Endophytes from Natural *Festuca spp.* in Southwest China and Their Compatibility with Tall Fescue Cultivars

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남서중국의 자생페스큐의 엔도파이트와 톨페스큐 품종과의 공생

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ABSTRACT

Investigating endophyte distribution, naturally occurring in native grasses, is important for understanding endophyte-grass associations and using the beneficial effects of endophytes in cultivated plants. The goal of this study was to investigate endophytes from natural *Festuca* spp. in Yunnan, Guizhou, and Sichuan Provinces of China, and to study the compatibility between the endophytes and turf type tall fescue (*Festuca arundinacea*) which is widely used for lawn and athletic fields in that region. Of 628 accessions in *F. ovina*, 421 had endophytes identified in leaf sheath from on-site microscopic examination. From *Festuca spp*, three isolates were obtained from the seeds and ninety isolates were obtained from seedlings established from the collected seeds.

The isolates from *F. ovina* and *F. stapfii* were tentatively identified as *Neotyphodium typhinum* and *Neotyphodiumstarii*, respectively. We tested compatibility of the two fungal species with seven tall fescue cultivars, Little Hero, Sub Boy, Eldorado, Arid

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III, Millennium, Crossfire, and Fawn. *N. typhinum* or *N. starii* did not infect 'Fawn'with either seed injection or seed soaking method. The highest infection rate byboth *N. typhinum* and *N. starii* was in 'Sun Boy' and 'Eldorado'. There were significant interaction effects between tall fescue cultivar and type of endophyte on infection.

Key words: stress tolerance, symbiosis, fungus, Turfgrass

INTRODUCTION

Endophytes are organismsthat live inside a plant. Endophytic fungi existin many grasses. Some of them, such as *Epichloe typhina* cause "choke" diseases, which prevent the infected inflorescences from developing. The fungus can infect other plants through flowers. Closely related species within the genus *Neotyphodium* can infect tall fescue and perennial ryegrass (*Lolium perenne*). The mycelia of *Neotyphodium* fungi remain within the plants and spread by seed transmission. *Neotyphodium* was neglected mycologically until the late 1970s, when the infection of grasses by the fungi was found to be responsible foranimal disorders (Bacon, 1977). Later it was determined that the endophytic fungi synthesized toxic alkaloids that resulted in toxicoses occurring in animals (Gallagher et al., 1984). These same fungi may also contribute to the tolerance of the host grasses to many biotic and abiotic stresses (Arachevaleta et al., 1989).

Li et al. (1996) conducted a survey of the endophyte infection from 11 grass species from Xinjiang Province, Northwest China and reported that the infection rate for *Festuca alatavica* and *Achnatherum inebriabs* was 100% and 96%, respectively. Using random amplified polymorphic DNA analysis, Li et al. (2006) investigated the genetic diversity of 13 isolates of *Epichloe* and 9 isolates of *Neotyphodium* from grasses and found that isolates indigenous to China were different from those originating from Europe. Wang et al. (2005) investigated 644 accessions of grass species in Dongying, Shangong Province and found that endophytes were detected more often from the grasses in areas with saline-alkali soils. There are estimated 200 genera and 1,500 species in Gramineae from China while the number of worldwide total grass genera and species are approximately 700 and 10,000, respectively (Keng and Wang, 1996). Very few grass species have been investigated for the distribution and diversity of endophytes.

Tall fescue is one of the most important low-maintenance turfgrass species and one that benefits a great deal from symbiotic endophytes (Arachevaleta et al., 1989 Bouton et al., 2001; Gwinn and Gavin, 1992; Koppenhofer and Eugene, 2003; Malinowski et al., 2000; West et al., 1993). There are 56 *Festuca* sp. widely distributed in China (Liu, 2002) with the most abundant concentrations in the Southwest, Northwest, and Northeast. Even though members of this particular grass genus are widely distributed throughout China, limited focus has been placed on the natural endophyte occurring within these species.

Indentifying new endophyte resources that can improve tall fescue stress tolerance is the first step toward their applications. Effective use of endophytes in agronomy and turfgrass management relies on better understanding of the host-endophyte interactions. Species of endophytes vary in their production of alkaloids, host-endophyte compatibility, and effects on grass growth (DeBattista et al., 1990; Glenn and Bacon, 1997 Hill et al., 1996). This concept has guided many researchers toward investigating the distribution of endophyte in different grass species and different geographical locations (Hoverland, 1997). The objective of this study was to investigate naturally occurring endophytes in *Festuca* spp. in the southwest of China and to test the compatibility of those endophytes with commonly adapted turf-type tall fescue cultivars.

MATERIALS AND METHODS

Three southwestern Chinese provinces, Guizhou, Sichuan, and Yunnan were chosen for investigation of endophytesdue to rich diversity in species of *Festuca* spp. and logistical reasons. A large part of Yunnan is 2,000 m above sea level. The climate is affected by a plateau monsoon spring. Many climates exist at different elevations within the same low latitude. Guizhou has a subtropical humid climate with average annual rainfall of 1000-1300 mm and an average temperature of 15 °C. In general, the western part of Sichuan is plateaus and mountainous, some 4,000 m above sea level, while the east consists of basin and hilly lands at an elevation of 1,000 to 3,000 m. The Sichuan Basin has a humid sub-tropical monsoonal climate, with mild winters, hot summers, and large amount of rainfall and high humidity. The Sichuan Plateau has a plateau climate with lower temperatures and less rainfall than the Sichuan Basin.

Locations within each province were chosen based on the records of *Festuca* species in the local flora (Fang, 1988; Sun et al., 2003; Wu, 1988) (Table 1). Grasses were

identified based on the morphological descriptions in floras (Iconographia Cormophytorum Sinicorum, 1983), and comparisons with specimens in herbaria of the Institute of Botany of Chinese Academy of Sciences, Kunming Institute of Botany, and Guizhou Institute of Botany. *Festuca* species were the main interest of collection, but other grass species from each location were also examined to determine the presence of endophytes. A total of 1,091 plants from 100 accessions representing five genera were examined microscopically with the leaf tissue method as described below. Endophyte-positive plants were removed from the ground and stored in anice box with roots wrapped with wet towels. The plants were quickly shipped in moist and aerated bags to Beijing, where the plants were transplanted in pots containing a mixture of soil:sand:peat at 4:4:2 ratio on a volumetric basis. These plant samples were maintained in a glasshouse for further study.

Table 1. Locations where the grass samples were collected from Yunnan, Guizhou, and Sichuan Provinces of China.

Accession Code [z]	Location	Latitude	Longitude	Elevation
GH	Hezhang, Guizhou	N27.13	E104.71	2900
GW	Weining Meadow, Guizhou	N27.13	E104.28	2300
GX	Weinin Mountain, Guizhou	N27.13	E104.20	2100
SM	Meigu, Shichuan	N28.33	E103.14	2900
SP	Puge, Shichuan	N28.33	E103.14	3500
LA02-03	Yulong, Yunnan	N26.86	E100.25	3000
LA04	Yulong, Yunnan			3700
LB01	Heqing, Yunnan	N26.55	E100.18	2700
LB02-06	Heqing, Yunnan			2000
LB07	Heqing, Yunnan	N26.55	E100.10	2700
LG	Lijiang Wenbi, Yunnan	N26.68	E100.20	1800
LW00-03	Lijiang Wenbi, Yunnan	N26.86	E100.25	2800
LY02-04	Yunshen, Yunnan	N26.71	E100.76	2700
YL	Lijiang Hailongtan, Yunnan			1000
YQ01-YQ13	Heqing, Yunnan			2000
YH01-YH10	Huizhe, Yunnan	N26.41	E103.27	3500
YJ	Jianchuan, Yunan	N26.53	E99.88	3000
YW	Lijiang Wenbi, Yunnan			2800
YX1	Hogrila, Yunnan	N27.78	E99.72	1800
YX2	Xiaozhong Dian, Yunnan			3200
YY01	Yunshen, Yunnan	N26.71	E100.76	2800
YY02	Yunshen, Yunnan			2500

[z] Location codes are first letters of the region name followed by the county number.

Seeds from plants within the genus *Festuca* were collected from the same locations where plants were examined and collected. The seeds were air dried before storing in plastic bags at 4C and 45% relative humidity. At least 200 seeds from each accession

were planted individually in pots with a media of 70% perlite and 30% peat. The plants in the glasshouse were watered to field capacity every 3 days with Knop nutrient solution containing 0.25g KH_2PO_4, 1g $Ca(NO_3)_2$, 0.25g $MgSO_4$, and 0.12g KCl in 1 L water (Shive, 1915).

Detection of endophytes in plants

The method for detection of endophytes in living plants was based on Latch (1984). A leaf sheath from the plant was removed and the adaxial epidermis was cut aseptically with a sterile scalpel and then stripped with sterile forceps. The epidermal tissue was placed on a microscope slide and dyed with a drop of 1 g L^{-1} cotton blue in lacto-phenol. Endophyte infections were examined under a microscope at the 4-tiller stage from the lowest three sheaths. Three leaves were sampled per plant. The plant was recorded as infected if endophytic mycelium was observed by microscopic examination with 250-400 of magnification. Infection rate was calculated from the number of plants with endophyte divided by total number of plants examined.

Detection of endophytes in seeds

Fifty seeds from each accession were soaked in 50g L^{-1} NaOH for 16 h, rinsed 3 times with distilled water, dehulled, and then stained with aniline blue-lactic acid for 8 h (1 g of aniline blue + 100mL of water + 200mL of 85% lactic acid). Each stained seed was crushed with a cover slip and examined microscopically for the presence of endophyte mycelium.Infection rate was derived from the number of entophyte positive seeds divided by the number of seeds examined.

Isolation and culture of endophytes from seeds

Caryopses were sterilized by soaking in 9% sodium hypochlorite (NaClO) for 1 h and then rinsed three times with sterile water. The caryopses were then cut into pieces and placed onto potato-dextrose agar (PDA) medium and cultured at 28 C in the dark for 20 d (Clark et al., 1983 Guo et al., 1998).

To check the effectiveness of surface sterilization, the surface-sterilized caryopses were also used to roll onto the surface a separate PDA medium and then removed, the medium was cultured under same conditions as those with caryopsis embedded (Sturz, 1999).

Isolation and culture of endophytes from leaves

Sections of the leaf blade tip, approximately 1 cm in length, were surface sterilized

with 2% sodium hypochlorite for 2 minutes. The leaf piece was then rinsed with sterile water three times before being placed on petri plates containing PDA medium. The presence of endophytes was verified after the medium was cultured at 28 C in the dark for 10 d.

To check the effectiveness of surface sterilization, the surface-sterilized leaf fragments were also used to press on the PDA medium and the medium was cultured under the same conditions as those with leaf blades emplaced (Sturz, 1999).

Induction of conidia and characterization of fungi isolated

Mycelia cultured from seeds or leaves on PDA medium were transferred to malt extract agar (MEA) (Bill and Polischook 1992) and microglia medium (MM) (ScienCell Research Laboratories, Carlsbad, CA). Leaf tissues from endophytic plants were surface sterilized and autoclaved and then cut into 1-cm pieces to culture on MEA and MM media. All those isolations were then cultured under two different conditions: a) 28 C in dark, b) 12-h UV light and 12-h visible light alternatively at 28 C (Guo et al., 1998). Conidia were studied under a microscope 15 d later. Colony size, color, and conidia spores were recorded once a week during the culture.

Inoculation of tall fescue with endophyte isolates

Endophytes isolated from *F. ovina* and *F. staffii*were used to inoculate seven tall fescue cultivars: Little Hero, Sub Boy, Eldorado, Arid III, Millennium, Crossfire, and Fawn. Seeds of these cultivars were soaked in glycine solution (glycine:water at 3:1 ratio) and stored at 40 °C for one month to kill any potential endophyte (Christensen, 1998). Three different methods were used to inoculate the endophyte-free plants.

1). Seedling slitting: The endophyte-free seeds were planted in plastic pots and three months after germination, the plants were checked for presence of endophyte. The endophyte-free plants were sterilized by consecutive immersion of stem for one min in 75% alcohol, 10 min in 65% commercial Chlorox (final concentration 3.25% aqueous sodium hypochlorite) and 30 sec in 75% ethanol. A small slit was cut at the sterilized area with a scalpel. Some mycelium was inserted with tweezers into each cut, which was then wrapped with sterilized film (Latch, 1997).

2). Seedling injection: Dehulled endophyte-free seeds were sterilized with 9% sodium hypochlorite for 1 h and the caryopses were planted on half-strength Murashige and Skoog (½ MS) medium for 7-10 days to get endophyte-free seedlings.
Sterilized water was added to the one-month-old mycelium grown on PDA under dark conditions. The mycelia were broken up with a needle, transferred to a

50-mL flask, and shaken for 24 h to make a mycelium suspension. Two drops of suspension were injected with a syringe and needle into each endophyte-free seedlingcultured on ½ MS medium. The injected seedlings were then transferred to ½ MS medium in 50-mL flasks with 5 seedlings in each flask. A total of 300 seedlings were inoculated for each cultivar. The seedlings were grown under alternating light conditions with 12 h dark and 12 h light at 25 °C for 20 d. Thereafter, the plants were transplanted to plastic pots with sterilized soil (perlite:peat at 7:3) and watered as needed. Fifty plants from each cultivar were observed for the presence of endophytes two months lateras described above (Latch and Christensen, 1985).

3). Seed Soaking: Endophyte-free seeds of the 7 cultivars were sterilized with 9% sodium hyperchloride and put in petri dishes with a piece of cheese cloth at the bottom. One milliliter of sterile water and 2 mL of mycelium suspension was added to the petri dish and sealed and cultured at 35 $\mu molm^{-2}s^{-1}$ light intensity and 25 °C. A total of 400 seeds for each cultivar were inoculated, with 40 seeds in each dish. The mycelial suspension was added every three days. Two weeks later, when the seeds germinated, the seedlings were transferred to ½ MS medium and incubated for 20 days. The seedlings were then transferred to plastic pots with sterilized soil (perlite:peat at 7:3) and watered as needed. Fifty plants from each cultivar were checked for endophyte presence two months later as previously described.

Infection rate data were analyzed with t-test and endophyte-host compatibility data were subjected to analysis of variance (ANOVA) using SAS GLM procedure (SAS Institute, 2008). Treatment means were separated with Duncan's least significant difference upon significant F-test.

RESULTS AND DISCUSSION

Endophytes in the field plants

Species identified and examined for presence of endophytes on site were *Festuca forestii* St.-Yves, *F. stapfii* E. Alexeev, *F. pratensis* Huds, *F. ovina* L., *F. rubra* L., *Bromus pseudoramosus* Keng, *Agrostis stolonifera* L., *Poa krylovii* Reverd., *Helictotrichon clelavayi*, *Brachypodium sylvaticum* (Huds) Beauv, *Danthonia cumminsii* Hook, *Arundinella khaseana* Nees ex Steud, and *Helictotrichon junghuhaii*. Of the genus *Festuca*, only *F. ovina* had endophytes in the sheath and infection rate varied from 45 to 78% among locations. The endophyte infection rate in seed was negatively

correlated with elevation (r^2=-0.67), while the infection of leaves was not correlated to the elevation.

Endophyte distribution in collected seeds and the regenerated plants

Endophytes were identified in whole plants and seeds of all the grass species examined except *F. forrestii* and *Danthonia cumminsii* Hook (Table 2). Infection rate in other *Festuca* species ranged from 13 to 82%. The checks on sterilization showed no growth of fungi indicating that the isolations were from inside the plants. Average seed infection rate was 51%, significantly higher than the 26% infection rate in leaves based on an *F*-test (p<0.001). With the exception of *F. stapfii* from Yunshen, Yunnan Province, endophyte infection rates were higher in seeds than in seedlings indicating that not all endophytes were alive or capable of growing into seedlings. Our results were similar to those reportedby Oliveira and Castro (1997) where the infection rate was higher in seeds than in seedlings.

Table 2. Endophyte infection rate in seeds collected from field accessions and in seedlings grown from the seeds.

Accession Code[z]	Species	Seedlings	Seeds
		%	
LA03	*Brachypodium sylvaticum*	--	5
LA02	*Danthonia cumminsii*	--	10
LA04	*Danthonia cumminsii*	--	0
LB01	*Arundinella khaseana*	--	82
LB07	*Helictotrichon junghuhaii.*	--	25
LB02	*Festuca forestii*	--	0
LB06	*F. rubra*	--	13
YQ01-YQ13	*F. ovina* L.	68	82
YH01-YH10	*F. ovina*	40	76
YJ	*F. ovina.*	20	36
YY01	*F. ovina.*	2	52
SM	*F. ovina*	58	63
SP	*F. ovina*	4	56
YW	*F. pratensis*	--	15
YL	*F. pratensis*	--	23
YY02	*F. pratensis*	--	35
LY02	*F. stapfii*	56	52
LW2	*F. stapfii*	46	68
$LSD_{0.05}$		27	29

[z]Accession codes are first letters of the region name followed by the county number same as locations code in table 1. 50 seeds per accession were tested.

Endophytic Fungi Morphology

White mycelium grew out of the seeds in the cut areas after about 30 d of culture.

Three endophytic isolates were obtained from the seeds of *Festuca* sp. The surface sterilized checks showed no fungal growth,indicating that the isolation was from inside the seeds.

Ninety endophytic isolates were obtained from the plants established from 41 accessions of *F. ovina* and *F. stapfii*. The isolates from *F. ovina* did not produce visible symptoms on the hosts. The fungal colonies were white and cottony with the bottom side of petri plate tan to brown on a PDA medium. Also, the fungal colonies were crowned in the center and flat at the margin. Conidiogenous cellsizes were 11.2-28.2 mm × 2.2-3.0 mm. Conidiogenous cells had a basal septum with an average ratio of length to diameter of 6.41 (Table 3). The isolates were tentatively identified as *Neotyphodium typhinum* because of the many morphological similarities to this species reported by White and Morgan-Jones (1987b).

The isolates from *F. stapfii* produced no symptoms on the hosts. After culturing on PDA at 28 °C, the colonies were white, cottony with the bottom side of petri plate pale brown. Aerial hyphae were hyaline, separate, smooth, and grew into bundles. The colonies grew slowly, approximately 0.7-1.5 mm d^{-1}, and reached an average diameter of 16 mm after 14 d incubation at 28°C. The average growth rate was 0.99 mm d^{-1}, significantly slower than 1.49 mm d^{-1} of the isolates from *F. ovina* (Table 3). Conidiogenous cells were cylindrical at the base, without septa, and attenuating towards the apex. Conidia were kidney-shaped, 5.8-6.8 mm × 2.0-2.7 mm. The conidia length to diameter ratio was 2.78, significantly higher than theratio of 1.78 of the isolates from *F. ovina* (Table 3). These isolates were tentatively identified as *Neotyphodium starii*due to the similarities to the species reported by White and Morgan-Jones (1987c).

The combination of MEA medium and alternative UV and visible light induced greater sporulation than the other cultural methods used in this study. *N. starii* had fewer hyphae, more compacted mycelia, and a slower rate of growth than *N.*

Table 3. Colony and conidium morphology of endophytic fungi isolated from two *Festuca* species.

Host	Number of isolates	Colony morphology on potato-dextrose agar				Conidia morphology on malt extract agar			Species
		Color	Texture	Mycelium	Growth rate[z]	Shape	Conidium length to diameter ratio	Conidiogenous cell length to diameter ratio	
					mm/d				
F. ovina	57	White	Cottony	Long, dense	1.49 (0.46)	Oblong	1.78	6.41	N. typhinum
F. stapfii	33	White	Cottony	Short, dense	0.99 (0.23)	Round	2.78	9.74	N. starii
t-test					**		**	**	

[z] Means followed by standard deviation in parenthesis.
*, ** Significant at 0.05 and 0.01 probability levels, respectively.

typhinum (Table 3), similar to earlier reports (Latch, et al., 1984; Morgan-Jones and Gams, 1982; White and Morgan-Jones, 1987a,b,c).

Compatibility of endophyte with tall fescue

None of the seven tall fescue cultivars were successfully inoculated with endophytes using the seedling slitting method. Only the results from seedling injection and seed soaking methods are reported here. The seedling survival rate post-inoculation rangedfrom 10 to 94.4%, but was not significantly affected by the different inoculation methods or edophytes. Similar endophyte-grass compatibility results were reported by Christensen et al. (1993).

Table 4. ANOVA of compatibility between seven tall fescue (*Festuca arundinacea*) cultivars and *N starii* and *N. typhinum* tested by two artificial inoculation methods.

Source of variation	df	*F*	*P*
Replication	2	0.28	0.7568
Inoculation Method (I)	1	1.79	0.1858
Cultivar (C)	6	52.15	<0.0001
Endophyte (E)	1	0.33	0.5684
I x C	6	1.08	0.3876
C x E	6	5.46	0.0001
I x E	1	0.02	0.8890
Error	60		

Infection rates differed among cultivars (Table 4). There was an interaction effect on infection rate between tall fescue cultivar andendophyte. Inoculation method had no effect on the infection rate.No interaction effect between inoculation method and cultivar was detected. There was no interaction between inoculation method and endophyte. *N. typhinum* did not infect 'Fawn' with either method. The highest infection rate by *N. typhinum* was in 'Sun Boy' and 'Eldorado'. The highest infection rate by *N. starii* was in 'Sun Boy' and 'Arid III' (Table 5).

Table 5. Compatibility between seven tall fescue (*Festuca arundinacea*) cultivars and *N starii* and *N. typhinum* tested by two artificial inoculation methods.

Tall fescue cultivar	Number of endophyte-positive plants in 50 samples.					
	N. typhinum			N. starii		
	SS[z]	SI[y]	Means[x]	SS	SI	Means
Millennium	3.0	3.0	3.0cb	4.0	4.4	4.2ab
Arid III	3.2	3.4	3.3b	4.5	6.0	5.3a
Eldorado	4.7	4.4	4.6a	3.8	3.7	3.8bc
Crossfire II	2.6	2.4	2.5cb	1.4	0.6	1.0d
Little Hero	1.8	3.1	2.5c	2.3	2.8	2.6c
Fawn	0	0	0d	0	0	0d
Sun Boy	5.0	5.6	5.3a	5.0	5.3	5.2a
Average	2.9	3.1		3.0	3.3	

[z]SS= Inoculation by seed soaking
[y]SI= Inoculation by seedling injection
[x]Within columns, means followed by the same letter are not significantly different according to LSD(0.05).

CONCLUSIONS

Endophytes were identified in 11 of 13 grass species tested from Yunnan, Guizhou, and Sichuan Provinces of China. There was a big pool of endophyte resources and variation among grass species and locations. Endophytes were identified more often from seeds or seedlings established from the collected seeds than from the plants in the field. Obvious different compatibility differences were noticed among the seven tall fescue cultivars both for *N. typhinum* and *N. starii*. This information may be used by breeders to incorporate endophytes into targeted cultivars. Further investigation is needed to test the influence of those endophytes on stress tolerance and turf quality. Comparison of the endophytes isolated from native grasses and from those isolated from tall fescue cultivars is also needed.

국문 요약

자생화본과 식물에서 자연적으로 생기는 엔도파이트 분포의 연구는 엔도파이트 공생에 대한 이해와 재배식물에서의 엔도파이트의 잇점에 대하여 중요하다. 본 연구는 중국 유난, 구이죠우, 시촨지역에서 자생하는 페스큐로부터 엔도파이트를 조사하고, 그 지역에서 널리 식재되는 잔디형 페스큐와의 융화에 대하여 연구하는데 목표를 두었다. 현장의 현미경 관찰 결과628개체의 sheep fescue 중 421개체가 엽초조직에서 엔도파이트가 동정되었다. 페스큐종에서 3개는 종자에서, 90개의 엔도파이트 분리체가 수집된 종자의 유묘에서 수집되었다. sheep fescue와 stapfii 페스큐의 엔도파이트는 *Neotyphodium typhinum* 와 *Neotyphodium starii*로 각각 동정되었다. Little Hero, Sub Boy, Eldorado, Arid III, Millennium, Crossfire, 그리고 Fawn 톨페스큐 7품종에서 위 2종의 균류의 융화성을 조사한 결과, *N. typhinum* 나 *N. starii*는' Fawn' 품종에서 종자주입방법이나 침지방법으로도 접종되지 않았다. *N. typhinum* and *N. starii* 는 'Sun Boy'와 'Eldorado'에서 가장 높은 접종율을 보였다. 톨페스큐 품종과 엔도파이트 타입과의 접종에서 매우 높은 유의성의 교호 효과를 보였다.

주요어 : 곰팡이류, 공생, 스트레스저항성, 잔디

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