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Research Paper

Development of PCR-based DNA marker for detection of white carrot contamination caused by *Y2* locus

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In carrot (*Daucus carota* L.), the taproot colors orange, yellow and white are determined mostly by the *Y*, *Y2*, and *Or* loci. One of the most severe issues in carrot seed production is contamination by wild white carrot. To evaluate the contamination ratio, easily detectable DNA markers for white carrot are desired. To develop PCR-based DNA markers for the *Y2* locus, we have re-sequenced two orange-colored carrot cultivars at our company (Fujii Seed, Japan), as well as six white- and one light-orange-colored carrots that contaminated our seed products. Within the candidate region previously reported for the *Y2* locus, only one DNA marker, Y2_7, clearly distinguished white carrots from orange ones in the re-sequenced samples. The Y2_7 marker was further examined in 12 of the most popular hybrid orange cultivars in Japan, as well as 'Nantes' and 'Chantenay Red Cored 2'. The Y2_7 marker showed that all of the orange cultivars examined had the orange allele except for 'Beta-441'. False white was detected in the orange-colored 'Beta-441'. The Y2_7 marker detected white root carrot contamination in an old open-pollinated Japanese cultivar, 'Nakamura Senkou Futo'. This marker would be a useful tool in a carrot seed quality control for some cultivars.

Key Words: carrot, *Daucus carota*, white carrot, seed contamination, DNA marker, *Y2*, re-sequence.

Introduction

Carrot (*D. carota* subsp. *sativus*) is one of the most important vegetables and is a main source of dietary provitamine A. Carrot seeds are produced worldwide in regions such as Southern Europe, America, and Australia (Magnussen and Hauser 2007, Mandel and Brunet 2019). The climate and environmental conditions of carrot seed production fields are suitable for not only parents of commercial carrot cultivars but also for wild carrot (*D. carota* subsp. *carota*). Wild carrot is the ancestor of cultivated carrots and frequently has white or pale-yellow taproots. Wild carrots often grow next to cultivated carrot fields, and wild and cultivated carrots can cross each other without apparent barriers via a large diversity of insects such as bee and fly (Koul *et al.* 1989, Lamborn and Ollerton 2000, Magnussen and Hauser 2007, Rong *et al.* 2010, Umehara *et al.* 2005). Therefore, contamination of commercial cultivars by wild carrot is well known (Hauser and Bjørn 2001, Hauser 2002, Umehara *et al.* 2005, Wijnheijmer *et al.* 1989). For carrot seed production by seed companies, contamination with white and wild carrots is one of the most important issues in seed quality control. Much effort is carried out to prevent contamination with wild carrots not only by removing them but also by covering parental lines of commercial cultivars with nets. In huge seed production fields, however, it is impossible to completely remove wild carrots and the insects carrying their pollens. A quality check of seed products is always carried out by growing plants from seed samples. However, as this takes a lot of time and requires a huge field, the number of test samples is limited. Therefore, DNA markers for examining the contamination of white carrots without growing them in a field are highly desired for carrot seed quality control.

A cross of wild white carrots and cultivated orange root carrots causes white root carrots in the next generation, because the white root phenotype is dominant over orange. The color difference in carrot roots between orange and white is determined mostly by two loci, *Y* and *Y2*, and carrots having recessive alleles at both loci show orange roots (Buishand and Gabelman 1979, Just *et al.* 2009). Furthermore, recently the *Or* gene, which also affects carrot root color, was identified (Ellison *et al.* 2018). Carrot roots were not white but orange, light orange, or yellow depending on the *Or* genotype, even in carrots having a homozygous orange allele at both the *Y* and *Y2* loci (Simon *et al.* 2019). The *Y*, *Y2*, and *Or* genes were located on different chromosomes,

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and no genetic linkage has been reported. Therefore, DNA markers that can be used to examine alleles of these three genes would be needed to detect non-orange carrots. The *Y* gene, which conditions carotenoid accumulation, and genomic sequences of recessive alleles of orange root have been identified, and the genotype of the *Y* allele can be identified by PCR using reported primers and by the examination of genomic sequences (Iorizzo *et al.* 2016). A single nucleotide polymorphism (SNP) on the *Or* which causes nonsynonymous mutation and co-segregates with carotenoid content has been identified (Ellison *et al.* 2018). Thus, DNA markers on the *Y* and *Or* genes could be developed according to previous reports (Ellison *et al.* 2018, Iorizzo *et al.* 2016). On the other hand, in the *Y2* locus, a PCR-based DNA marker has been reported (Bradeen and Simon 1998). This is a co-dominant PCR-based marker followed by standard agarose gel electrophoresis. However, false-positive and false-negative results would sometimes happen because of the 6.6 cM genetic distance between the *Y2* locus and the marker. Recently, the *Y2* locus was fine-mapped to an approximately 650 kb region on chromosome 7, and two co-dominant cleaved amplified polymorphic sequence (CAPS) DNA markers within the candidate region were reported (Ellison *et al.* 2017). The reported CAPS markers were very effective for genotyping the *Y2* locus, as they identified the root color in a diverse panel of accessions. However, CAPS markers are not suitable for genotyping when many samples are used, such as in cases of seed quality control, because they are more expensive and time-consuming than simple PCR-based markers. Conversion of CAPS markers to fluorescence-detectable markers could be very effective in high-throughput screening, but it is expensive initially and during operation. Therefore, in the present study a simple PCR-based insertion/deletion (InDel) DNA marker that is detectable in agarose gels within the *Y2* candidate region was developed using re-sequencing technology. The developed DNA marker was examined in re-sequenced orange, white, and light-orange carrots and in 15 carrot cultivars together with previously reported DNA markers and a SNP for carrot root color.

Materials and Methods

Plant materials

Two orange root hybrid carrot cultivars, 'Ayahomare' and 'Syousai' (Fujii Seed, Osaka, Japan), bred by Fujii Seed, as well as six white (named Pro_4, Pro_6, Pro_8, Pro_10, Pro_17, and Pro_25) and one light-orange root (Pro_9) carrots contaminated by orange root seeds harvested at seed production fields in Melipilla and Cachapoal, Chile, were used as plant materials for re-sequencing. A developed InDel marker was examined for its ability to identify root color in 12 popular commercially available Japanese hybrid cultivars: 'Tensyou' (Takii, Kyoto, Japan), 'Bantyu Tensyou' (Takii), 'Syouma' (Takii), 'Koimusume' (Takii), 'Aikou' (Sumika Agrotech, Osaka, Japan), 'Benihinata' (Sumika Agrotech), 'Beta-441' (Sakata Seed, Kanagawa, Japan), 'Christine' (Mikado Kyowa Seed, Chiba, Japan), 'Akemigosun' (Kaneko Seeds, Gunma, Japan), 'Aroma Red' (Tohoku Seed, Tochigi, Japan), 'Ayahomare' (Fujii Seed), and 'Syousai' (Fujii Seed), as well as commercially available 'Nantes' (Green Field Project, Kumamoto, Japan), 'Chantenay Red Cored 2' (Johnsons, Suffolk, UK), and a Japanese old open-pollinated cultivar, 'Nakamura Senkou Futo' (Genebank Project, NARO, JP No. 25816).

DNA extraction and re-sequencing analysis

Total genomic DNA was extracted from young leaves of sample plants using the DNeasy Plant Mini Kit (Qiagen Inc., Hilden, Germany). In 'Ayahomare' and 'Syousai' used for re-sequencing, 10 plants were bulked and used for DNA extraction to avoid genomic bias within a cultivar. Paired-end sequencing libraries with an insert size of approx. 400 bp were prepared by using the TrueSeq DNA PCR-free library prep kit according to the manufacturer's protocol (Illumina, San Diego, CA, USA). The nucleotide sequences were determined using massively parallel sequencing by synthesis on an Illumina HiSeqX in paired-end 150 bp mode.

Computational data processing

Primary processing of sequence data, such as deleting low-quality bases and trimming adapters, as well as mapping onto the carrot genome *Daucus carota* v2.0 (Iorizzo *et al.* 2016), were performed as described previously (Arafa *et al.* 2017). For InDel detection, the resultant binary sequence alignment/map format (BAM) files were subjected to 50-300 bp InDel calling with Manta (Chen *et al.* 2016). For SNP calling on the *Or* gene, the resultant BAM files were converted to an mpileup file with SAMtools (version 0.1.19) (Li *et al.* 2009) and subjected to SNP calling with VarScan (version 2.3.9) (Koboldt *et al.* 2009). SNPs were further filtered with VCFtools (version 0.1.13) (Danecek *et al.* 2011) parameters of --minDP 10, --max-missing 0.75, --maf 0.05.

InDel marker validation

Twelve InDels detected within the previously reported *Y2* candidate region, at physical positions from Chr7 33,175,032 to 33,851,849 (Ellison *et al.* 2017), were chosen for the development of InDel markers and validated by PCR in re-sequenced samples. The composition of PCR amplification followed Shirasawa *et al.* (2010), and amplification was performed after an initial denaturation step for 2 min at 94°C, 35 cycles (45 sec at 94°C, 45 sec at 55°C, and 2 min at 72°C), followed by 10 min at 72°C. PCR fragments were separated on 2% agarose gels.

Genotyping of Y and Y2 loci with previously reported markers

Y and *Y2* loci of sample plants were examined with

previously reported DNA markers, yY-W for the *Y* locus (Iorizzo *et al.* 2016), and 4135c for the *Y2* locus (Ellison *et al.* 2017).

Results

Totals ranging from 40.0 to 166.6 million high-quality reads for each sample were obtained and mapped onto carrot genome *Daucus carota* v2.0 (Iorizzo *et al.* 2016), with alignment rates ranging from 85.1 to 88.8% and genome coverage of total aligned reads from ×11.1 to ×47.2, with the average at ×31.4 (**Supplemental Table 1**). In the whole carrot genome, Manta detected 20,699 InDels whose sizes ranged from 50 to 300 bp. Among them, 22 InDels were detected on the approx. 650 kb previously reported *Y2* candidate region (Ellison *et al.* 2017), and 12 InDels were chosen for experimental PCR validation according to physical position and InDel size (**Supplemental Tables 2**, **3**). A threshold cutoff based on the quality value for each InDel by Manta was not used because the physical position of the desired DNA marker was restricted, and the validation of InDels by PCR was planned. A PCR experiment demonstrated that only one InDel marker, Y2_7, could clearly distinguish white root carrots from orange root carrots (**Fig. 1A**, **Supplemental Fig. 1**, and **Table 1**). Y2_7 is a co-dominant marker and 196 bp is inserted in orange root carrots. The amplified product sizes were 361 bp and 165 bp for orange and white root alleles, respectively. All examined white root samples that had contaminated our orange root seed products were heterozygous for Y2_7. The Y2_7 alleles corresponded to those of a previously reported CAPS marker, 4135c for the *Y2* locus (Ellison *et al.* 2017), in re-sequenced samples (**Fig. 1A**, **1B**). The physical position of Y2_7 was 33.6 Mb on chromosome 7 and about 200 kb downstream from 4135c (**Fig. 2**). A sample named Pro_9 contaminated our orange seed products and had light-orange-colored root easily distinguished from the other orange-colored roots (**Fig. 1D**). This Pro_9 sample showed the orange genotype by Y2_7 and 4135c for the *Y2* locus and yY-W for the *Y* locus (**Fig. 1A–1C**, **Table 1**). It has been reported that the *Or* allele affects carrot color even in carrots having the homozygous orange alleles of both *Y* and *Y2* (Simon *et al.* 2019). Therefore, an SNP was examined in the *Or* gene in exon 5 at the physical position of 5,197,361 on chromosome 3, which causes nonsynonymous mutation and co-segregates with carotenoid content, and the genotype of this SNP mostly correlates with those of wild and orange domesticated carrots (Ellison *et al.* 2018). The re-sequenced results showed that Pro_9 had the heterozygote *Or* allele, which causes the light orange root

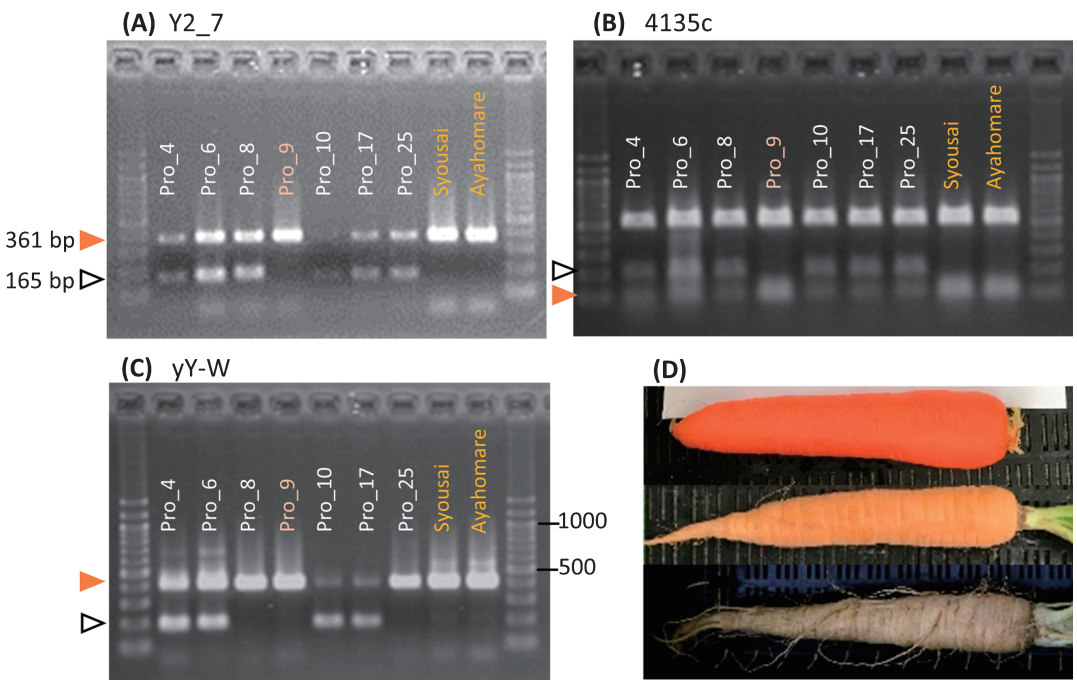

Fig. 1. Gel electrophoresis images of the carrot taproot color DNA markers and taproot colors of re-sequenced carrots. The InDel detected by re-sequencing within the previously reported *Y2* candidate region (Ellison *et al.* 2017) was validated by PCR with Y2_7 primer in re-sequenced carrots (A). Orange and white alleles of amplified products are shown by orange and white arrowheads, respectively. Genotypes of *Y* and *Y2* of re-sequenced carrots were examined using previously reported DNA markers *Y2* for 4135c (Ellison *et al.* 2017) (B) and *Y* for yY-W (Iorizzo *et al.* 2016) (C), respectively. PCR fragments were separated on 2% agarose gels with a 100 bp DNA ladder marker. The migration of size markers (bp) is shown to the right of (C). The taproot colors of re-sequenced carrots are shown in (D). Top is orange in 'Ayahomare', middle is light orange in the Pro_9 sample, and bottom is white in the contaminated carrot. The colors of the sample names show the taproot colors of sample carrots: Pro_4, Pro_6, Pro_8, Pro_10, Pro_17, and Pro_25 are white; Pro_9 is light orange; and 'Syousai' and 'Ayahomare' are orange.

Table 1. Taproot color, genotypes of DNA markers, and SNP correlated to taproot color in re-sequenced carrots

	Y2_7	4135c	yY-W	Or_SNP_Chr3_5197361[a]	Taproot color
Pro_4	H	H	H	Y	white
Pro_6	H	H	H	Y	white
Pro_8	H	H	O	Y	white
Pro_9	O	O	O	Y	light orange
Pro_10	H	H	H	Y	white
Pro_17	H	H	H	Y	white
Pro_25	H	H	O	Y	white
Syousai	O	O	O	T	orange
Ayahomare	O	O	O	T	orange

"H" and "O" indicate heterozygote for white and orange alleles and homozygote for orange allele, respectively. "Y" and "T" indicate a nucleotide of a previously reported SNP on the *Or* gene causing a nonsynonymous mutation and co-segregating with carotenoid content (Ellison *et al.* 2018) using IUPAC. Non-orange alleles are shaded in gray. 4135c and yY-W are previously reported DNA markers for *Y2* (Ellison *et al.* 2017) and *Y* (Iorizzo *et al.* 2016) correlated to carrot taproot color, respectively.
[a] Physical position in carrot genome Daucus carota v2.0 (Iorizzo *et al.* 2016).

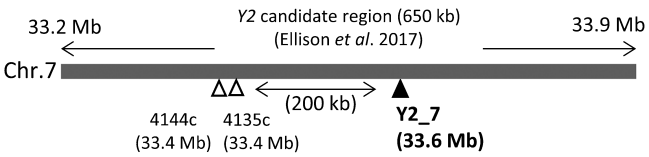

Fig. 2. Schematic image of physical positions of Y2_7 and previously reported CAPS markers (4144c and 4135c) in the *Y2* candidate region (Ellison *et al.* 2017).

color with the homozygous orange alleles of both *Y* and *Y2* (Simon *et al.* 2019) (**Table 1**). All contaminated re-sequenced white root samples had the heterozygote allele for this SNP, and two orange cultivars had the homozygous orange allele, consistent with a previous report (Ellison *et al.* 2018).

To study the versatility of Y2_7, 12 popular orange-colored hybrid Japanese carrot cultivars, as well as 'Nantes' and 'Chantenay Red Cored 2', were examined (**Fig. 3**). Y2_7 showed the orange allele in all of the carrots examined except 'Beta-441', suggesting that Y2_7 could be used to detect white-colored carrots caused by *Y2* in all of the examined cultivars except for 'Beta-441', though more plants would be needed for a precise validation. On the other hand, Y2_7 showed the white root allele in 'Beta-441' even though 'Beta-441' is an orange-colored cultivar. This result showed that Y2_7 gives false white results in 'Beta-441'. The 12 popular orange-colored hybrid Japanese carrot cultivars, along with 'Nantes' and 'Chantenay Red Cored 2', were also examined by 4135c (Ellison *et al.* 2017). 4135c showed the orange allele in all examined samples including 'Beta-441', suggesting that 4135c is more specific for root color than Y2_7 (**Supplemental Fig. 2**). In 'Nakamura Senkou Futo', an old open-pollinated Japanese cultivar, Y2_7 clearly detected contaminated white root carrot (**Fig. 4**).

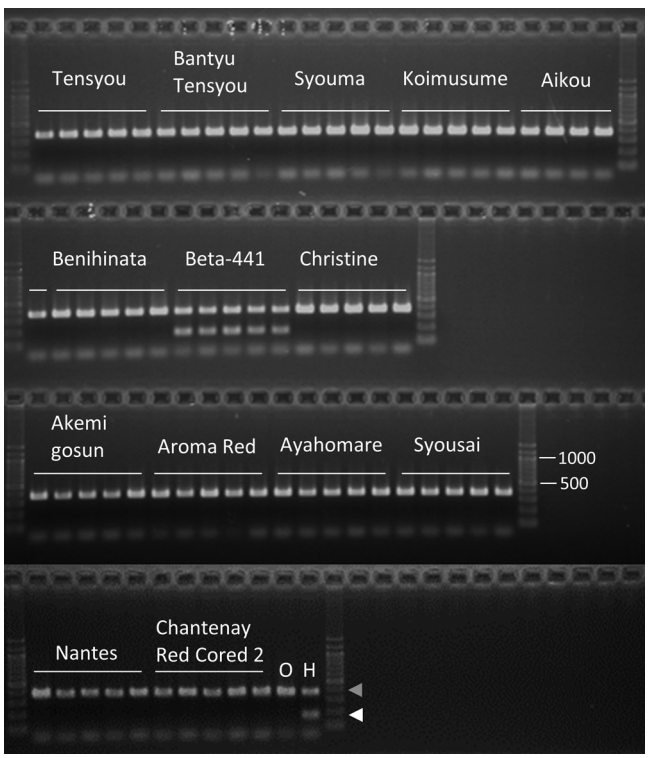

Fig. 3. Examination of Y2_7 in popular Japanese hybrid orange cultivars ('Tensyou', 'Bantyu Tensyou', 'Syouma', 'Koimusume', 'Aikou', 'Benihinata', 'Beta-441', 'Christine', 'Akemigosun', 'Aroma Red', 'Ayahomare', and 'Syousai') as well as 'Nantes' and 'Chantenay Red Cored 2'. All carrot samples except for 'Beta-441' showed orange allele. Orange and white alleles of amplified products are shown by gray and white arrowheads, respectively. PCR fragments were separated on 2% agarose gels with a 100 bp DNA ladder marker. The migration of the size markers (bp) is shown at the right in the gel. O: homozygous orange allele control. H: heterozygous control. Five plants of each cultivar were examined.

Discussion

We developed a co-dominant InDel DNA marker, Y2_7, for the *Y2* locus by re-sequencing technology (**Fig. 1**, **Table 1**). The developed marker is located within a 650 kb region of the *Y2* candidate region (Ellison *et al.* 2017)

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Fig. 4. White carrot detection by Y2_7 marker in 'Nakamura Senkou Futo'. A: Taproot color of examined carrots. B: Gel electrophoresis images of the Y2_7 PCR products of carrots shown in (A). Sample numbers in (A) and (B) are identical. PCR fragments were separated on 2% agarose gels with a 100 bp DNA ladder marker. The migration of size markers (bp) is shown to the right of the gels. O: homozygous orange allele control. H: heterozygous control.

(**Fig. 2**). In carrots, most of the differences between orange and white taproot color are explained by two loci, *Y* and *Y2* (Buishand and Gabelman 1979, Just *et al.* 2009). Therefore, the combination of DNA markers for the *Y* locus (Iorizzo *et al.* 2016) and Y2_7 for the *Y2* locus would be a strong tool for the quality control of orange root carrot seeds. In fact, the combination detected all of the white root carrots in the re-sequenced samples (**Table 1**). The use of these markers can reduce the number of samples needed to grow in order to examine the contamination of white root carrots and can save both cost and time in seed quality control. Light-orange and yellow root colors were caused by the *Or* gene even in the carrots having homozygous orange alleles at both the *Y* and *Y2* loci (Ellison *et al.* 2018, Simon *et al.* 2019). The addition of the *Or* marker to seed quality control markers would be more effective (**Table 1**).

There were no homozygous white alleles in contaminated white-colored carrots for the *Y*, *Y2*, and *Or* loci (**Fig. 1**, **Table 1**). These results would be caused by the cross of seed parents for commercial orange carrot seeds and wild white carrots. In seed production fields, seed and pollen parents for commercial seeds are planted in rows with equal spacing, allowing us to easily identify them from wild and contaminated carrots, which grow in irregular positions. We can therefore remove wild and contaminated carrots from the fields. However, we cannot identify a seed parent crossed with wild carrot during flowering in the fields. Therefore, all contaminated white root carrots examined would have been heterozygotes between orange and wild carrots.

In the Pro_8, Pro_9, and Pro_25 samples, the genotypes of *Y2*, *Y*, and *Or* did not correspond with these three markers (**Fig. 1**, **Table 1**). These results showed that the use of only one DNA marker in orange carrot seed quality control could not detect all contaminated white carrots. The use of the *Or* genotype alone in this study could detect all white and pale-orange root carrots (**Table 1**). However, as the cross between parents of cultivars and wild carrots progressed, the *Or* genotype alone cannot detect all white carrots because of the increase in the number of recombination events between wild plants and cultivars. Therefore, it is necessary to use several DNA markers for seed quality control.

As shown in **Fig. 3**, the white root allele of Y2_7 was not correlated to the root color phenotype in 'Beta-441', which is an orange root cultivar. However, if a seed parent of 'Beta-441' has the allele that is the same as that of wild carrot, and the pollen parent of 'Beta-441' has the orange allele for Y2_7, then the contaminated F_1 plant will show a homozygote for Y2_7, and Y2_7 can be used in this cultivar for white carrot detection. On the *Y* gene, several nonfunctional alleles that cause orange-colored roots have been reported (Iorizzo *et al.* 2016). The *Y2* gene might have several nonfunctional orange alleles like the *Y* gene, and Y2_7 might link to only one orange allele in the *Y2* gene. The previously reported DNA marker 4135c detected white-colored carrots without false-white results in all examined carrots including 'Beta-441' (**Fig. 1**, **Supplemental Fig. 2**). 4135c is therefore more specific than Y2_7 for white carrot detection. However, it is more expensive and time-consuming than Y2_7, because 4135c is a CAPS marker.

According to NARO Genebank, 'Nakamura Senkou Futo' originated and was donated from Hokkaido, northern Japan (Genebank Project, NARO, JP No. 25816, https://www.gene.affrc.go.jp/index_en.php) where wild white carrots, known as Queen Anne's lace, are frequently observed (Umehara *et al.* 2005). The examination of Y2_7 in 'Nakamura Senkou Futo' (**Fig. 4**) suggested that Y2_7 could detect wild white carrots grown not only in Melipilla and Cachapoal, Chile, but also in Hokkaido, Japan. We evaluated Y2_7 in contaminated white carrots harvested in only three regions: Melipilla and Cachapoal in Chile and Hokkaido in Japan. Wild white carrot is native to temperate regions of Europe and western Asia, and it has been introduced into the United States, New Zealand, Australia, Japan, and all continents except for Antarctica (Grzebelus *et al.* 2011, Hauser *et al.* 2004, Mandel and Brunet 2019, Umehara *et al.* 2005), and it is genetically diverse (Iorizzo *et al.* 2016). Moreover, when a recombination event between Y2_7 and the *Y2* gene happens, Y2_7 does not show a correlation to the white root phenotype. Therefore, it is necessary to keep examining the correspondence between the Y2_7 genotype and the root color phenotype in the use of Y2_7 in seed quality control. The *Y2* gene has not been identified, although a candidate gene has been reported (Ellison *et al.* 2017). The identification of the *Y2*

gene would be very helpful and the use of a DNA marker on the gene would be the best way to examine *Y2* in seed quality control. However, at present, Y2_7 would be an easy and simple DNA marker for genotyping the *Y2* locus in some cultivars for individual genotyping.

To evaluate the contamination ratio approximately in a seed product for seed quality control, a dominant marker might be more sensitive to the heterozygous allele in bulk DNA samples, which consist of DNA from many samples. Development of a primer at regions adjacent to the ends of the orange-allele insertion of Y2_7 to amplify only the white allele would provide a dominant DNA marker. However, as this dominant marker does not have positive control for PCR amplification, it must be used with care in order to minimize false negative results. Moreover, because Y2_7 shows co-dominant segregation, the contamination ratio in a bulk DNA sample can be determined more precisely with digital PCR targeting the InDel used in Y2_7 when fluorescent-labeled probes are developed. In this case, users can identify the contamination ratios of large-scale samples at once. The selection of a suitable combination of DNA markers for detecting contaminated non-orange-colored wild carrots depending on the cultivars and experimental environments would provide efficient quality control in carrot seed production.

Author Contribution Statement

T.S. designed the experiment and wrote the manuscript; S.I. provided direction for the study and correction to the manuscript; C.M. and A.O. performed the experiment; S.N. performed NGS data analysis; C.K. and T.F. provided plant materials and information for plant materials.

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