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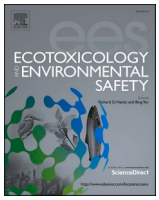

Uncovering the diversity and contents of gene cassettes in class 1 integrons from the endophytes of raw vegetables

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ABSTRACT

Rapid spread of antibiotic resistance genes (ARGs) in pathogens is threatening human health. Integrons allow bacteria to integrate and express foreign genes, facilitating horizontal transfer of ARGs in environments. Consumption of raw vegetables represents a pathway for human exposure to environmental ARGs. However, few studies have focused on integron-associated ARGs in the endophytes of raw vegetables. Here, based on the approach of qPCR and clone library, we quantified the abundance of integrase genes and analyzed the diversity and contents of resistance gene cassettes in class 1 integrons from the endophytes of six common raw vegetables. The results revealed that integrase genes for class 1 integron were most prevalent compared with class 2 and class 3 integron integrase genes (1–2 order magnitude, $P < 0.05$). The cucumber endophytes harbored a higher absolute abundance of integrase genes than other vegetables, while the highest bacterial abundance was detected in cabbage and cucumber endophytes. Thirty-two unique resistance gene cassettes were detected, the majority of which were associated with the genes encoding resistance to beta-lactam and aminoglycoside. Antibiotic resistance gene cassettes accounted for 52.5 % of the functionally annotated gene cassettes, and $bla_{TEM-157}$ and *aadA2* were the most frequently detected resistance cassettes. Additionally, carrot endophytes harbored the highest proportion of antibiotic resistance gene cassettes in the class 1 integrons. Collectively, these results provide an in-depth view of acquired resistance genes by integrons in the raw vegetable endophytes and highlight the potential health risk of the transmission of ARGs via the food chain.

1. Introduction

The emergence and spread of antibiotic resistance in pathogens is a serious threat to public health, and antibiotic resistance genes (ARGs) have been listed as a top health risk by World Health Organization (Levy and Marshall, 2004; Ma et al., 2017; Fresia et al., 2019). Numerous evidence have shown that ARGs can be transferred to other microorganisms through horizontal gene transfer (HGT) mediated by mobile genetic elements (MGEs), facilitating the emergence and evolution of multi-resistant bacteria (Wiedenbeck and Cohan, 2011; Liu et al., 2016; San Millan, 2018). Previous studies have revealed that many clinically relevant ARGs carried on MGEs can be colonized the gastrointestinal tract and then transferred to other bacterial members (Holzel et al., 2018; Zhang et al., 2021). Therefore, understanding the distribution of MGEs and identifying their potential contribution in ARGs dissemination are particularly important for mitigating the antibiotic resistance.

Integrons are one of the genetic elements with the ability to acquire, express and disseminate exogenous genes, e.g., ARGs (White et al., 2001). Integrons are composed of three essential core elements. The first element is the *intI* gene, which can encode integron integrase. Integrase can catalyze site-spectific recombination between the second core element *attI* site and/or the promoterless cassette-associated *attC* site, resulting in the integration or excision of cassettes (Escudero et al., 2015; Ma et al., 2017). Once a newly acquired gene cassette is inserted, the third core element, Pc promoter, can drive its expression (Gillings, 2014). Gene cassettes are individually mobilizable elements, which

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commonly consist of an open reading frame (ORF) bounded by a site-specific *attC* site (Partridge et al., 2009). Gene cassettes can be integrated into chromosomes or plasmids, and their mobility allows genes to infiltrate into new organisms (White et al., 2001; Gillings et al., 2008; Partridge et al., 2009). Thus, integrons can acquire a vast pool of different functional gene cassettes conferring favorable phenotypes. Based on the amino acid sequences of integrases, integron can be classified into many classes, of which class 1, class 2 and class 3 integrons were generally associated with the horizontal transfer of antibiotic resistance (Gillings, 2014; An et al., 2018b). Class 1 integron-integrase genes (*intI1*) are more prevalent than the other two integrase genes in various environments, e.g., wastewater, sludge, soil and agricultural irrigation water (Barraud et al., 2013; Stalder et al., 2014; Paraoan et al., 2017). Studies have shown that *intI1* is commonly associated with genes conferring resistance to the majority of antibiotics (e.g. the antibiotics controlling gram-negative pathogens), disinfectants (e.g. quaternary ammonium compounds) and heavy metals (e.g. Cu) and has been considered as a proxy for anthropogenic pollution (Gillings et al., 2015; Zheng et al., 2020). Additionally, class 1 integrons are highly conserved and closely associated with Tn*402*-like transposons (Gillings, 2014). As for class 2 integrons, they are probably captured by Tn*7* transposon, whose transposition activity is directed at a specific binding site on plasmids or chromosomes (Alieda et al., 2007; Jones-Dias et al., 2016; An et al., 2018b). Previous studies have shown that the presence of the premature in-frame stop codon could contribute to the inactive characteristics of class 2 integron integrase, and most of their cassette arrays are highly conserved with a limited range of cassette functions (Marquez et al., 2008; Wang et al., 2021). Class 3 integrons are also associated with the Tn*402* transposon, but in the reverse orientation to the class 1 capture event (Gillings, 2014). Less is known about Class 3 integrons, which have been described as rare and found only in a limited number of isolates (Uyaguari et al., 2013; Fernandez Rivas et al., 2021).

According to the Food and Agriculture Organization (FAO), the global vegetable production stood at 1.56 billion tons in 2018 (FAO, 2018) and weighted mean vegetable intake was 186 g/day (56–349 g/day) (Kalmpourtzidou et al., 2020). Consumption of the produce (especially raw and unprocessed fruits and vegetables) represents a main pathway for human exposure to environmental antibiotic resistance. The environmental ARGs can be introduced to agricultural produce surfaces through agricultural runoff, wind, soil microorganisms and animal activities and other external forces. Numerous studies have reported that anthropogenic activities such as organic fertilizer application or reclaimed water irrigation could facilitate the enrichment of ARGs in the aboveground phyllosphere microorganisms (Chen et al., 2017, 2019; Holzel et al., 2018) and a large variety of ARGs have been detected in the raw vegetable phyllosphere or salads (Zhu et al., 2017; Zhou et al., 2020). Recent studies have also shown that the diversity of antibiotic resistance gene cassettes of class 1 integrons in the phyllosphere was increased with the application of fertilizer, and the surface/phyllosphere of produce could be a potential pool of integrase genes and resistance gene cassettes (An et al., 2018a). These surface ARB/ARGs could enter the plant tissues via direct penetration, wound sites and natural plant openings, and even transfer to endophytic pathogenic microorganisms via horizontal transfer (Seo and Frank, 1999; Ben Said et al., 2015; Jones-Dias et al., 2016). Thus, the risk of human illness associated with the endophytic ARB and ARGs can be increased by ingesting the raw or uncooked produce (Wiedemann et al., 2014; Holzel et al., 2018; Chen et al., 2019), despite that cleaning or sanitizing procedures for produce can physically remove the majority of the surface-attached microbial contaminants (Ssemanda et al., 2018). However, the knowledge of the prevalence of integrase genes and the diversity of resistance gene cassettes in endophytic bacteria of produce (especially raw vegetables) is still elusive.

In this study, six different raw vegetables were collected, and the abundance of class 1, class 2 and class 3 integrase genes in endophytic bacteria was quantified using qPCR analysis. Additionally, the contents of class 1 integrons-carried gene cassettes in the endophytes of raw vegetables were characterized based on the clone library analysis. The aims of this study are to (1) characterize the variations in the abundance of class 1, class 2 and class 3 integrase genes, (2) explore the contents of gene cassettes pool and (3) compare the diversity and abundance of antibiotic resistance gene cassettes carried by class 1 integrons in the different vegetable endophytes. This study will provide data for evaluating the human health risk of the spread of antibiotic resistance gene cassettes carried by integrons in produce.

2. Materials and methods

2.1. Sampling and DNA extraction

Six types of common vegetables that can be eaten raw, including 10 cucumbers (*Cucumis sativus* Linn.), 6 cherry tomatoes (*Lycopersicon esculentum* var. *cerasiforme* Alef.), 5 cabbages (*Brassica oleracea* var. *capitata* L.,1753), 7 carrots (*Daucus carota* var. *sativus* Hoffm.), 5 purple cabbages (*Brassica oleracea* var . *capitata rubra*) and 4 lettuces (*Lactuca sativa* var. *ramosa* Hort.), were collected from ten large markets. These vegetables are commonly used for ready-to-eat salad, especially in the diet of western countries. All samples were collected from Xiamen, which is one of the typical cities with the high consumption of vegetables and fruits in China. One specimen for each vegetable was collected in each market, only if the vegetable was not available in the market. All samples were stored at 4 °C for no more than 12 h before DNA extraction. For the leafy vegetables, 5 g of fresh leaf tissue was used for endophytic DNA extraction. To remove the microbial pollution of the phyllosphere, surface cleaning and disinfection were performed according to previous studies (Ruiz-Perez and Zambrano, 2017; Zhu et al., 2017). Briefly, fresh leaf tissue were rinsed with deionized water, wiped with 70 % ethanol under aseptic conditions, and then soaked in 70 % ethanol for 3 min, 2.5 % sodium hypochlorite for 2 min and 70 % alcohol for 3 min. After each soaking, a four-time rinse with sterile water was performed. To check if surface microbial pollutants were completely removed, the last eluent was coated on LB plate, and the plates were incubated at 37 ℃ overnight. For cucumbers, cherry tomatoes and carrots, pericarp fraction was removed with a sterile blade, and 8 g internal tissues were used for endophytic DNA extraction. A FastDNA® SPIN Kit for Soil (MP Biomedicals, USA) was used for DNA extraction and quality and concentration of DNA were assessed by a NanoDrop ND-1000 (Nanodrop ND-1000, Thermo Scientific, Waltham, MA) and QuantiFluor dsDNA system (Promega, Madison, WI), respectively.

2.2. Quantitative PCR (qPCR) for integrase genes

qPCR assays were applied to assess the abundance of total bacterial (16S rRNA), class 1 (*intI1*), class 2 (*intI2*) and class 3 (*intI3*) integron-integrase genes. To reduce the co-amplification of plant Ct16S and Mt18S genes, the primer sets 799F (AACMGGATTAGATACCCKG) and 1193R (ACGTCATCCCCACCTTCC) were used in the present study (Bai et al., 2015; Bulgarelli et al., 2015; Chen et al., 2021). qPCR analysis was performed using a LightCycler Roche 480 (Roche Inc., USA) with a reaction system consisting of 10 μL SYBR® Premix Ex Taq™ II (Takara, Japan), 0.8 μL of each primer, 0.5 μL of bovine serum albumin (BSA) and 2 μL of DNA. The amplification conditions of these genes were consistent with those previously reported (Barraud et al., 2013; Chen et al., 2016; An et al., 2018b), and the amplification reactions of all samples were analyzed in triplicate. A 10-fold serial dilution for plasmids carrying the corresponding gene fragments was performed to construct standard curves. Relative abundance of integrase genes (copies/copy of 16S rRNA gene) was calculated by dividing the copy numbers of the bacterial 16S rRNA gene by the copy number of integrase genes.

2.3. Construction and analysis of clone libraries for gene cassettes

The variable regions of class 1 integron were amplified using the primer pairs (5′CS: 5′-GGCATCCAAGCAGCAAG-3′ and 3′CS: 5′-AAGCAGACTTGACCTGA-3′) (Chang et al., 2007; An et al., 2018b). PCR mixtures (50 μL) consisted of 20 ng of DNA, 0.8 μL of each primer, 2.5 μL of dimethyl sulfoxide (DMSO), 32.4 μL PCR grade water, 0.5 μL LA Taq™ (Takara, Japan) and 5 μL 10 ×LA Buffer and 8 μL dNTP Mix. qPCR conditions were set as following: initial enzyme activation at 95 ℃ for 10 min, 30 cycles of 94 ℃ 30 s, 55 ℃ 30 s, and then 72 ℃ for 2 min 30 s, with a final extension at 72 ℃ for 10 min. Each DNA sample was amplified in duplicate. The amplicons of each sample were pooled and then purified with a Wizard® gel and PCR clean-up system (Promega, USA). The purified PCR products were quantified using a Qubit™ dsDNA HS Assay Kit (Invitrogen, USA) according to the manufacturer′s protocol.

PCR amplicons were ligated into pMD19-T vector at 16 °C overnight and then transferred into competent cells *Escherichia coli* DH5α according to manufacturer's instructions (Takara, Japan). For each library, 130 positive clones identified by Blue-White screening were randomly picked into 25 μL amplification mixture. Because 5′CS primer could target nonspecific sites on metagenome DNA, we used the couple of the 3′CS primer and the internal primer MRG284 adjacent to the 5′CS primer hybridization site to confirm the presence of a class 1 integron in the picked colonies according to the previously reported studies (Gillings et al., 2009; Stalder et al., 2014; An et al., 2018b). Then the same PCR program was used to verify the clone with at least one gene cassette (> 153 bp). PCR products were analyzed by electrophoresis on 1 % (w/v) agarose gel at 120 V for 18 min. Clones with gene cassettes were Sanger-sequenced using M13 forward and reverse primers.

Raw sequences were assembled, and primers were trimmed. Putative *attC* sites were detected using IntegronFinder (v1.5) with the use of cmsearch, hmmsearch and infernal profiles against an *attC* database. The ORF prediction was performed using Prodigal v2.6.3. If a potential ORF was found between two putative *attC* sites, the ORF was considered to be a potential gene cassette. The predicted ORFs were then annotated by BLASTX against non-redundant NCBI NR database and CARD database with an E-value $\leq 1e^{-10}$. A "variant" of a known gene cassette was defined when the sequence showed an identity and Qcover of 90–99 % with a reference gene.

2.4. Statistical analysis

One-way analysis of variance (ANOVA) and significance testing were performed using SPSS v26.0 (IBM, USA), and nonparametric Kruskal-Wallis tests were applied to assess any statistically significant ($P < 0.05$) differences when data were from a suspected non-normal population. The diversity of gene cassettes (Shannon index and richness) was analyzed by Past 3. Beta-diversity distribution of antibiotic resistance gene cassettes was assessed using Bray-Curtis dissimilarity-based Non-metric Multidimensional Scaling (NMDS) analysis. Anosim test was applied to evaluate the significance of dissimilarity in beta-diversity distribution of antibiotic resistance gene cassettes. Other graphs in this study were produced by Origin 2018. All sequences have been deposited in the Sequence Read Archive (SRA) with the accession no. SUB10648651.

3. Results

3.1. Abundance of integron integrase

The absolute abundance of *intI1* ranged from 3.1×10^5 to 1.2×10^8 copies/g, with the highest abundance in cucumber and the lowest in cabbage. The abundance of *intI2* and *intI3* in vegetables ranged from 5.2×10^3 to 7.7×10^7 copies/g and 5.2×10^3 to 1.1×10^7 copies/g, respectively. The abundance of *intI1* was significantly higher than that of *intI2* and *intI3* in the vegetable endophytes. The abundance of *intI1* was observed to be nearly equal to the abundance of *intI3* in cabbage and purple cabbage. Among these vegetable samples, the bacterial abundance varied from 9.5×10^7 to 6.1×10^8 copies/g with the highest copy number in cabbage (Fig. 1).

Compared with *intI2* and *intI3*, the relative abundance of *intI1* was higher with the range from 9.0×10^{-3} to 3.2×10^{-1} copies/copy of 16S rRNA gene. The lowest relative abundance of *intI1* gene was found in purple cabbage, whereas the highest abundance of *intI1* was detected in lettuce (Fig. 2**A**). The relative abundances of *intI2* and *intI3* were relatively low, ranging from 6.1×10^{-5} to 1.9×10^{-1} and from 5.0×10^{-5} to 3.2×10^{-2} copies/copy of 16S rRNA gene, respectively (Figs. 2**B and** 2**C**). The highest relative abundances of *intI2* and *intI3* were observed in cucumber and cabbage, separately. Kruskal-Wallis test showed that there were significant differences in the abundance of *intI3* ($P = 0.003$) among six vegetables. Additionally, we found that cherry tomato harbored a similar abundance of *intI2* (4.6×10^{-4} copies/copy of 16S rRNA gene) to cabbage (4.7×10^{-4} copies/copy of 16S rRNA gene) (Fig. 2**B**). The relatively low abundance of *intI3* was found in cherry tomato (5.6×10^{-5} copies/copy of 16S rRNA gene) and lettuce (5.0×10^{-5} copies/copy of 16S rRNA gene) (Fig. 2**C**).

3.2. Gene cassettes carried by class 1 integron

The gene cassettes contained in the vegetable endophytes were further evaluated by clone library analysis. For each sample, we randomly picked at least 130 clones targeting class 1 integron gene cassettes, and we observed that 8–49 % of integrons were empty. A total of 2024 integron gene cassettes were identified from the 37 vegetable samples, ranging from 1 to 115 cassettes for each sample, and approximately 22–53 % of clones carried ≥ 1 gene cassette. Totally, 101 types of unique gene cassettes were detected in the vegetable endophytes, including 44 types in carrot, 39 in cabbage, and no more than 30 in other vegetables (Appendix Table S1). Among the identified gene cassettes, the genes associated with antibiotic resistance (44–61 %), hypothetical proteins (6–29 %), and transport (5–23 %) occupied a large proportion (Fig. 3). The endophytes of carrot (59%) and cherry tomato (55 %) were observed to carry more ARGs-related cassettes than other four vegetables. Other gene cassettes encoding biosynthesis, cell synthesis, DNA

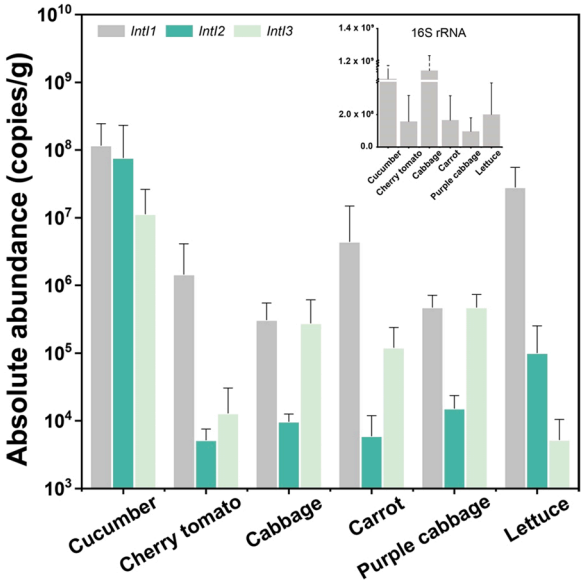


Fig. 1. Variation in the absolute abundance of class 1, class 2, and class 3 integron integrase genes (*intI1*, *intI2* and *intI3*) in the endophytes of six vegetables. The embedded bar plot shows the abundance of 16S rRNA gene. Means ± SDs are shown.

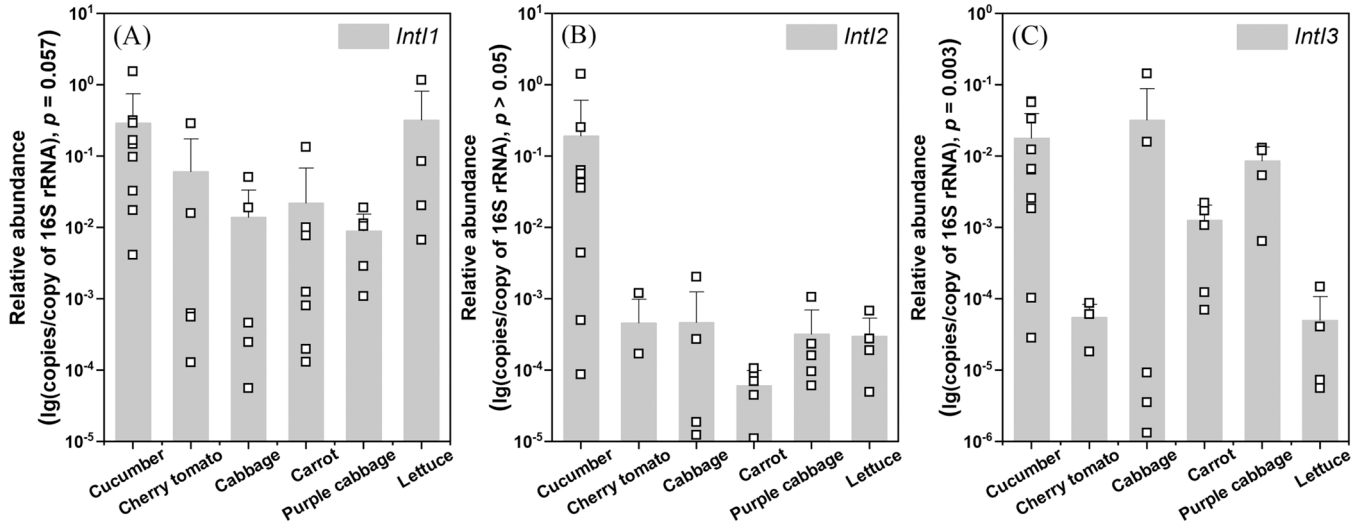


Fig. 2. Normalized relative abundance (copies per copy of 16S rRNA gene) of integrase genes *IntI1* (A), *IntI2* (B), and *IntI3* (C) in the endophytes of different vegetable samples. Means ± SDs are shown. Kruskal-Wallis test (A and C) and ANOVA (B) was performed to test the significance of difference.

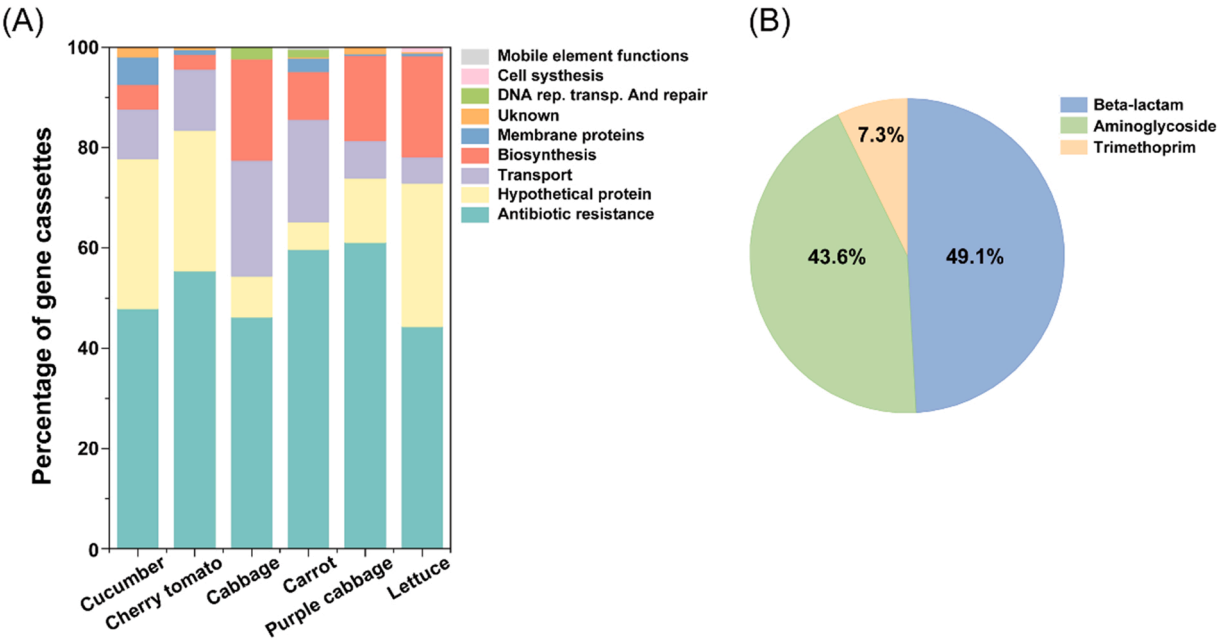


Fig. 3. Percentage of integron-associated genes identified by blastx against NCBI non-redundant (nr) database and CARD database. (A) Percentage of the functionally annotated gene cassettes in the vegetable endophytes; (B) Percentage of antibiotic resistance gene cassettes in all vegetable samples. DNA rep., transp. and repair: DNA replication, transport and repair.

replication, transport and repair, membrane proteins, mobile element functions and mobility were also detected in these vegetable endophytes. The gene cassettes encoding WAT1-related protein At5g13670 was the most prevalent among the vegetable endophytes, especially in carrot. ATP-binding cassette (ABC) transporter genes were only detected in lettuce. The gene cassette related to $bla_{\text{TEM-157}}$ was also frequently detected in the vegetable endophytes.

3.3. Diversity of antibiotic resistance gene cassette

Clone library analysis showed that the class 1 integron gene cassettes harbored 32 unique ARGs, including genes encoding resistance to aminoglycoside, beta-lactam and trimethoprim. Beta-lactam resistance genes were the most frequently observed, accounting for 49.1 % of all ARG cassettes, followed by aminoglycoside resistance genes (43.6 %) and trimethoprim resistance genes (7.3 %). In addition, only aminoglycoside resistance gene cassettes were detected in all kinds of vegetables (Table 1). The number of detected antibiotic resistance gene cassettes in carrot was significantly higher than that in lettuce (Fig. 4**A**). The cassettes richness (the number of unique gene cassettes) and the Shannon index of gene cassettes revealed that there was no significant difference in the diversity of antibiotic resistance gene cassettes among the six vegetables, except that the cassette diversity of lettuce was lower than that of cherry tomato ($P < 0.05$) (Figs. 4**B and** 4**C**). Non-metric Multidimensional Scaling (NMDS) analysis also showed that the overall patterns of antibiotic resistance gene cassettes in different samples were not significantly different (Anosim test, $P > 0.05$) (Fig. 5).

$bla_{\text{TEM-157}}$ and *aadA2* were the most frequently detected cassettes in the endophytes. bla_{OXA} gene was only detected in cherry tomato and cabbage. Some different resistance gene alleles were found to be likely

Table 1
The number of clones for antibiotic resistance gene cassettes detected in all samples (Sequences of a cucumber sample were completely filtered due to its identity and Qcover < 90 %).

Family	Antibiotic resistance associated GCs	Cucumber (n = 9)									Cherry tomato (n = 6)										Cabbage (n = 5)										Carrot (n = 7)										Purple cabbage (n = 5)					Lettuce (n = 4)			
Aminoglycoside	AAC(6')-IIa	0	0	0	0	0	0	0	0	0	0	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Aminoglycoside	AAC(6')-Ib7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5
Aminoglycoside	AAC(6')-Ib-cr	0	0	0	0	0	0	9	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Aminoglycoside	ANT(3'')-IIa	0	0	0	1	0	0	0	3	0	0	0	0	0	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	10	0	0	0	0	0	0	0	0	0	0	0	0
Aminoglycoside	aadA2	25	10	4	1	0	0	0	0	28	0	0	0	6	13	0	0	0	0	0	0	0	0	0	3	0	0	43	0	34	2	1	0	0	2	0	2	4	0	0	0	0	0	0	0	0	0	0
Aminoglycoside	aadA2_variant	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Aminoglycoside	aadA3	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Aminoglycoside	aadA3_variant	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Aminoglycoside	aadA5	0	0	0	0	0	0	0	8	0	0	0	0	0	0	0	5	0	0	0	0	4	0	0	0	102	0	4	0	0	0	0	15	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Aminoglycoside	aadA5_variant	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Aminoglycoside	aadA9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Beta-lactam	blaTEM-57	0	1	0	0	0	0	0	0	0	0	0	0	11	0	0	0	2	0	0	0	1	0	7	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Beta-lactam	blaTEM-57_variant	0	0	0	0	0	0	0	1	0	0	0	0	8	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Beta-lactam	blaTEM-116	0	3	0	0	0	0	0	0	0	0	0	6	0	0	0	0	0	0	1	1	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Beta-lactam	blaTEM-150	0	1	6	0	0	0	0	0	0	1	2	3	0	0	0	0	0	0	0	0	2	1	11	0	0	0	0	3	0	0	0	0	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Beta-lactam	blaTEM-150_variant	0	0	0	0	0	0	0	0	0	0	3	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Beta-lactam	blaTEM-157	0	10	29	0	0	0	0	0	0	33	2	18	1	0	0	13	0	0	2	32	32	38	1	0	0	0	20	0	0	0	0	0	21	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Beta-lactam	blaTEM-157_variant	0	0	0	0	0	0	0	0	0	0	10	5	2	0	0	0	0	1	1	16	2	7	0	0	0	0	4	0	0	0	0	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Beta-lactam	blaTEM-162	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Beta-lactam	blaTEM-162_variant	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Beta-lactam	blaTEM-171	0	0	0	0	0	0	0	0	0	9	1	2	8	0	1	0	1	8	3	5	1	2	12	10	0	0	5	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Beta-lactam	blaTEM-171_variant	0	0	2	0	0	0	0	0	0	1	0	0	11	0	0	0	0	3	0	6	0	0	4	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Beta-lactam	blaTEM-197	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	2	2	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Beta-lactam	blaTEM-197_variant	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Beta-lactam	blaTEM-220	0	1	1	0	0	0	6	1	0	5	2	12	1	0	0	0	0	0	28	3	0	11	2	0	0	0	2	0	0	0	0	0	25	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Beta-lactam	blaTEM-220_variant	0	0	0	0	0	0	4	0	0	1	1	17	3	0	0	0	0	0	2	7	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Beta-lactam	blaOXA-4	0	0	0	0	0	0	0	0	0	0	6	0	0	0	0	0	0	0	0	0	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Beta-lactam	blaOXA-224	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Trimethoprim	dfrA1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Trimethoprim	dfrA12	0	0	0	0	0	0	0	0	29	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Trimethoprim	dfrA27	0	0	0	0	0	0	8	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Trimethoprim	dfrA27_variant	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

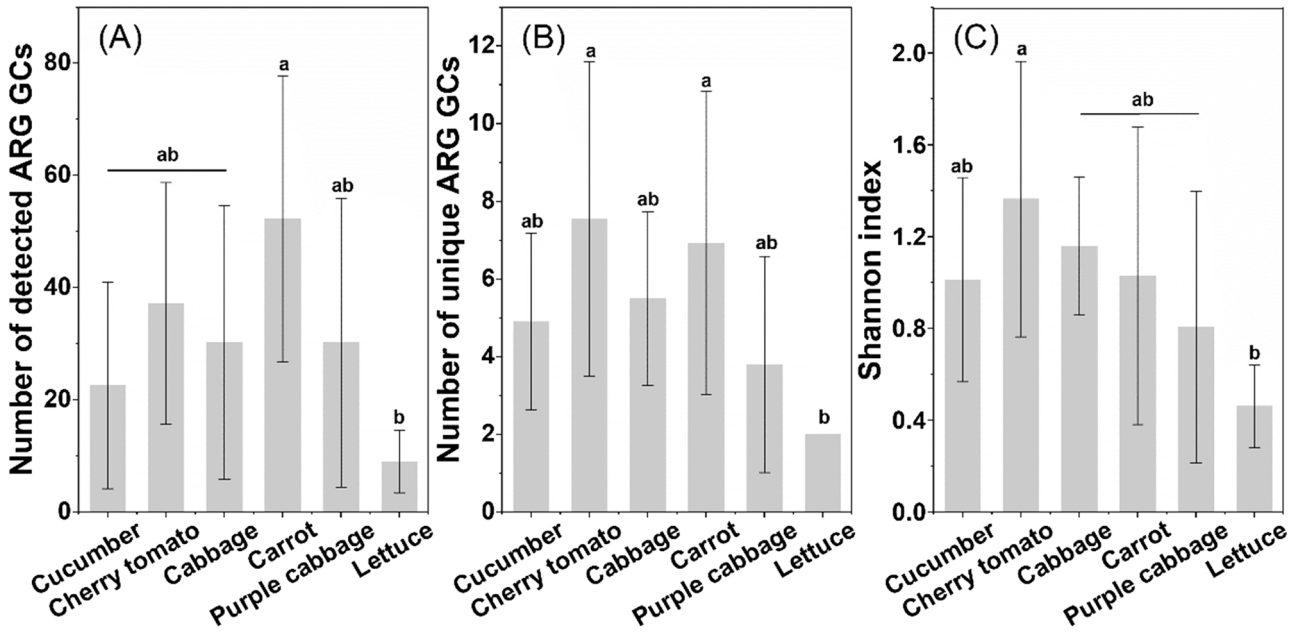


Fig. 4. The number (A) and α-diversity (B and C) of antibiotic resistance gene cassettes in class 1 integrons in each sample. GCs represents gene cassettes. Means ± SDs are shown. ANOVA was performed to test the significance of difference.

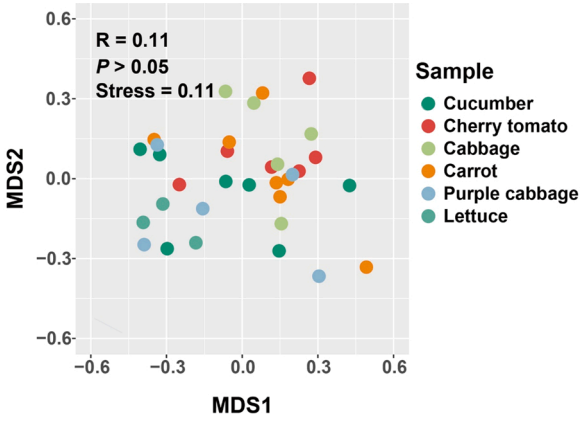


Fig. 5. Non-metric Multidimensional Scaling (NMDS) analysis depicting the overall distribution pattern of antibiotic resistance gene cassettes in each sample.

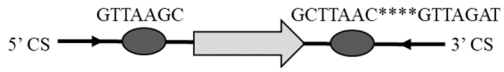
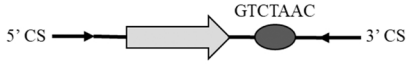
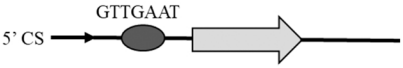
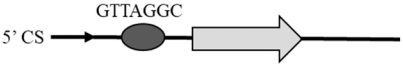
resistance gene variants (90–99 % identity with known resistance gene cassettes), e.g., *aadA* alleles, bla_{TEM} alleles and *dfrA* alleles (Table 2). We found 20 unique resistance gene cassettes in carrot, 18 in cherry tomato, and no more than 17 in other vegetables. *aac(6')-Ib7* and *aadA9* were the genes encoding aminoglycoside resistance, which were detected in lettuce and purple cabbage, respectively (Table 1). In addition, gene cassette array carrying more than one gene cassette was not observed in this study.

4. Discussion

Integrons are the genetic platform enabling bacteria to capture, store and reorder antibiotic resistance cassettes through site-specific recombination, facilitating the dissemination of antibiotic resistance (Stalder et al., 2012; Chainier et al., 2017; Adelowo et al., 2018). The antibiotic resistomes could enter human body with the intake of plant endophytes and even be transferred to human pathogens by MGEs (e.g. integrons), possibly colonizing human gut and altering the trajectory of human health (Zhu et al., 2017; Zhou et al., 2020; Rincon and Neelam, 2021). Our study provided a comprehensive profile of the abundance and distribution of integrase genes and their resistance gene cassettes in the endophytes of six vegetables. To our best knowledge, this is the first study that confirms the widespread prevalence of integrase genes and antibiotic resistance gene cassettes in the vegetable endophytes. In this study, all samples were tested positive for *intI1*, *intI2* and *intI3* genes by qPCR. *IntI1* was more abundant than *intI2* and *intI3*, which is possibly attributed to the low activity of truncated integrase of class 2 and class 3 integrons (Ghaly et al., 2019; Fernandez Rivas et al., 2021). The abundance of class 1 integron integrase gene (3.1×10^5–1.2×10^8 copies/g) detected in the endophytes were lower than those previously reported in sediment and swine manure (Wright et al., 2008; Uyaguari et al., 2013; Xiong et al., 2019). This might be attributed to the low bacterial biomass (the abundance of 16S rRNA gene) in the vegetable endophytes (An et al., 2018a, 2018b). Different vegetable endophytes carried distinct abundances of integrase genes. For example, the relative abundances of *intI1, intI2* and *intI3* in cucumber were higher than those in the other vegetables, and lettuce endophytes also harbored high relative abundance of *intI1*. This was consistent with previous studies (Yang et al., 2017; Xiong et al., 2019). The different concentrations of drug-resistant bacteria in the vegetable endophytes possibly explained the variations in the abundance of integrase genes.

Our analysis of gene cassette contents embedded in class 1 integrons showed that many cassettes exhibited homology with the genes encoding conserved hypothetical proteins and biosynthesis-related proteins, and these data are consistent with those in other environments, such as wastewater treatment plants, deep-sea hydrothermal vents and plant phyllosphere (Elsaied et al., 2007; An et al., 2018a, 2018b). The most frequently detected and functionally characterized gene cassette was the one encoding the potential WAT1-related protein At5g13670, which was reported to play an essential role in plant growth and development and anti-stress defense response by regulating hormone metabolism (Ranocha et al., 2010; Denance et al., 2013). These data further demonstrate that endophytic microbiomes possibly assist their host to sustain different biotic and abiotic stresses and affect plant fitness (Nie et al., 2015; Vandana et al., 2021). Additionally, bacterial ATP-binding cassette (ABC) transporter genes, one of the largest transporter families, were also detected in our study, some members of which are associated with multidrug resistance (MDR) of pathogens and disease resistance

Table 2
Class 1 integron GC_variant and their structures detected in the vegetable endophytes.[a]

GC name	GC structure	No. of GCs
aadA2_variant		7
aadA3_variant		2
aadA5_variant		1
blaTEM-57_variant		10
blaTEM-150_variant		7
blaTEM-157_variant		58
blaTEM-162_variant		1
blaTEM-171_variant		29
blaTEM-197_variant		2
blaTEM-220_variant		37
dfrA27_variant		2

[a] Black circles represent the *attC* sites, and arrows indicate the corresponding GC_variant. Nucleotide sequences with asterisks indicate the inverted repeats. GCs: gene cassettes.

and detoxification of plants (Guerreiro et al., 2018; Peng et al., 2021; Yan et al., 2021). Recent evidences have also shown that these ATP-binding cassette (ABC) transporters were important to bacterial phytopathogenesis (Xu et al., 2018; Zeng and Charkowski, 2021). This indicated that the gene cassettes carried class 1 integrons could be closely related to the interactions between plant and endophytes.

Antibiotic resistance-associated cassettes were the most prevalent functionally annotated genes among the vegetable endophytes, indicating a high incidence of ARGs-transfer events. In this study, all vegetable samples contained various numbers of antibiotic resistance gene cassettes, while the frequency and the type of these cassettes were lower than those in other environments (e.g. water, sediment and sludge). The lower frequency may be attributed to the low level of selective pressure by antibiotics, the plant host immune system and the oligotrophic environment in the plant endophytes (Ma et al., 2017; An et al., 2018a, 2018b). These antibiotic gene cassettes were significantly associated with acquired resistance to aminoglycoside, beta-lactam and trimethoprim, which was consistent with previous studies of foodborne gene cassettes, e.g., meat and shellfish (Xiong et al., 2019; Van et al., 2007). These findings emphasize the close association of these resistant genes with class 1 integron and the importance of integrons in the development of resistant strains via acquirement and expression of various resistance genes. The low fitness cost of these resistance cassettes could favor their maintenance and prevalence in many bacterial species (Kheiri and Akhtari, 2016; Lacotte et al., 2017). Thus, these resistance genes should be given priority when assessing the risk of ARGs to human health owing to their potential mobility. Furthermore, our study found that all resistance-related sequences carried only one resistance gene cassette, and gene cassette array carrying more than one gene cassette was not observed. One of the possible explanations is that the cloning efficiency of longer products (> 5 kb) into T-vectors is low. The response and the high cost-adaption of integron-carrying communities to the oligotrophic condition in the vegetables may also contribute to the limited number of gene cassettes in the array (Lévesque et al., 1995; Elsaied et al., 2007; Stalder et al., 2014). Our analysis revealed that $bla_{\text{TEM-157}}$ and *aadA2* were the frequently detected genes in the resistance gene cassettes. The $bla_{\text{TEM-157}}$ was a gene encoding the extended-spectrum beta-lactamases (ESBLs), which have become a significant clinical and epidemiologic concern. *aadA2* was an aminoglycoside nucleotidyltransferase gene, which was commonly detected in the gene cassettes of the rice field, agriculture effluent and soil samples (Ali et al., 2020). Previous studies reported that these two gene cassettes were commonly detected in the opportunistic pathogens of Enterobacteriaceae (Van Hoek et al., 2015; Abatcha et al., 2018; Richter et al., 2020). Additionally, *bla*OXA genes (*bla*OXA-4 and *bla*OXA-224) were detected in the cherry tomato and cabbage endophytes, which were previously reported to be associated with the resistance to extended spectrum β-lactamases and carbapenemases (Heritier et al., 2005; Kingsley and Verghese, 2013; Leulmi et al., 2019). Additionally, some gene cassette variants were also identified in this study, indicating that there were genetic variations and diversity in the pattern of resistance in plant (Gillings, 2014). Noticeably, the integron-carried ARGs possibly transfer into or colonize human gut microbiome, once the vegetable endophytes were consumed by the food chain (Melotto et al., 2008;

Tyler and Triplett, 2008). Hence, it is important to monitor antibiotic resistance among the foodborne bacteria. In this study, the sampling depth of the clone library might be insufficient to capture the overall variation of gene cassette contents in these vegetables. Further studies of integron gene cassettes with deep sequencing are necessary for the comprehensive understanding of integron-mediated HGT.

5. Conclusions

The study provided a preliminary baseline for the prevalence of integrase genes and antibiotic resistance gene cassettes in class 1 integrons from the vegetable endophytes. The prevalence of antibiotic resistance cassettes in the endophytes confirmed that the endophytes of the raw vegetables were also a potential reservoir of integrase genes and antibiotic resistance gene cassettes. The uptake of raw vegetables possibly provided a potential route for the dissemination and colonization of antibiotic resistance gene cassettes in human gut microbiome. These data further enhance our understanding of endophytic antibiotic resistomes in common raw vegetables, and highlight the importance of continuous monitoring of the resistomes in produce for food safety.

CRediT authorship contribution statement

Cai-Xia Zhao: Investigation, Software, Formal analysis, Visualization, Writing – original draft. **Xiao-Xuan Su:** Data curation. **Mei-Rong Xu:** Investigation. **Jian-Qiang Su:** Writing – review & editing. **Xin-Li An:** Conceptualization, Methodology, Supervision, Writing – review & editing.

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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

The data that has been used is confidential.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2022.114282.

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