AtNRT2.6, CAB89321.1; AtNRT2.7, CAB87624.1; *Cucumis sativus* CsNRT2.1, AAS93686.3; CsNRT2.2, AGO64298.1; CsNRT2.3, AGO64299.1; *Glycine max* GmNRT2, AAC09320.1; *Lotus japonicus* LjNRT2, CAC35729.1; *Nicotiana plumbaginifolia* NpNRT2.1, CAA69387.1; *Prunus persica* PrpNRT2.1, BAD02939.1; PrpNRT2.2, BAD04063.1; *Solanum lycopersicum* SlNRT2.1, AAF00053.1; SlNRT2.2, AAF00054.1; SlNRT2.3, AAK72402.1; *Egeria densa* EdNRT2, BAK51923.1; *Hordeum vulgare* HvNRT2.1, AAC49531.1; HvNRT2.2, AAC49532.1; HvNRT2.3, AAD28363.1; HvNRT2.4, AAD28364.1; HvNRT2.5, ABG20828.1; HvNRT2.6, ABG20829.1; *Oryza sativa* OsNRT2.1, BAA33382.1; OsNRT2.2, BAG98877.1; OsNRT2.3a, BAG98894.1; OsNRT2.3b, BAD81572.1; OsNRT2.4, NP_918250.1; *Phragmites australis* PaNRT2, BAC76606.1; *Zea mays* ZmNRT2.1, AAN05088.1; ZmNRT2.2, AAT66252.1; *Physcomitrella patens* PpNRT2.1, BAD00097.1; PpNRT2.2, BAD00098.1; PpNRT2.3, BAD00099.2; PpNRT2.4, BAD00100.1; PpNRT2.5, BAD00101.1; PpNRT2.6, BAF42657.1; PpNRT2.7, BAF42658.1; PpNRT2.8, BAF42659.1; *Chlamydomonas reinhardtii* CrNRT2.1, CAA80925.1.

2003; Orsel et al. 2002), although *NRT2* is a single-copy gene in *E. densa* (Takayanagi et al. 2012). A full-length *RnNRT2* sequence would permit a functional analysis of RnNRT2 and determine whether or not *R. nipponicus* has other *NRT2(s)*. Recently, it has been reported that some NRT2 members require a partner protein, NAR2, for high-affinity nitrate transport (Xu et al. 2012). Further investigation is necessary to examine whether *R. nipponicus* has an *NAR2* ortholog responsible for nitrate uptake.

To evaluate the levels of *RnNRT2* and *EdNRT2* transcripts in each species, the shoots of small plants were fixed and then pre-treatment and nitrate treatment were applied under the same conditions described for ^{15}N-nitrate feeding experiments. Shoots were collected 0, 1, 3, 6 and 12 h after treatment initiation. Total RNA was isolated, cDNA was synthesised and real-time RT-PCR reactions were performed according to the method described by Takayanagi et al. (2011, 2012). The cDNA of *R. nipponicus* (1 μl of a 1 : 10 dilution), which had been synthesised from 1.5 μg aliquots of total RNA, was prepared as the template. The P3-forward primer and RT-reverse primer (5′-CCC TAT ATC GCC TTT CAC CA-3′) were used to examine the levels of *RnNRT2* transcripts (Figure 2). The levels of *RnNRT2* transcript was normalised against that of *RnActin* (accession no. AB778033), an internal reference gene, using the Act1-forward primer (5′-ATG CTC TCC CAC ACG CTA TC-3′) and the Act2-reverse primer (5′-TCG GCT GTT GTT GTG AAC AT-3′).

When nitrate was fed to shoots of both species at 15°C, the transcripts of *RnNRT2* and *EdNRT2* were induced within 1 h after the initiation of 0.2 mM nitrate treatment (Figure 4). The levels of *RnNRT2* transcripts rapidly increased, reached a maximum at 6 h and then decreased rapidly from 6 to 12 h after treatment initiation, whereas the levels of *EdNRT2* transcripts increased gradually up to 6 h and then decreased gradually (Figure 4). The induction fold of *RnNRT2* at 6 h after the initiation of nitrate treatment was 148.1 or approximately ten times higher than that of *EdNRT2* (14.8; Figure 4). This result is in agreement with the results of ^{15}N-nitrate feeding experiments (Table 1). These observations suggest that

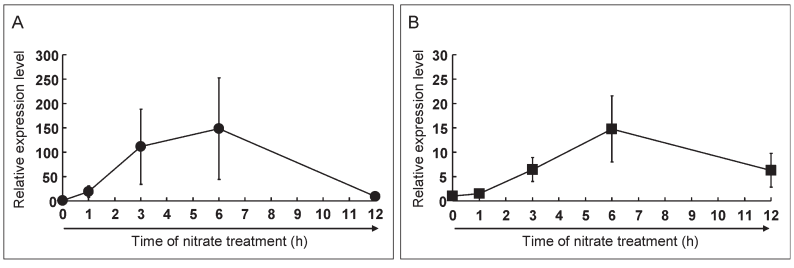

Figure 4. Changes in the levels of transcript of (A) *RnNRT2* and (B) *EdNRT2* in the shoots of *Ranunculus nipponicus* and *Egeria densa*, respectively, following nitrate treatment at 15°C. Shoots were treated with 0.2 mM KNO_3 containing 0.4 mM $CaSO_4$. Expression levels were presented as values relative to untreated control samples at 0 h after normalisation to actin levels. Values are mean of three to four replicates, each with three shoots of small plants. Bars indicate standard deviation.

the high induction fold of *RnNRT2* transcripts relative to *EdNRT2* transcripts contributes to active nitrate uptake at 15°C. Further investigations are needed to elucidate the relationship between the levels of *RnNRT2* transcripts and high-affinity nitrate uptake.

In conclusion, the shoots of *R. nipponicus* can actively take up nitrate at 15°C and are expected to be useful for the clean-up of nitrate-contaminated groundwater. This is supported by the following facts: under outdoor conditions, in several submerged species of Ranunculaceae, removal of the roots had no negative impact on the relative growth rate and activity of nitrate reductase, the primary nitrate reducing enzyme, was detected not only in roots but also in shoots (Cedergreen and Madsen 2003; Madsen and Cedergreen 2002). Therefore, the shoots are expected to be useful for the clean-up of nitrate-contaminated groundwater, although it is still necessary to estimate the abilities of nitrate storage and metabolism in the shoot tissues. Our results also suggest that, similar to nitrate uptake, the shoots of *R. nipponicus* can actively take up various nutrient ions and harmful substances such as radiocesium in cool water. The evaluation of the uptake ability of various ions by this plant awaits further investigation.

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