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Japanese bunching onion line with a high resistance to the stone leek leafminer, *Liriomyza chinensis* from the 'Beicong' population: evaluating the inheritance of resistance

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Abstract The Japanese bunching onion, *Allium fistulosum* L., is an important vegetable in East Asia. However, the stone leek leafminer, *Liriomyza chinensis* (Kato), is a serious insect pest that invades the *Allium* species. As the feeding punctures on the leaf surface caused by the female adults as well as the larval mining inside the unifacial leaves reduce the commercial value of *A. fistulosum*, it is essential to control *L. chinensis* during its growth. The accession 'Beicong' has shown resistance to *L. chinensis* due to its egg-killing defense, but the degree of resistance varies within the 'Beicong' population. Therefore, in the present study, we selected highly resistant selfed lines from the 'Beicong' population by artificially inoculating *L. chinensis* eggs into the leaves to breed *A. fistulosum* with a resistance to *L. chinensis*. A highly resistant line was obtained by continuously self-pollinating the resistant 'Beicong' individual among the 191 individuals, which was inherited in the F_1 hybrid of the resistant line and the susceptible variety. However, the F_1 hybrid's degree of resistance was an intermediate of the two parents'. It was also revealed that the resistance of 'Beicong' was due to both egg- and larval-killing defense mechanisms by artificially inoculating the eggs and larvae into the leaves. Hence, developing a highly resistant line could contribute to integrated pest management in *A. fistulosum* cultivation by decreasing the population of *L. chinensis*.

Keywords Japanese bunching onion · *Allium fistulosum* L. · Stone leek leafminer · *Liriomyza chinensis* (Kato) · Beicong · Insect pest resistance

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Introduction

The Japanese bunching onion, *Allium fistulosum* L., is one of the most economically important crops among the *Allium* species in East Asia, especially in Japan, Korea, and China (Kumazawa and Katsumata 1965; Ford-Lloyd and Armstrong 1993). In Japan, the 2018 production volume of *A. fistulosum* was approximately 146.6 billion Japanese Yen and is ranked the fourth highest among the vegetables (MAFF 2018). Both leaf parts, including the blade and sheath, are edible, so it is important to cultivate them by controlling infections and pests during their growth. The stone leek leafminer, *Liriomyza chinensis* (Kato) (Diptera: Agromyzidae), is a harmful insect pest that invades *A. fistulosum* in Japan, Korea, China, and other Asian countries (Chen et al. 2003; Choi et al. 2003; Tokumaru 2016; Tran and Takagi 2005). Furthermore, *L. chinensis* feeds on *Allium* plants and has been expanding its habitat in Eastern Europe (Martinez 1982; Martynov et al. 2016; Papp and Cerny 2017). Female adults puncture the epidermis by the ovipositor and cause feeding punctures. In addition, they insert the ovipositor in the leaves for oviposition, where the hatched larvae in the unifacial leaves feed on the inner parts of the leaves. In essence, the feeding punctures generated by the female adults and the larval mining reduce the production value of *A. fistulosum*. The number of next-generation *L. chinensis* individuals produced by one female adult was the largest at 25 °C (Tokumaru 2016), and the *L. chinensis* epidemic in Japan appears between spring and autumn. Therefore, there is concern regarding the extended occurrence period caused by global warming, which leads to an average temperature rise in Japan (+ 1.49 °C/100 years from March to May and + 1.23 °C/100 years from September to November; Japan Meteorological Agency 2020). Furthermore, since a different biotype of *L.chinensis* (biotype B) was identified in Japan from 2016 and the larval mining is more severe than that of the conventional *L. chinensis* (biotype A), it is necessary to control *L. chinensis* more precisely (Tokumaru and Uesugi 2019).

The insect pest control methods of *L. chinensis* in *A. fistulosum* were performed with a risk of producing pesticide-resistant individuals. To control the pest more sustainably, integrated pest management (IPM) practices should be encouraged. Oida and Kawana (2017) reported that hymenopterous parasitoids of Agromyzid leafminers (Diptera: Agromyzidae) on the leaves and flowers of *Phacelia tanacetifolia* acted as natural enemies of *L. chinensis*, whereas *P. tanacetifolia* can potentially act as a banker or insectary plant for the parasitoids to control *L. chinensis* in the *A. fistulosum* fields. In terms of cultural control, solarization using plastic mulching in summer effectively kills *L. chinensis* pupae in the soil (Kai and Morita 2001). To promote IPM when cultivating *A. fistulosum*, it is effective and necessary to develop *A. fistulosum* varieties resistant to the pest and introduce natural enemies. For instance, 'Beicong', an *A. fistulosum* accession, showed resistance to *L. chinensis* in terms of the adult's non-preference (Sueyoshi et al. 2006) as well as egg-killing, which was revealed in our previous study when the pupation rates were evaluated by artificially inoculating the eggs (Takeda et al. 2020). However, the degree of resistance varies across the 'Beicong' population as 'Beicong' was propagated via an outcrossing approach to avoid any inbreeding depression. In addition, no other factor affecting the pupation rates, except for egg-killing, has been revealed to date.

In the present study, we selected *A. fistulosum* lines from the 'Beicong' progeny that showed a high antibiosis resistance to *L. chinensis* (biotype A) as described by Takeda et al. (2020). Furthermore, we investigated the inheritance of resistance in the progeny to develop breeding materials. In addition, we attempted to elucidate the insecticidal ability at the larval stage to identify resistant factors besides egg-killing.

Materials and methods

Plant material

'Chouetsu', 'Kujou futo', 'Kokusen natsuyo', and 'Fuyuwarabe' are commercial varieties of *A. fistulosum* in Japan, where 'Beicong' is an accession of *A. fistulosum* that is preserved in the NARO Genebank. 'Beicong' and 'Kokusen natsuyo' were observed to show resistance to *L. chinensis* based on the pupation rate of the eggs (Takeda et al. 2020). Thus, 'Beicong' and 'Kokusen natsuyo' as well as their progenies were used to evaluate their resistance to *L. chinensis* (Table 1). The plants were grown in greenhouses at the Institute of Vegetable and Floriculture Science, NARO, Japan (N34°61′, E136°25′). To prepare the plants, seeds of *A. fistulosum* were sown in 200-cell plug trays, while the seedlings were grown by regularly spraying the bioinsecticide (*Beauveria bassiana*) BotaniGard® ES (Arysta LifeScience, Tokyo, Japan) without any synthetic pesticides. The bioinsecticide was sprayed three times or once a week from May to September or from October to April, respectively, to protect from the onion thrips (*Thrips tabaci*). Approximately three months after germination, the seedlings were individually transplanted into

Table 1 Information regarding tested plant materials

Variety or line	Accession Number[a]	Generation[b]	Source
Beicong	JP138785	OP	NARO
Kujou futo	JP133928	OP	Takii Seed Co., Ltd
Chouetsu	JP133892	OP	Mikado Kyowa Seed Co., Ltd
Kokusen natsuyo	JP133881	OP	Mikado Kyowa Seed Co., Ltd
Fuyuwarabe	JP253180	OP	NARO
D8s-10s	–	S_2	NARO
D8s-11s	–	S_2	NARO
D8s-12s	–	S_2	NARO
D8s-19s	–	S_2	NARO
D8s-20s	–	S_2	NARO
D8s-10s-1s	–	S_3	NARO
D8s-12s-2s	–	S_3	NARO
D8s-19s-1s	–	S_3	NARO
D8s-19s × Chouetsu	–	$F_1(S_2 \times OP)$	NARO

[a] Accession number for MAFF Genebank

[b] OP = "open pollinated"

a pipe container (φ22 mm, 80 mm high) and placed on the tray with water, as described by Takeda et al. (2020). To avoid spraying before the experiment, the test plants were transplanted into the pipe container 2 days before the experiment.

Insect material

Liriomyza chinensis (biotype A; Tokumaru and Uesugi 2019) was collected from a colony that was established on several varieties of *A. fistulosum* plants at the National Institute of Vegetable and Tea Science (present name: Institute of Vegetable and Floriculture Science, NARO) in October 2011, which were reared with an *A. fistulosum* variety 'Kujou futo' in a vinyl chloride cage (300 × 150 × 200 mm high). The rearing cage was placed in a growth chamber (TAM131AM-SV; Toshiba Carrier, Co., Ltd., Japan) that was set to 22 °C and a photoperiod of 14 h/10 h light/dark (14L10D), following our previous study (Takeda et al. 2020). Mass-rearing of *L. chinensis* was performed according to Urairi et al. (2020); for oviposition, *A. fistulosum* plants (six plastic pots with six plants per pot) were introduced in the rearing cage with two bottles containing a solution of 20 % hydromel and hundreds of emerged adults. The exchange with a new set of plants was carried out three times a week.

Evaluating *A. fistulosum* resistance to *L. chinensis*

Evaluating the resistance of *A. fistulosum* to *L. chinensis* was conducted by artificially inoculating the eggs into the newest leaf, as described by Takeda et al. (2020). To collect thousands of fresh eggs, 200 adult flies in two plastic pots, with six plants of 'Kujou futo' per pot, were introduced into a vinyl chloride cage (300 × 150 × 200 mm) at approximately 9 AM. Female flies were left to lay eggs in the leaves for 24 h (24 °C, 14L10D); it is necessary to let adult females oviposit for 24 h to successfully collect thousands of fresh eggs. Thereafter, at approximately 9 AM the next day, the eggs were collected from the inner parts of the unifacial leaves and placed in a petri dish filled with water. Five eggs were inoculated with a plunger microsyringe (MS-GAN050 0.5 ml volume, Ito Co., Ltd., Japan) into four points, respectively, resulting in 20 eggs inoculated into the newest leaf (leaf length was longer than 20 cm) of each *A. fistulosum* sample, as shown in Fig. 1. Immediately after inoculation, each tested plant was placed in a testing pipe cage, covered individually with a nylon mesh (φ22 mm, 320 mm high), firmly sealed with adhesive tape, and then placed in a growth chamber set to 24 °C and 14L10D. Here, the temperature was set to 24 °C for mass-rearing and not 22 °C because of the shortened experimental period. After 14 days of inoculation, the soil pupae were placed in a tea strainer containing water, and the floated pupae were collected and counted.

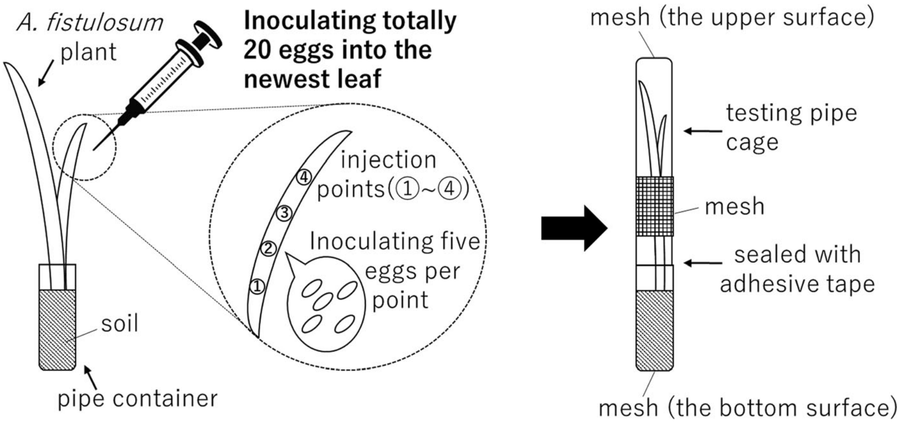

Fig. 1 The procedure of artificially inoculating *Allium fistulosum* L. leaves with *Liriomyza chinensis* eggs

Selecting highly resistant 'Beicong' lines and elucidating the resistance level of the F_1 hybrid

To select the lines with a high resistance to *L. chinensis*, the resistance of the 'Beicong' individuals was evaluated in 2014. A total of 200 seeds of 'Beicong' were sown on a 200-cell tray (one seed per cell), while the seedlings were grown in a greenhouse. In winter, the greenhouse was heated such that the temperature was maintained at or above 15 °C. Approximately 3 months after germination, each seedling was transplanted into a pipe container, where the resistance to *L. chinensis* was individually evaluated by artificially inoculating the eggs into the newest leaf and investigating the pupation rate according to Takeda et al. (2020). After the evaluation, individuals showing high resistance (i.e., pupation rate: 0 %) were self-pollinated to investigate the inheritance of the resistance in the next progeny. Similarly, the evaluation of resistance to *L. chinensis* was conducted on 20 individuals of each self-pollinated progeny in 2015. Thus, the selected 'Beicong' plant with a high resistance to *L. chinensis* was self-pollinated several times (described as S_x in Table 1). To elucidate the inheritance of the resistance in the F_1 hybrid between the resistant line and the susceptible variety, the D8s S_2 progeny plant was crossed with 'Chouetsu'. During the experiments conducted in 2016 and 2017, evaluating the resistance to *L. chinensis* of the obtained F_1 hybrid was conducted by artificially inoculating the eggs, as described by Takeda et al. (2020).

Investigating the effect of D8s on larval growth

During the experiments in 2018, the hatched larvae of *L. chinensis* were inoculated into the leaves of *A. fistulosum* using a method described by Takeda et al. (2020) to elucidate the insecticidal ability at the larval stage of *L. chinensis*. The eggs were collected from the 'Kujou futo' inner leaf parts and placed in a petri dish filled with water. To hatch the collected eggs, the petri dish was placed in a growth chamber (24 °C, 14L10D) for 3 days. Within 24 h after hatching, the five newly hatched larvae were inoculated into each of the four parts of the tested *A. fistulosum*'s newest leaf using the plunger microsyringe (MS-GAN050 0.5 ml volume, Ito Co., Ltd., Japan) similar to the egg inoculation. After inoculation, each plant was placed in a testing pipe cage, individually covered with a nylon mesh (φ22 mm, 320 mm high), firmly sealed with adhesive tape (as shown in Fig. 1), and then placed in a growth chamber set to 24 °C and 14L10D. Here, the temperature was set to 24 °C for mass-rearing and not 22 °C because of the shortened experimental period. After 14 days of inoculation, the soil pupae were placed in a tea strainer containing water, and the floated pupae were collected and counted.

Statistical analysis

The pupation rates of the eggs or the larvae to pupae were compared among the tested *A. fistulosum* lines or varieties using the Steel-Dwass test. All statistical analyses were conducted using the JMP statistical package (SAS Institute 2020, USA).

Results

Selecting highly resistant lines from ‘Beicong’

Out of the 200 ‘Beicong’ individuals, 191 were germinated normally, in which the resistance to *L. chinensis* of the 191 individuals was evaluated in 2014 to select the highly resistant plants, which included A1–50, B1 – 50, C1–50, and D1–41. As a result, 14 individuals (A13, A19, A20, A29, A30, A34, A36, A37, A38, A42, A43, C30, C33, and D8) showed high resistance to *L. chinensis* with a pupation rate of 0 % (Fig. 2). However, C33 was excluded due to its limited growth; therefore, the other 13 individuals were self-pollinated, where 20 S_1 progeny plants per individual were used in the following experiment in 2015. As a result of evaluating the S_1 progeny, the line which showed the lowest mean of pupation rate was selected as the highly resistant line (D8s; Fig. 3). Moreover, during the experiments conducted in 2016 and 2017, the resistance of the D8s progeny plants (S_2 and S_3) and the plant material were evaluated. As a result of these experiments, it was revealed that the D8s progeny plants (S_2 and S_3) showed significantly higher resistance to *L. chinensis* when compared to ‘Kujou futo’ and ‘Chouetsu’ ($p < 0.05$; Table 2). Finally, the pupation rate of ‘Kokusen natsuyo’ was found to be 6.5 %, reflecting no significant difference between the D8s progeny and ‘Kokusen natsuyo’.

Investigating the inheritance of resistance to *L. chinensis*

The resistance of the F_1 hybrid was tested in 2017 that was generated from the highly resistant line, the D8s progeny (S_2), and the susceptible variety, ‘Chouetsu’, to *L. chinensis*. The pupation rate of the F_1 hybrid, ‘D8s-19s × Chouetsu’, was 12.0 %, which was significantly lower than that of ‘Kujou futo’ and ‘Chouetsu’ ($p < 0.05$). Although there was no significant difference between the F_1 hybrid and the S_3 progeny, the mean values of the F_1 hybrid were higher than those of the S_3 progeny (Table 2).

Effects of the D8s on larval growth after artificially inoculating the hatched larvae

The D8s progeny (S_2) and the commercial varieties (‘Kujou futo’, ‘Fuyuwarabe’, and ‘Kokusen natsuyo’) were used in the experiment conducted in 2018. As a result of the artificial larval inoculation into the newest leaf, the pupation rate values (from larvae to pupae) of D8s-19s and D8s-20s were found to be 5.9 % and 1.3 %, respectively (Table 3). In addition, a significant difference was observed between the D8s progeny plants and both ‘Kujou futo’ and ‘Fuyuwarabe’ ($p < 0.05$) individually. Moreover, the pupation rate of ‘Kokusen natsuyo’ was found to be 9.6 %, where no significant difference was observed between the D8s progeny plants and ‘Kokusen natsuyo’.

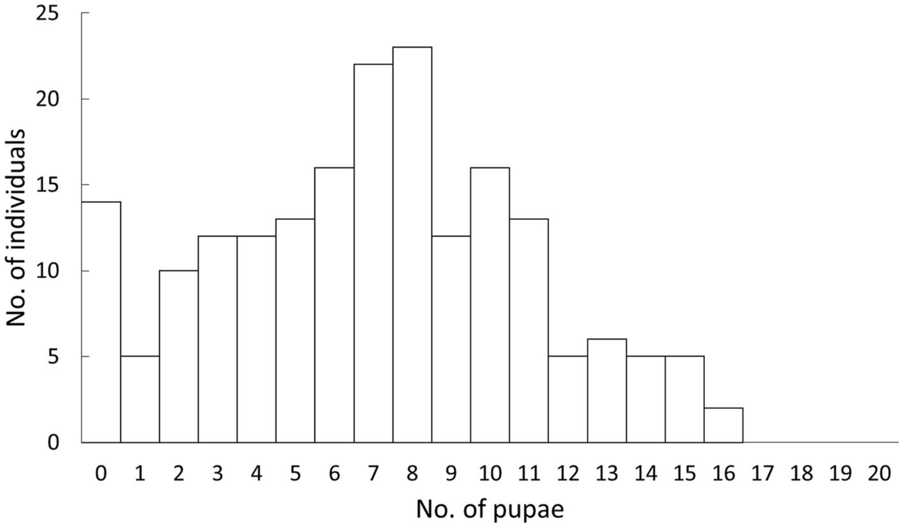

Fig. 2 Histograms showing the number of pupae among the 191 individuals of ‘Beicong’

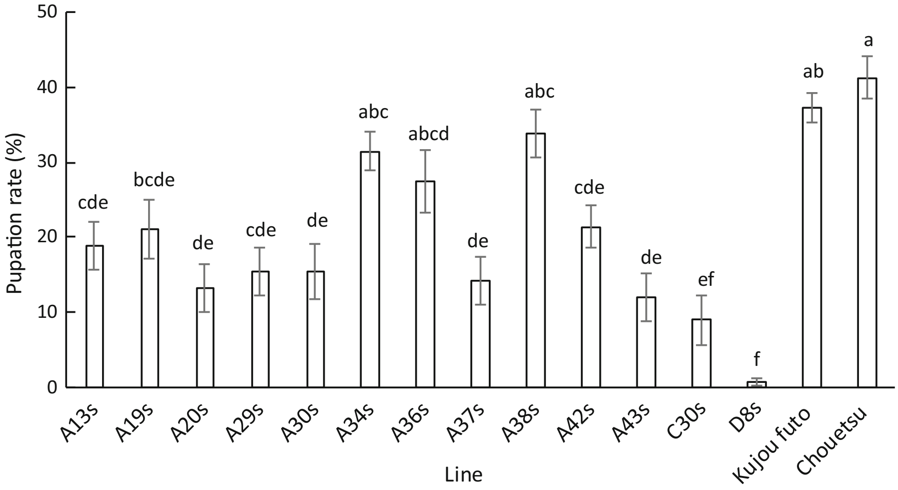

Fig. 3 Comparison of the pupation rate (from eggs to pupae) of *Liriomyza chinensis* among the 13 S_1 lines of 'Beicong' and the commercial varieties (mean ± standard error). Columns with different letters are significantly different ($p < 0.05$), as determined via the Steel-Dwass test (n = 19 ± 1)

Table 2 Comparison of pupation rate (from eggs to pupae; mean ± SD) among highly resistant lines (D8s progeny), 'Beicong', commercial varieties, and the F_1 hybrid

Variety or Line	Pupation rate (%)	Year
D8s-10s	5.3 ± 8.3c	2016
D8s-11s	5.0 ± 7.8c	
D8s-12s	6.3 ± 12.2c	
D8s-19s	6.0 ± 13.9c	
D8s-20s	4.5 ± 8.7c	
Beicong	17.5 ± 10.1b	
Chouetsu	44.3 ± 13.1a	
Kujou futo	44.5 ± 12.4a	
D8s-10s-1s	1.0 ± 3.5yz	2017
D8s-12s-2s	0.3 ± 1.1z	
D8s-19s-1s	1.0 ± 3.5yz	
Kujou futo	38.3 ± 16.0w	
Kokusen natsuyo	6.5 ± 11.1yz	
Chouetsu	25.3 ± 19.5wx	
D8s-19s × Chouetsu	12.0 ± 16.3xy	

Means followed by different letters within columns are significantly different ($p < 0.05$) by Steel-Dwass test (n = 20)

Table 3 Comparison of pupation rate (from larvae to pupae; mean ± SD) among highly resistant lines (D8s progeny), 'Beicong', and commercial varieties

Variety or Line	Pupation rate (%)
D8s-19s	5.9 ± 8.4bc
D8s-20s	1.3 ± 3.4c
Beicong	8.5 ± 10.5b
Kokusen natsuyo	9.6 ± 11.6b
Kujou futo	40.4 ± 19.8a
Fuyuwarabe	33.9 ± 20.2a

Means followed by different letters are significantly different ($p < 0.05$) by Steel-Dwass test (n = 23)

Pupation rate was assessed in 2018

Discussion

To develop breeding materials with a high resistance to *L. chinensis*, the individual numbered as D8, which showed a high resistance with a pupation rate of 0 %, was selected from the 'Beicong' population according to Takeda et al. (2020). In addition, the D8 selfed lines showed lower pupation rate values relative to the commercial varieties; thus, the resistance of D8 was stably inherited into the progeny. By continuously self-pollinating the D8 individual, the D8s line with a fixed high resistance to *L. chinensis* was developed. As 'Kokusen natsuyo' showed a resistance as high as that of 'Beicong' in our previous study (Takeda et al. 2020) and the present study, 'Kokusen natsuyo' may be also a candidate for breeding the Japanese bunching onion. Moreover, the F_1 hybrid between the D8s line and the commercial variety 'Chouetsu' also showed high resistance relative to the parental variety 'Chouetsu', but the pupation rate was higher than of the D8s line; thus, the F_1 hybrid resistance was an intermediate between the D8s line and the susceptible variety. Hence, these results demonstrate that the mode of inheriting resistance is an incomplete dominance. For this reason, the genome region relevant to resistance must be homozygous in the D8 allele to maintain the high resistance. Furthermore, to reveal the number of genes that are related to the resistance in detail, it is necessary to investigate the segregation ratio in the F_2 population between the D8s line and the susceptible variety.

Takeda et al. (2020) reported that the hatchability of *L. chinensis* was significantly different between 'Beicong' and 'Kujou futo' when artificially inoculating the eggs. In the present study, the pupation rate from the larvae to the pupae of the D8s line was also investigated to reveal the effects of resistance on the hatched larvae. Moreover, 2 weeks after inoculating the newly developed leaves with the hatched larvae, the pupation rate of the D8s line was found to be significantly lower relative to the susceptible varieties, 'Kujou futo' and 'Fuyuwarabe' ($p < 0.05$). In addition, the varietal resistance to the larvae showed almost the same tendency as observed with regards to the eggs, suggesting that the D8s line has both egg- and larval-killing defense mechanisms.

Furthermore, De Jong and Rademaker (1991) reported varietal differences in the susceptibility of chrysanthemum to *L. trifoli* (Burgess) (Diptera: Agromyzidae) after a no-choice oviposition, in which the varietal differences were considered to involve the larval-killing defense mechanism on the leaves. This study, however, has not reported the mechanism or the inheritance of resistance. Moreover, Sueyoshi et al. (2006) reported that resistance to *L. chinensis* on 'Beicong' was not caused by a physical defense mechanism, such as the hardness of leaves, but by other factors. Since the inoculated eggs and larvae were unaffected by the physical factor when using the artificial inoculation methods, the egg- and larval-killing defense mechanisms could be caused by chemical factors, such as chemical compounds found in the leaves. However, it remains unclear whether the same factors in antibiosis resistance were also associated with egg- and larval-killing. Comparing the chemical compositions among the D8s line and other *A. fistulosum* varieties and conducting the bioassay with *L. chinensis* eggs and larvae will aid identification of the antibiotic chemical components.

In addition to the antibiotic effects of 'Beicong', it was also reported that the number of feeding punctures generated by the female adults on 'Beicong' was lower than that of other varieties of *A. fistulosum* in the choice tests (Sueyoshi et al. 2006). Moreover, the D8s line would also reduce the feeding puncture damage generated by female adults. Thus, it was assumed that the D8s line decreased the feeding behavior of adults and suppressed the generation cycles of *L. chinensis* by killing the eggs and larvae, and finally, preventing large outbreaks.

It is known that other leafminer flies such as South American leafminer (*Liriomyza huidobrensis*) and garden pea leafminer (*Chromatomyia horticola*) also attack *Allium* spp. (Schrameyer 2001; Scheffer 2000). Furthermore, a new genotype of *L. chinensis* (biotype B) was recently identified in Japan, where the number of larvae in the leaves and the larval mining surface area of the unifacial leaves were mostly larger than those of the conventional strain (biotype A) (Tokumaru and Uesugi 2019). However, in the present study, the ovicidal and larvicidal effect of D8s on the biotype A eggs and larvae were elucidated. To develop the breeding materials with a high resistance to both biotypes A and B and other leafminer species attacking *Allium* spp., it is necessary to reveal whether the D8s line shows the same resistance to *L. chinensis* biotype B and those other leafminer species attacking *Allium* spp. such as *L. chinensis* biotype A by artificially inoculating the eggs or larvae in future studies. In addition, field resistance to these leafminer flies should be confirmed by conducting field experiments. We believe that by developing the resistant varieties to both biotypes and these other leafminer species could contribute to IPM by decreasing the population of *L. chinensis* and using less amount of pesticides.

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Authors' contributions Conceptualization, S. F., K. Y., T. W., and M. T.; Methodology, S. F., K. Y., T. W., and M. T.; Investigation, S. F., C. U., K. Y., and A. K.; Writing-Original Draft, S. F.; Writing-Review and Editing, S. F., C. U., K. Y., T. W., A. K., and M. T.

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Conflicts of interest The authors declare that they have no competing interests.

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