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Does invasion involve alternation of germination requirements? A comparative study between native and introduced strains of an annual Brassicaceae, *Cardamine hirsuta*

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Abstract *Cardamine hirsuta* is a European annual weed that has been naturalized in Japan. Although the species is a widespread weed in Europe, its introduction to the Japanese Islands occurred recently. We hypothesized that the introduction of *Cardamine hirsuta* required adaptation that has delayed its spread in Japan. The aim of this study is to determine whether there is a difference in temperature requirements for seed germination between Japanese and European strains of *Cardamine hirsuta*. We compared temperature requirements for seed germination, because it is known to be a critical determinant of phenology and thus is expected to be important in the success of introduction into different climates. Seeds of six and five strains from Japan and Europe, respectively, were used in seed germination experiments under five different temperature regimes. Japanese strains generally showed stronger initial seed dormancy and were characterized by suppressed germination in higher temperature regimes. European strains showed variable patterns of temperature-dependent seed germination. It turned out that these temperature-dependent dormancies are mediated by abscisic acid (ABA), because dormant seeds germinated under the presence of an inhibitor of ABA synthesis. Seed germination characteristics shared by Japanese strains presumably enhance long transport and autumn germination, while

these characteristics are not necessarily common among European strains. The results are supportive for the idea that adaptation has altered germination characteristics of *Cardamine hirsuta* through the introduction process into Japan.

Keywords Adaptation · Abscisic acid · Plant invasion · Seed dormancy · Temperature dependence

Introduction

Species introductions beyond their natural distribution ranges became frequent events, and the majority of them are related with human activities. Explosive spread of an alien plant has been referred to as plant invasion. Certain characteristics of plant life history, such as production of numerous seeds, rapid life cycles and extensive clonal spread, have been considered to allow the species to expand their ranges explosively (Baker 1974; Mack 1996; Williamson and Fitter 1996). In Japan, more than 1,400 species are considered to have been introduced over the past 200 years (Ecological Society of Japan 2002), providing numbers of opportunities to study plant invasion.

The invasion process itself may require alternation of life-history characteristics of alien species (Reznick and Ghalambor 2001; Lee 2002). Several recent studies have reported that introduced plants exhibit modified life-history characteristics compared with plants from their native ranges (Thébaud and Abbott 1995), and some of them are considered to be the results of adaptation to the new environments (Blossey and Nötzold 1995; García-Ramos and Rodríguez 2002; Maron et al. 2004). Therefore, all of the genotypes within species are not necessarily invasive, and in some cases, successful invasion is allowed only for minority genotypes in the native populations. Consequently, successful invasion may be delayed until arrival of fitted genotypes (Ellstrand and Schierenbeck 2000).

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Cardamine hirsuta L. is a European annual weed that has been naturalized into Japan (Kudoh et al. 1992). Although the species is a widespread weed in Europe, its introduction to the Japanese Islands occurred recently. The earliest specimen in Japan was collected in 1974, and the distribution in Japan was localized when it was first reported (Kudoh et al. 1992). *Cardamine hirsuta* rapidly expanded its distribution range during the 1990s in Japan. It became a common roadside weed over most of Honshu Island, the main island of Japan, and is considered to be continually spreading also to Kyushu and Hokkaido (Yatsu et al. 2003; Kudoh et al. 2006; Lihová et al. 2006). This is an exceptional case among widespread European weeds that usually had been introduced and spread in Japan much earlier (Ecological Society of Japan 2002). This fact raises a question as to what had delayed the introduction of *Cardamine hirsuta*. We hypothesize that, if the introduction of *Cardamine hirsuta* required adaptation, there should be ecological differences between Japanese and European plants.

The aim of this study is to determine whether there is a difference in temperature requirements for seed germination between Japanese and European *Cardamine hirsuta*. As a candidate character for adaptation, we selected the seed germination requirement, because it is expected to be critical both in survival during long-distance transport and in adaptation to different climates. Rapid and high germination rates have often been associated with invasiveness (Baker 1974; Radford and Cousens 2000; Mandák 2003), but it has been reported that invasive species do not necessarily show rapid and high germination rates (Mandák 2003; Milbau et al. 2003). Furthermore, only a few studies really compared seed germination characteristics between invasive and native populations (Erfmeier and Bruelheide 2005). We predicted that seeds with deeper dormancy would have a greater chance to be transported into a distant area. We also predicted that there would be a specific temperature requirement shared by Japanese strains if adaptation to local seasonal environments was required prior to the recent spreads in Japan.

Materials and methods

Plants

Cardamine hirsuta is an annual weedy species distributed across Europe. Although its distribution history within Europe is unknown, European distribution should have been formed earlier and should include the natural distribution range (Jalas and Suominen 1994; Marhold 1996). It has been reported that *Cardamine hirsuta* behaves most typically as a winter annual in Europe, but summer-annual populations also exist (Grime et al. 1988). Recent introduction to Japan, North America, Australia, North Africa and other areas has been reported (Kudoh et al. 1992, 2006; Lihová et al. 2006). In Japan, the germination period starts in September and

plants form rosettes to overwinter (Yatsu et al. 2003). The flowering period extends from February to April in Japan, and the plants produce numerous seeds through auto-pollination (Yatsu et al. 2003). Seeds are mechanically dispersed by dehiscent fruits, but dispersal distances are within a couple of meters. Most long distance dispersals are obviously attributable to human activity accompanied by transport of soils (H. Kudoh, unpublished observations). It is known to be a diploid species ($2n = 16$, Marhold 1994; Kučera et al. 2005).

Seed preparation

Six and five strains from Japan and Europe, respectively, were used in the experiments (Table 1). Seeds from a single individual from each locality were sown and germinated in a greenhouse at Kobe University (N 34°44', E 135°14') in June 2005. Germinated individuals were transplanted in pots and were grown in a growth chamber (12 h day length and 25°C/15°C of day/night temperature) for 3 months. Four to six individuals per locality were obtained. Three-week cold treatment (5°C and dark) on these grown rosettes to promote flowering was applied. After the cold treatment, plants were placed in the greenhouse and kept until they produced seeds. Plants were covered with nets prior to flowering, and seeds were produced through auto-pollination. Matured fruits dehisced within nets and released seeds. Collected seeds were put in micro tubes and stored in desiccators at room temperature until the germination experiments. Seeds from a single individual for each locality that produced enough numbers of seeds were used in the following experiments.

Germination experiments

Germination experiments were started between 1 and 2 months after seed collection for all strains. Germination experiments were conducted under five different temperature regimes, i.e., 35°C/25°C, 30°C/20°C, 25°C/15°C, 20°C/10°C and 15°C/5°C of day/night temperature (12 h day length), provided by a temperature gradient incubator (TG-200-AD, Nihon-Ikakiki Co., Osaka, Japan). For the strain from Slovakia, germination experiments were conducted under four temperature regimes (the 25°C/15°C treatment was excluded) because we failed to obtain enough seeds to conduct experiments under five temperature regimes. Approximately 50 seeds were sown on 40-g crystal sands in a Petri dish (9 cm in diameter) and kept moistened by distilled water during the germination experiments. Three replicates of dishes were used for each strain and temperature regime. Petri dishes were observed at 2-3-day intervals, and germinated seeds were recorded and removed. Cumulative ratios of germinated seeds to the tested seeds were determined for each Petri dish at 1, 2 and 5 weeks after seed sowing. For seeds that had not germinated by

Table 1 Localities, latitude, longitude and altitude where six Japanese and five European strains used in this study were originally collected

Strains	Locality	Latitude (N)	Longitude (E)	Altitude (m)
Japanese strains				
Miyagi	Miyagi Prefecture: Sendai-shi, Aoba-ku, katahira	38°15'	140°52'	50
Gunma	Gunma Prefecture: Tone-gun, Katashina-mura, Higashiogawa	36°47'	139°15'	860
Ibaraki	Ibaraki Prefecture: Ishioka-shi, Takahama	36°10'	140°18'	5
Tokyo	Tokyo: Hachioji-shi, Minami-osawa	35°37'	139°23'	140
Shiga	Shiga Prefecture: Ohtsu-shi, Kitahira	35°13'	135°56'	100
Hyogo	Hyogo Prefecture: Kobe-shi, Nada-ku, Rokkodaicho	34°44'	135°14'	120
European strains				
Russia	Krasnodarskii krai: Sochi, near village of Krasnaya Skala	43°40'	39°55'	120
Slovakia	Velká Fatra Mts., Mt. Borišov (collected in the Botanical Garden of Comenius University in Blatnica)	48°56'	18°55'	1,400
Slovenia	Subpanonsko območje: between Ptuj and Placar	46°29'	15°53'	240
Italy	Province Piedmont: Torino, Stupinigi	44°59'	7°35'	250
Spain	Province Guipuzcoa: near village Pikoketa, South of Irun	43°21'	1°47' (W)	400

5 weeks after seed sowing, we continued observation for an additional 5 weeks, and the minority of seeds was germinated during this period. Then, we applied 10 μ M fluridone, an inhibitor of abscisic acid (ABA) biosynthesis (Bartels and Watson 1978; Xu and Bewley 1995), to each Petri dish. We applied these treatments primarily to know whether ungerminated seeds were alive or not and then secondarily to know if the seeds were in the ABA-mediated dormant state. We recorded seed germination for an additional 5 weeks, and the final ratios of germinated seeds were recorded. During this period, after 2 weeks of the fluridone applications, all Petri dishes were moved into the 25°C/15°C temperature regime to facilitate seed germinations. Most of seeds germinated by the end of the experiments, and the final germination ratios ranged from 96 to 100%.

Data analyses

The ratio of germinated seeds was determined for each Petri dish at 1, 2 and 5 weeks after the seed sowing and at the end of the experiment after the fluridone application. The ratio of germinated seeds was calculated as the cumulative number of germinated seeds during given periods divided by the initial number of tested seeds. The ratios of germinated seeds at 1, 2 and 5 weeks after seed sowing were analyzed separately using two-way nested ANOVA. We tested the effects of temperature, strain and origin (Japan or Europe). Interaction terms between temperature and origin and between temperature and strain were included in the model. Strain term and temperature by strain interaction were nested into the origin. The data were arcsine-square root transformed prior to the statistical analyses.

Results

Germination responses to different temperature regimes varied both between origins and among strains (Fig. 1),

and all of the interaction terms in the nested ANOVA were statistically significant for the ratio of germinated seeds at 1, 2 and 5 weeks after seed sowing (Table 2). In the nested ANOVA, all of the strain and temperature terms were highly significant, and the origin term was significant for the ratio of germinated seeds at 5 weeks (Table 2). Generally, Japanese strains tended to show stronger dormancy than European strains (Fig. 1). Three strains from Japan, i.e., Miyagi, Ibaraki and Hyogo, showed almost no germination during 5-week observation periods across all five temperature regimes (Fig. 1). The other three Japanese strains showed partial germination of seeds at lower temperature regimes, but still the highest ratios of germinated seeds within 5 weeks were 0.62 and 0.29 on average for Gunma and Tokyo, respectively. A strain from Shiga exhibited relatively higher germination ratios, and approximately half of seeds germinated at the three lowest temperature regimes within 2 weeks and almost all of the seeds germinated within 5 weeks except for the highest temperature regime, 35°C/25°C (Fig. 1). Among the European strains, temperature regimes with the highest germination ratio varied, and two strains, i.e., Slovakia and Spain, showed nearly 100% germinations even in the highest temperature regime. Rapid germination was observed in these two strains, and germination started within the 1st week of the experiments under all temperature regimes (Fig. 1). In the two strains, more than 80% of seeds germinated by 2 weeks after seed sowing except for the Spanish strain in 35°C/25°C, and nearly all of the seeds germinated across all temperature regimes within 5 weeks (Fig. 1). Few seeds germinated within the first 2 weeks for the other three strains from Europe, but strains from Slovenia and Italy showed a high germination rate in intermediate and low temperature regimes at 5 weeks (Fig. 1). A strain from Russia showed a generally lower germination ratio at 5 weeks compared with other European strains (Fig. 1). Most of all ungerminated seeds germinated within 5 weeks after the application of fluridone (Fig. 1).

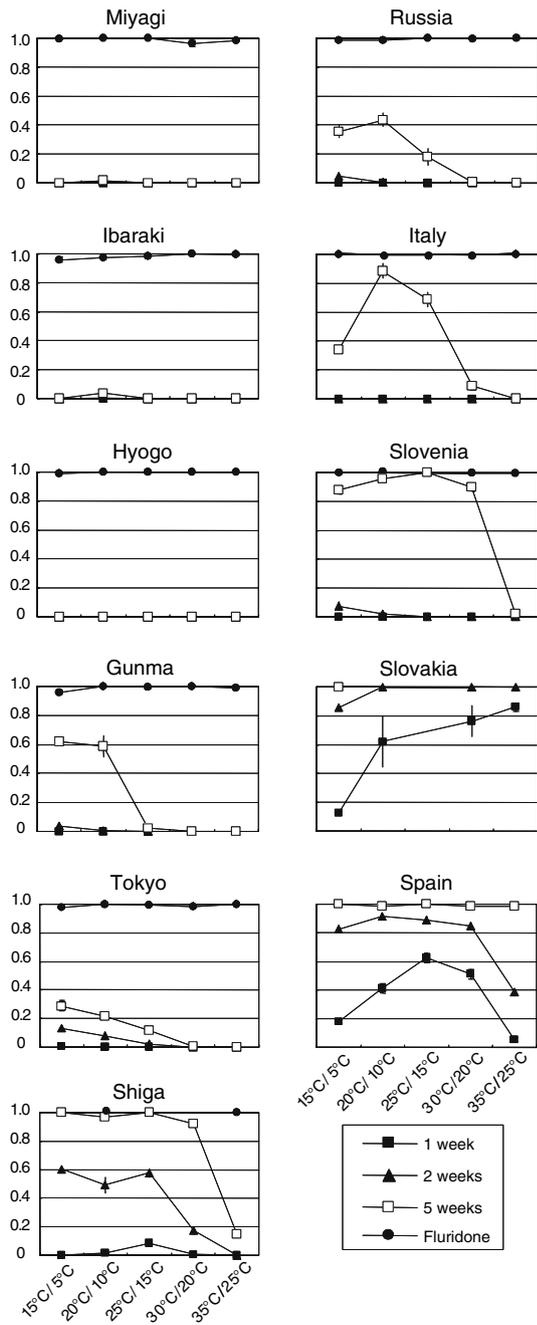


Fig. 1 Cumulative ratios of germinated seeds under five different temperature regimes for six Japanese (left column) and five European strains (right column) at 1 (closed square), 2 (closed triangle) and 5 weeks (open squares) after seed sowing. The final ratios of germinated seeds after fluridone applications are also shown (closed circles). Vertical bars indicate standard errors. Note that symbols for the latest timing appear when ratios of germination are unchanged. When all of seeds germinated either within 2 or 5 weeks and/or fluridone application were not made and therefore do not appear on the figures

Discussion

Japan is one of the excellent systems to study plant invasion because of its geography and human history.

Plant invasion is relatively easy to identify in the islands when compared with the continental area. When we find continental species with low seed dispersal abilities in Japan, we judge that the seeds had been brought to Japan by human activities. Furthermore, because of governmental policy, travels and trades with European and North American countries were minimal by the end of 19th century. Therefore, the majority of plant species with European and North American origins are expected to have been naturalized during the 20th century (Ecological Society of Japan 2002). *Cardamine hirsuta* is one of the most recently introduced European plants that is widely spreading into Japan. The first record is the specimen from Tottori Prefecture collected in 1974 (Kudoh et al. 1992), and the species was rapidly spreading across Honshu during the 1990s (Yatsu et al. 2003).

We found variation in seed germination responses among Japanese as well as European strains. Despite the recent introduction, it seems that there is a certain level of genetic variation in seed germination characteristics among Japanese plants. Although seed germination responses are known to be under the influence of maternal environments (Munir et al. 2001; Donohue et al. 2005a), we tried to minimize the maternal effects by obtaining seeds from plants grown in the common garden. The existence of genetic variation in seed germination characters among Japanese strains may be explained by multiple introductions from independent sources, but further molecular studies are required to test this speculation. Lihová et al. (2006) reported low genetic diversity in *Cardamine hirsuta* even among European strains based on nuclear ITS and cpDNA sequences. Although we have found multiple ITS haplotypes among Japanese strains in our preliminary molecular study (H. Kudoh et al., unpublished data), more variable DNA markers are required to resolve historical migration patterns of the species. Previous studies reported that introduced plant populations often had reduced genetic variation compared with those of native populations (e.g., Husband and Barrett 1991; Amsellem et al. 2000). Recent studies, however, have shown that introduced populations may have comparable or even increased genetic variation (Neuffer and Hurka 1999; Lee et al. 2004; Maron et al. 2004; Genton et al. 2005).

We expected that we would find differences between Japanese and European strains if the introduction process requires adaptation. For invasive species, introduction to new environments may alter strength and direction of natural selection that have maintained life-history characteristics in native environments (Reznick and Ghalambor 2001). Under strong selection, it is expected that populations go extinct unless there is enough genetic variance for introduced populations to respond to new environments (Gomukiewicz and Holt 1995). Multiple introductions have been considered to be required for introduced species to become invasive through adaptation (Ellstrand and Schierenbeck 2000). Therefore, in the case of *Cardamine hirsuta*, we expected

Table 2 The results of two-way nested ANOVAs on ratios of germinated seeds at 1, 2 and 5 weeks after seed sowing

Dependent	Factor (nested in)	Degree of freedom	Sum of squares	Mean square	F	P-value
Germination after 1 week	Origin	1	3.11	3.11	3.0	0.12
	Strain (origin)	9	9.31	1.03	81.1	<0.0001
	Temperature	4	0.28	0.071	5.6	0.0004
	Origin × temperature	4	0.23	0.058	4.5	0.0020
	Strain × temperature (origin)	35	1.86	0.053	4.2	<0.0001
	Residual	107	1.36	0.013		
Germination after 2 weeks	Origin	1	6.19	6.19	1.9	0.20
	Strain (origin)	9	28.9	3.21	654	<0.0001
	Temperature	4	0.62	0.15	31.4	<0.0001
	Origin × temperature	4	0.13	0.033	6.7	<0.0001
	Strain × temperature (origin)	35	2.49	0.071	14.5	<0.0001
	Residual	107	0.53	0.005		
Germination after 5 weeks	Origin	1	20.1	20.1	5.4	0.045
	Strain (origin)	9	33.5	3.73	417	<0.0001
	Temperature	4	6.09	1.52	170	<0.0001
	Origin × temperature	4	0.62	0.15	17.3	<0.0001
	Strain × temperature (origin)	35	7.98	0.23	25.5	<0.0001
	Residual	107	0.96	0.009		

The effects of origin (Japan or Europe), strain (nested in the origin), temperature and their interactions were tested. Data were arcsine-square root transformed in the analyses

that two types of germination characteristics have been important during introduction process, i.e., characteristics that have enhanced arrivals to Japan and those for local adaptation to the climates in Japan.

In our experiments, Japanese strains were characterized by strong initial seed dormancy and also by suppressed germination in higher temperature regimes. Three strains from Miyagi, Ibaraki and Hyogo showed almost no germination within 5 weeks across all temperature regimes. Strong initial dormancy is expected to allow *Cardamine hirsuta* to form persistent seed banks that may have been transported with soils (Jalloq 1974) and may have increased the probability of arrival to Japan from distant areas. Strong initial dormancy together with suppressed germination in higher temperature regimes presumably enhances autumn germination, and these characteristics are not necessarily common ones among European strains. Postponing germination of spring-dispersed seeds to autumn may be adaptive in seasonal climates in Japan. *Cardamine hirsuta* behaves in Japan as a strict winter annual, and no summer-annual life-cycle is observed (Yatsu et al. 2003; H. Kudoh, personal observation). Not only initial seed dormancy investigated in this study, but also conditions that release or re-induce seed dormancy are critical determinants of germination timing in natural habitats (Baskin and Baskin 1998). For instance, three Japanese strains (Miyagi, Ibaraki and Hyogo) did not germinate under all temperature regimes within 5 weeks of the experimental periods of this study, and there should be a certain environmental cue to release seed dormancy in their natural habitats. The study on germination characteristics of an introduced population of *Cardamine hirsuta* in Kentucky, North America (Baskin and Baskin 1986), may be relevant to mention here. The Kentucky strain showed a similar pattern of seed germination and dormancy with those for Japanese strains in this study,

i.e., strong initial dormancy and suppressed germination in higher temperature regimes. Furthermore, it turned out that, in the Kentucky strains, seeds expanded their germination ranges toward higher temperature after the seeds experienced summer temperature regimes.

On the other hand, the patterns of temperature-dependent suppression of germination were variable among European strains. Although we do not know how the European strains used in this study behave in fields, there may be variation in their life cycles. For example, the Russian strain showed a similar pattern with those found among Japanese strains and is likely to behave as a winter annual. It is noteworthy to mention that the Sochi region where the Russian strain used in this study was collected has similar climates to those observed in Honshu, Japan (Rybak et al. 1994). The Sochi region is on the coast of the Black Sea with 3–5°C and 22–26°C of winter and summer monthly mean temperature, respectively, 100–200 mm monthly precipitation, and ca. 60 days snow cover (Rybak et al. 1994). The Slovakian strain showed enhanced seed germination in higher temperature regimes that is likely to result in spring–summer germination. The Slovakian strain originated from high altitude (ca. 1,400 m, Table 1) and may either behave as a summer annual or require longer vegetative growth before winter when it acts as a winter annual.

In previous studies, Erfmeier and Bruelheide (2005) compared germination characteristics of *Rhododendron ponticum* between invasive and native populations. Although invasive populations germinated earlier than native populations did, differences in the maximum germination rate and optimum germination temperature were not detected in *R. ponticum* (Erfmeier and Bruelheide 2005). Adaptation to new climates has been reported in several studies, although they did not examine germination characteristics. In *Solidago*

species, non-native European populations exhibited latitudinal differentiation in phenology and growth traits (Weber and Schmid 1998). In *Capsella bursa-pastoris*, it is reported that multiple introductions allowed the pre-adapted European ecotypes to form clines along climatic variation in new Californian environments (Neuffer and Hurka 1999). More recently, Maron et al. (2004) compared native European and introduced North American populations of *Hypericum perforatum* using common garden experiments and AFLP analyses, and concluded that adaptive evolution played important roles for introduced populations to form a latitudinal cline.

It turned out that seed dormancy observed in this study is a physiological one mediated by ABA, because dormant seeds became germinated under the presence of an inhibitor of ABA synthesis, fluridone. It is known that ABA-mediated dormancy is one of the major mechanisms of temperature-dependant seed germination that controls timing of plant emergence in the seasonal environments (Yoshioka et al. 1998; Ali-Rachedi et al. 2004). For example, one of the *Arabidopsis thaliana* strains has been reported to exhibit ABA-mediated dormancy after dispersal and suppressed germination at high temperature regimes (Ali-Rachedi et al. 2004). These characteristics are similar to those of all Japanese and some European strains of *Cardamine hirsuta* tested in this study and are considered to work as a mechanism for spring dispersed seeds to stay as dormant seeds during summer and to germinate in autumn (Baskin and Baskin 1972, 1986).

This study revealed that germination characteristics of introduced *Cardamine hirsuta* in Japan do not necessarily represent those found among European strains. Natural selections acting on germination timing have often been identified to be strong and vary depending on climates of the habitats (Griffith et al. 2004; Donohue et al. 2005b). Therefore, adaptation in germination timing may constrain the speed of geographical range expansion of plant species. There is a study that showed a single mutation was responsible for the appearance of plant invasiveness (Jain and Martin 1979). In the case of seed germination characteristics, however, recent genetic studies suggested that many genes are involved in the variation of seed dormancy (Koornneef et al. 1998; Alonso-Blanco et al. 2003). Although rigorous studies using larger numbers of strains are required before reaching solid conclusions, our results are supportive of the idea that the lag phase between the first arrival and rapid expansions of *Cardamine hirsuta* in Japan is attributable to adaptation. The process may involve phenomena such as multiple introductions that allow arrivals of the pre-adapted genotypes (Neuffer and Hurka 1999; Ellstrand and Schierenbeck 2000) and hybridizations between strains that create new germination characteristics through transgressive segregation (Ellstrand and Schierenbeck 2000; Donohue et al. 2005b). Although estimation of past evolutionary responses is not a simple task, future studies should

quantify additive genetic variance, epistasis, genetic trade-offs and the number of genes involved in variation of germination characteristics of European and Japanese strains of *Cardamine hirsuta*.

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