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Detection of a novel intramolecular rearrangement during gramine biosynthesis in barley using stable isotope-labeled tryptophan

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ABSTRACT

Plants accumulate various secondary metabolites, and the biosynthetic reactions responsible for their scaffold construction are the key steps that characterize their structural categories. Gramine, an indole alkaloid, is a defensive secondary metabolite biosynthesized in barley (*Hordeum vulgare*) from tryptophan (Trp) via aminomethylindole (AMI). While the two sequential *N*-methylation steps following the formation of AMI have already been characterized both genetically and enzymatically, the step preceding AMI formation, which includes the Trp side chain-shortening, has not yet been revealed. To gain further insight into these biosynthetic reactions, barley seedlings were fed Trp labeled with stable isotopes (^{13}C and ^{15}N) at various positions, and the isotope incorporation into gramine was analyzed by liquid chromatography/mass spectrometry. Significant increases in the abundance of isotopic gramine were detected in experimental sets in which Trp was labeled at either the indole ring, the β-carbon, or the amino group, whereas the isotopolog composition was not affected by α-carbon-labeled Trp. Although absorbed Trp presumably undergoes transamination in plants, this reaction did not seem to be related to gramine productivity. The data indicated that AMI directly inherited the amino group from Trp, while the α-carbon was removed, suggesting that the Trp–AMI conversion includes a novel intramolecular rearrangement reaction. The results of this study provide novel insights into scaffold formation in plant secondary-metabolite synthesis.

1. Introduction

Plants produce a wide variety of secondary metabolites that are key components of the plant defense system against pathogens and herbivores. The structural diversity of these metabolites is brought about by the diversification of the biosynthetic pathways that has occurred in the course of evolution, and some secondary metabolites can be found in only a few plant species. Particularly, among *Hordeum* spp., gramine (3-[*N, N*-dimethylaminomethyl]indole) is found in a limited number of cultivars of domesticated barley (*Hordeum vulgare*) and wild barley (*H. vulgare* subsp. *spontaneum*) [1,2], in which it is involved in plant resistance responses against aphids and pathogens [3–5]. Although gramine constitutively accumulates in barley, its concentration increases upon biotic or abiotic stimuli such as infection, heat, and drought [3,6–8].

In the biosynthetic pathways of secondary metabolites, reactions such as cyclization, rearrangement, and carbon chain elongation or shortening are crucial for the formation of the scaffold of metabolites. Similar to other indole alkaloids, gramine is believed to be biosynthesized from tryptophan (Trp) via 3-aminomethylindole (AMI) [9–11]. Although the *N*-methyltransferase (HvNMT) involved in the two sequential *N*-methylations of AMI to produce gramine has already been identified [12,13], the step(s) before *N*-methylation, namely, the scaffold construction step from Trp to AMI, has not been revealed to date. The step between Trp–AMI includes side chain-shortening of Trp by two carbon atoms (C2) (Supplementary Fig. S1). A previous study using isotope-labeled Trp suggested that the indole ring and β-carbon atom were retained through the reaction [9,10]. Hence, Wenkert (1962) and O'Donovan and Leete (1963) proposed a reaction mechanism whereby the C–C bond between α- and β-carbons is cleaved [9,14]. According to the hypothesis, Trp undergoes retro-Michael degradation aided by pyridoxal phosphate (PLP), leading to the elimination of the amino group, α-carbon, and carboxyl group of Trp, whereby protonated 3-methyleneindolenine ion is generated (Supplementary Fig. S1). However, no data indicating the elimination of the α-carbon and amino group from Trp during the conversion have been reported. Furthermore, no evidence of the involvement of PLP or PLP-related enzymes has been reported.

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Side chain-shortening of plant aromatic amino acids has been most studied for the secondary metabolites originating from phenylalanine (Phe). In the corresponding pathway, the side-chain amino group is removed as ammonia by phenylalanine ammonia lyase to produce cinnamate. In turn, cinnamate and its derivatives, such as ferulate, undergo C2 removal through several pathways, including the β-oxidative pathway and the hydratase/lyase pathway [15]. Vanillylamine, an intermediate of capsaicin biosynthesis, possesses an amino group separated from the aromatic ring by one carbon atom, which is similar to the structure of AMI. Since vanillylamine is synthesized from vanillin, which is synthesized from ferulate by a hydratase/lyase (vanillin synthase; VpVAN) [16], the amino group in vanillylamine is not derived from the precursor Phe. Alternatively, a two-step shortening may be considered: the carboxyl group is first cleaved by a decarboxylase, followed by further carbon elimination. However, even in this case, the amino group should be removed before the second carbon removal-step. Another possible reaction that can locate a nitrogen atom one carbon apart from the indole ring may resemble isothiocyanate formation from indole glucosinolates [17]. However, glucosinolate accumulates specifically in Brassicaceae. Therefore, if AMI inherits the amino-group nitrogen from Trp, the pathway would involve a novel enzymatic reaction.

In this study, we fed barley seedlings Trp labeled with stable isotopes (^{13}C and ^{15}N) at various positions and analyzed their incorporation into gramine by LC/MS, as this technology allows the high-resolution separation of target metabolites from their structural analogs, and the evaluation of the incorporation of the isotopes based on the fragment ions as well as protonated molecules, which may provide more information than what can be acquired by classical radioisotope-tracer experiments. Subsequently, we inferred the gramine biosynthesis by focusing on the metabolism of the amino-group nitrogen of Trp.

2. Materials and methods

2.1. Chemicals

L-Trp was purchased from Fujifilm Wako (Tokyo, Japan). [Indole-2-^{13}C]-, [α-^{13}C]-, [amino-^{15}N]-, and [$^{15}N_2$]-Trp were purchased from Sigma-Aldrich (St. Louis, MO, USA). [β-^{13}C]-Trp was chemically synthesized from indole and *N*,*N*-dimethyl-[^{13}C]-formamide following the procedure described by Takatori et al. [18]. Detailed method of the synthesis is described in Supplementary materials.

2.2. Plant growth conditions

Barley seeds were washed in 70% (v/v) ethanol for a minute and sterilized in sodium hypochlorite solution for 15 min. The seeds were then rinsed with sterilized water and imbibed in water for 2 h at room temperature. Subsequently, they were placed on 0.8% (w/v) agar in a plant box (φ87 mm × 120 mm; Bio Medical Science, Tokyo, Japan) and grown in a controlled environment at 25 °C (12 h light [15,000 lx]/12 h dark). For the stable isotope tracer experiments, seeds were placed on four layers of paper towels immersed in 5 mL water containing 1 mM of isotope-labeled Trp and grown under the same conditions for 72 h.

2.3. Analysis of plant metabolites and evaluation of isotope incorporation into gramine

To examine gramine accumulation in various barley cultivars, shoots were quickly weighed and frozen in liquid nitrogen 72-h after imbibition. The frozen tissue was ground into a powder using mortar and pestle, and the metabolites were extracted using 10 vol (v/w) of methanol. To the extract was added 0.2% (v/v) acetic acid in the same volume as the extract, followed by refrigeration at −20 °C for 20 min and centrifugation at 15,000 rpm for 15 min at 4 °C. The resulting supernatant was subjected to LC/MS analysis as described below. To analyze the distribution of gramine in different plant parts, leaves, coleoptiles, and roots were separated, and gramine concentration in the individual plant was measured using the same procedure described above.

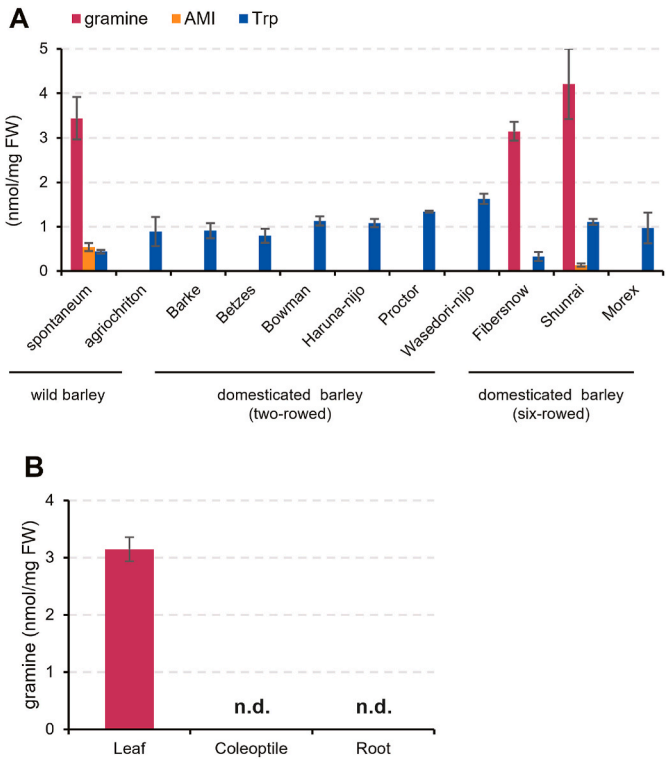

Fig. 1. Accumulation of gramine, aminomethylindole (AMI), and tryptophan (Trp) in wild and domesticated barley. (**A**) Accumulation profiles in various barley cultivars and subspecies. The metabolites were extracted from the leaves of 3-day-old plants. (**B**) Distribution of gramine in barley (cv. Fibersnow). Data shown are means ± SD of three individual plants. n.d., not detected.

Gramine, Trp, and aminomethylindole (AMI) were determined using an Acquity UPLC H-Class system equipped with a photodiode array (PDA) detector and a QDa mass detector (Waters; Milford, MA). Chromatography was performed on an Acquity UPLC BEH C18 column (1.7 μm, 2.1 × 50 mm, Waters) with 0.1% (v/v) formic acid/H_2O (solvent A) and acetonitrile (solvent B). Separation was performed with a series of linear gradients: 3–20% B (0–2 min) and 20–50% B (2–3 min) at a flow rate of 0.6 mL/min at 40 °C. MS analyses were performed in positvie ion mode with the following conditions; electrospray ionization; detection range, 100–300 *m/z*; capillary voltage, 0.3 kV; source temperature, 120 °C; probe temperature, 600 °C; sampling cone voltage, 3.0 V. Additionally, gramine ($C_{11}H_{14}N_2$; exact mass 174.12) and Trp ($C_{11}H_{12}N_2O_2$; exact mass 204.09) were quantified by selected ion recording (SIR) in positive ion mode at *m/z* 175 $[M+H]^+$ and 205 $[M+H]^+$, respectively. As the protonated molecule of AMI ($C_9H_{10}N_2$; exact mass 146.08; *m/z* 147 $[M+H]^+$) could not be detected, AMI was quantified by detecting its fragment ion at *m/z* 130. Monitoring at *m/z* 130 was also used to detect the fragment of gramine because the fragment consisted of the indole ring and methylene carbon of gramine. In tracer experiments, the relative abundances of the isotopologs of gramine and Trp were evaluated based on the total ion chromatogram (TIC) with a mass detection range of 100–300. Statistical analyses were performed using SigmaPlot 14.5 (SYSTAT Software, CA).

3. Results and discussion

3.1. Gramine accumulation

To select barley cultivars that are suitable for the investigation of gramine biosynthesis, we examined the accumulation of gramine, Trp,

Table 1
Incorporation of ^{13}C and ^{15}N into Trp and gramine in barley.

	Tryptophan	Gramine	Fragment of gramine
	(%)	(%)	(%)
Calculated	12.7	12.1	9.8
Control	13.1 ± 1.6	12.1 ± 1.1	9.7 ± 1.0
[Indole-2-^{13}C]-Trp	21.7 ± 4.9 *	17.9 ± 2.7 *	14.1 ± 1.9 *
[α-^{13}C]-Trp	23.3 ± 6.7 *	12.2 ± 1.5	9.3 ± 0.8
[β-^{13}C]-Trp	23.9 ± 6.9 *	17.7 ± 2.4 *	15.0 ± 1.7 *
[$^{15}NH_2$]-Trp	22.2 ± 9.5 *	16.3 ± 3.4 *	10.5 ± 1.5

Values represent the intensity (%) of the isotopic ions relative to the monoisotopic ions. Gramine (exact mass: 174.12) was detected as a protonated molecule (*m/z* 175 $[M+H]^+$ and 176 $[M+1+H]^+$) and fragment (*m/z* 130 and 131). Values are means ± SD. Asterisks indicate that the values are significantly different from the control ($p < 0.01$; Welch's *t*-test or Mann-Whitney *U* test).

and AMI in several cultivars of domesticated and wild barley (*H. vulgare* subsp. *spontaneum*) cultivar (Fig. 1). While Trp was detected in all cultivars tested, gramine accumulation was limited to subsp. *spontaneum* and some members of the six-rowed barley cultivars, Fibersnow and Shunrai (~4.21 nmol/mg FW, Fig. 1A). While examining its distribution among the different plant parts, gramine was detected in leaves but neither in coleoptiles nor roots (Fig. 1B). In contrast, Trp was present in coleoptiles, roots, and leaves, indicating that the biosynthetic pathway of gramine succeeding Trp is leaf-specific (Supplementary Fig. S2).

Aminomethylindole (AMI), the precursor of gramine, was also found only in the gramine-producing cultivar Shunrai and the subsp. *spontaneum*, although at much lower concentration than that of gramine (0.54 and 0.14 nmol/mg FW in *spontaneum* and Shunrai, respectively). Ube et al. reported AMI accumulation in barley roots following *Fusarium culmorum* inoculation [19]. However, those results do not conflict with the data reported herein, because the AMI level was reported to be undetectable in intact roots. Meanwhile, in Fibersnow, AMI was not detected, while the concentration of gramine was similar to that found in the other two gramine-producing barley materials. As the AMI level was much lower than that of gramine, and as its concentration might be cultivar dependent, its concentration in Fibersnow might be too low for detection under the experimental conditions used herein.

Among the six-rowed barley cultivars tested, neither gramine nor AMI were detected in Morex. Larsson et al. reported that the *N*-methyltransferase, which is responsible for the sequential methylation of AMI to generate gramine, was not detected in Morex at the transcript or protein levels [12]. If the lack of gramine accumulation in Morex is solely attributable to the deficiency of *N*-methyltransferase, and Morex possesses the pathway preceding AMI, then the plant would accumulate some AMI. Therefore, Morex might actually be deficient in AMI biosynthetic enzymes and *N*-methyltransferase. Considering the gramine content and seed availability in our laboratory, the leaves of Fibersnow were used as the plant material in subsequent experiments.

3.2. Cleavage between α- and β-carbons in Trp occurs during gramine biosynthesis

To confirm which carbons are removed from Trp during gramine biosynthesis, we fed barley seedlings several species of ^{13}C-Trp labeled at any of the indole ring and α- and β-positions. The mass spectrum of gramine had a fragment ion peak at *m/z* 130, which represented the 3-methyleneindolium ion ($C_6H_8N^+$; exact mass 130.07), in addition to the protonated molecule (*m/z* 175 $[M+H]^+$) (Supplementary Fig. S3). The incorporation of the indole ring and β-carbon of Trp into gramine may be assessed by detecting the fragment. In this study, isotope incorporation was evaluated based on the intensity (%) of isotopic ions relative to the most abundant ions (monoisotopic ions). The abundance of the M+1 isotopic ions of Trp, gramine, and 3-methyleneindolium was calculated to be 12.7, 12.1, and 9.8%, respectively, relative to the monoisotopic ions (Table 1). Therefore, significantly increased relative intensities

Table 2
Incorporation of [$^{15}N_2$]-Trp into barley and wheat.

	Tryptophan		Gramine	
	+1	+2	+1	+2
	(%)	(%)	(%)	(%)
Fibersnow				
Control	13.1 ± 1.6	n.d.	12.1 ± 1.1	n.d.
$^{15}N_2$-Trp	25.5 ± 3.6*	24.1 ± 8.8*	17.7 ± 2.5*	7.7 ± 3.6*
Morex				
Control	10.7 ± 1.1	1.02 ± 0.31	n.d.	n.d.
$^{15}N_2$-Trp	21.0 ± 4.2*	20.7 ± 12.7*	n.d.	n.d.
Wasedori-nijo				
Control	11.9 ± 1.8	1.03 ± 0.27	n.d.	n.d.
$^{15}N_2$-Trp	20.1 ± 1.7*	18.7 ± 3.2*	n.d.	n.d.
Wheat				
Control	13.0 ± 0.47	1.06 ± 0.43	n.d.	n.d.
$^{15}N_2$-Trp	27.7 ± 2.2*	27.4 ± 5.7*	n.d.	n.d.

Values represent the intensity (%) of the isotopic ions relative to the monoisotopic ions (tryptophan, *m/z* 205 $[M+H]^+$; gramine, *m/z* 175 $[M+H]^+$). Fiber snow, Morex, and Wasedori-nijo are cultivars of barley. Values are means ± SD. +1 and + 2 are the data for M+1 and M+2 isotopologs, respectively. Asterisks indicate that the values are significantly different from the control ($p < 0.01$; Welch's *t*-test). n.d., not detected.

indicate the incorporation of isotopes. Hereafter, the relative intensity (%) of, for example, the M+1 isotopolog is denoted simply as a +1 value.

As shown in Table 1, the +1 values of Trp rose significantly (≈1.9 fold) by feeding on any species of ^{13}C-labeled Trp, indicating that the labeled Trp was effectively absorbed by barley under the experimental conditions. When seedlings were fed [indole-2-^{13}C]- and [β-^{13}C]-Trp, the +1 values of gramine and its fragment increased by approximately 5%, compared to the control; contrastingly, no significant changes in the values were observed for the [α-^{13}C]-Trp-fed plants (12.2% ± 1.5% and 9.3% ± 0.8% for gramine and its fragment, respectively). These results clearly demonstrate that the indole ring and β-carbon of Trp are inherited by gramine, while the α-carbon is eliminated through the Trp–AMI conversion. These data also imply that the carbon atom of the carboxyl group was removed, because the only carbon atom present in the side chain of AMI originates from the β-carbon in Trp. Hence, the information on the origin of the carbon atoms is consistent with the hypothesis proposed in previous reports [9,10].

3.3. The nitrogen atom of the gramine side-chain is inherited from the amino group of Trp

The elimination of the α-carbon of Trp implies the possibility that the amino group connected to the α-carbon is also detached from the Trp backbone during AMI biosynthesis. Therefore, feeding experiments using ^{15}N-labeled Trp were performed to verify this hypothesis. As shown in Table 1, feeding the seedlings [amino-^{15}N]-Trp significantly increased the +1 value of gramine from 12.1% ± 1.1%–16.3% ± 3.4% ($p < 0.01$), while that of the fragment ion was not affected, indicating the incorporation of the amino-group nitrogen of Trp into gramine. Such incorporation was more clearly illustrated when the +1 values of gramine of the individual plants were plotted against those of Trp, which showed a positive correlation between the amounts of ^{15}N-gramine and ^{15}N-Trp (Supplementary Fig. S4), implying that the more labeled Trp was absorbed by the plant, the more isotopic gramine accumulated. On the other hand, an obvious correlation was not observed when [α-^{13}C]-Trp was fed.

If the nitrogen atom of the $-NH_2$ group is passed from Trp to gramine, the [$^{15}N_2$]-Trp labeled at both the indole ring and the amino group can be metabolized into gramine with a mass of M+2. The feeding of barley with [$^{15}N_2$]-Trp induced gramine formation at *m/z* 177 $[M+2+H]^+$ (+2 value: 7.7% ± 3.6%) that was not detected in the controls (Supplementary Fig. S5 and Table 2). Notably, the M+1 isotopolog of gramine also increased due to [$^{15}N_2$]-Trp feeding. The variation in the +1

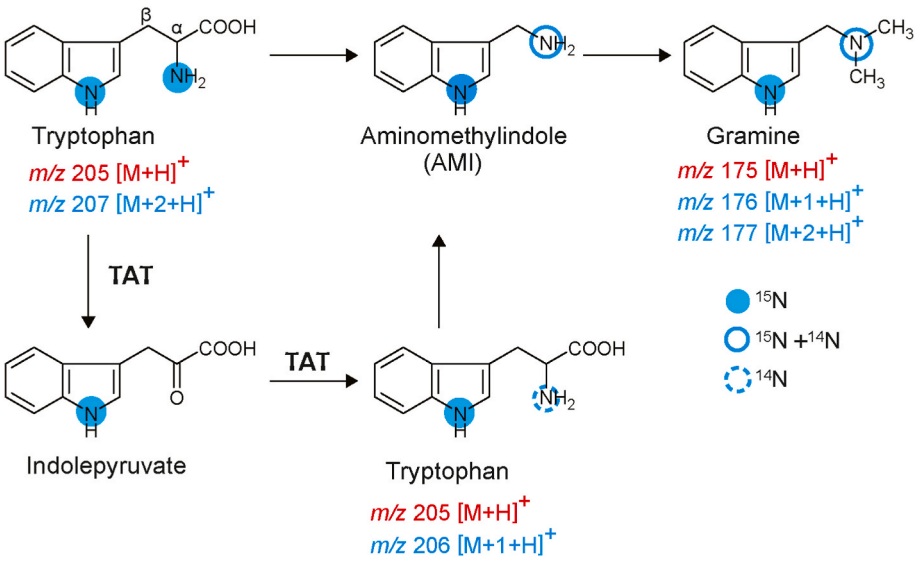

Fig. 2. Incorporation of the nitrogen atoms in Trp forming gramine. Feeding barley [$^{15}N_2$]-Trp (*m/z* 207 [M+2 + H]$^+$) resulted in the detection of gramine at *m/z* 177 [M+2 + H]$^+$ and 176 [M+1 + H]$^+$ as well as *m/z* 175 [M+H]$^+$ originated from endogenous Trp (*m/z* 205 [M+H]$^+$). The α- and carboxyl carbons were eliminated from Trp during AMI biosynthesis. TAT, tryptophan aminotransferase.

value of the control (17.7%–12.1% = 5.6%) was less than that of the +2 value (7.7%). Additionally, the +1 value of Trp increased, similar to that of gramine. Therefore, it is reasonable to consider that M+1 Trp is first generated by two exchanges of the amino group of M+2 Trp, that is, deamination of [^{15}N]-NH_2 and re-amination with [^{14}N]-NH_2 by a tryptophan aminotransferase that has not yet been identified in barley, and then M+1 gramine was synthesized from M+1 Trp (Fig. 2).

To explore whether the observed amino group exchange might be associated with gramine productivity, we examined the mass spectra of Trp in wheat (cv. Chinese Spring) and two barley cultivars, Morex and Wasedori-nijo, which do not accumulate gramine. When plants were fed [$^{15}N_2$]-Trp (*m/z* 207), an ion representing M+1 isotopolog (*m/z* 206) was detected with an intensity comparable to that of M+2 (Table 2), as observed in Fibersnow, a gramine-accumulating barley cultivar. Therefore, the remarkable exchange of the amino group of Trp was not a phenomenon characteristic of gramine-accumulating plants, and the phenomenon did not seem to be associated with gramine productivity. Consistently, Breccia and Marion showed that [β-^{14}C]-labeled indole-3-pyruvate was metabolized into radioactive gramine, and hence, proposed that it might be an intermediate within the gramine biosynthetic pathway [20]. Indole-3-pyruvate is a product of the deamination of Trp and serves as a substrate for an aminotransferase that produces Trp (Fig. 2). Therefore, incorporation of radioactivity from indole-3-pyruvate to gramine does not indicate that the compounds are intermediates of gramine biosynthesis.

In this study, the side chain-shortening process preceding AMI and gramine synthesis was demonstrated to be associated with α-carbon elimination without the removal of the amino group (Fig. 2). These data suggest that the reaction proceeds via an intramolecular rearrangement of the amino group to allow the nitrogen atom to attach to the α-carbon, although the mechanism remains unknown. Side-chain rearrangements of Trp derivatives have been studied with respect to glucosinolate-derived phytoalexins in Brassicaceae, such as brassinin, rapalexin A, and isocyalexin A, whose biosynthetic pathways have been proposed to include Lossen, Neber, and Beckmann-type rearrangements [17,21]. However, they also require glucosinolate-related structures (e.g., thiohydroximate, oxime, and sulfate groups) that are found only within Brassicaceae, and the reactions presumably proceed spontaneously or are catalyzed by unidentified enzymes.

This study revealed a novel reaction in the gramine biosynthesis pathway in barley. Elucidating the pathways and mechanisms involved in AMI biosynthesis will provide novel information about the scaffold construction of secondary metabolites. Ongoing research using RNA-seq and QTL analysis focuses on a comprehensive study to identify the gene (s) responsible for these steps.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2023.101439.

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