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EXAMINATION OF PUNGENCY CHARACTERISTICS IN PEPPER BY "OPTION 1" APPROACH

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Examination of Pungency Characteristics in Pepper by "Option 1" Approach

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Introduction

Pepper (*Capsicum annuum* L.) is cultivated worldwide mainly as a fresh vegetable or a food additive. Presently, two cultivation types of pepper are largely distinguished based on the presence of pungency, that is, chili (hot) pepper and sweet pepper (paprika), which account for both consumption types. Chili pepper has a strong pungency and is used as a food additive (or a spice) to flavor food. Pungency, a major factor for consumption of the *Capsicum* fruits, is caused by capsaicinoids, which are unique alkaloids restricted to the genus *Capsicum*. Conversely, sweet pepper is non-pungent with larger fruits and is used as a vegetable. Sweet peppers are known to have resulted from the domestication of wild chili peppers which are usually hot (Andrews, 1995).

Regardless of the type of pepper, accurate selection of the pungency trait is a major concern for breeders, and also for DUS examiners. Currently, the phenotyping of pungency relies on taste tests, HPLC analysis or gas chromatography. These methods, however, cannot be easily utilized due to physical, technical or economical limitations.

In the UPOV Test Guidelines for pepper, the pungency trait (capsaicin in placenta) is described as a qualitative type characteristic (absent or present in characteristic No. 45, document TG/76/8) and defined by taste testing pepper flesh together with the locules in the placenta area. However, the testing is very limited to a few varieties at a time for each person. Also, weak pungency can be misinterpreted as little (null value), still more in latter trials, which shows general situations resulting from sensory test. Marker-assisted detection may provide an alternative to the phenotyping, and allows assessment in large populations in an earlier, easier, faster and more economical manner, even before fruit setting.

Pungency is known to depend on a single dominant gene, *C*, now also known as *Pun1* (Stewart et al., 2005 and references therein; Andrews, 1995) that. in a homozygous recessive condition, results in the absence of pungency. Molecular genetic mapping positioned the *C* locus on chromosome 2 (Blum et al., 2002; Lefebvre et al., 2002). Many genes on the capsicinoid biosynthetic pathway have been identified (Curry et al., 1999). Kim et al. (2001) isolated a cDNA clone encoding a putative capsaicinoid synthase (*CS*), which was identified as AT3 gene, a putative acyltransferase on *Pun1* locus (Stewart et al., 2005). A large deletion at this locus resulted in the loss-of-function allele known as *Pun1*.

The *Pun1* gene encoding a putative acyltransferase on the locus provides a key to disclose a secret in long breeding history of pepper, and thereby suggests a possible way to an "Option

1" approach* for pungency detection in pepper. We have now examined the locus-derived specific (LDS) marker system in pepper F_1 hybrids to test the feasibility of pungency detection.

Materials and Methods

Plant materials

A total of 136 varieties of pepper including both types of chili pepper and sweet pepper, together with two ornamental varieties and a few breeding lines were used for this examination. Most of those were hybrids, except for nine inbred lines. The pungency test was carried out by tasting or by HPLC, as a DUS testing procedure through 2003 to 2006. Plant samples were collected directly from the testing field, ground and stored at -70˚C until DNA isolation.

DNA extraction and PCR analysis

Genomic DNA was extracted from frozen powder with NucleoSpin Plant kit (Macherey-Nagel, Germany). The PCR amplification was performed in 25 μl of a uniform reaction mixture, 3mM $MgCl_2$, 0.2mM of each of the dNTP, 50ng of template DNA, 10 pmole of each primer, 1 unit of *nTaq*^{tenuto} DNA polymerase and 1× commercial buffer (Enzynomics, Korea). Following the preliminary denaturation step of 5 min at 94˚C, 35 amplification cycles of 1 min at 94˚C, 1 min at 62˚C, 2 min at 72˚C, followed by a final step of 10 min at 72˚C were performed by using a PTC-200 thermocycler (MJ Research, Chatham, NJ, USA). The resulting amplicons were analyzed on 0.8% agarose gel.

Detection of capsaicinoids by HPLC

Ten fruits from each variety were harvested at about 100 days after anthesis in August. After removing pedicels, whole fruits were bulked, dried in 50˚C and ground. Detection of capsaicinoids was carried out according to Blum et al (2003). One gram of dried powder was extracted with 10ml of acetonitril shaken at room temperature for 4 hours. A 20 μl membrane-filtered extract was injected for analysis in a Jasco 970 (Japan). The mobile phase was CH_3OH:H_2O=65:35 and the solid phase was a CresPak C18S(150*46 mm) column. Detection was at 280 nm and retention times were 6.2 minutes for capsaicin and 9.3 minutes for dihydrocapsaicin. Capsaicin and dihyrocapsaicin external standards (Sigma, M2028 and M1022, respectively) were prepared as 50 ppm stocks in absolute methanol. Peak areas of capsaicin and dihydrocapsaicin were converted to mg/g(dry weight).

Experimental Results

Lee et al. (2005) originally developed six SCAR markers from a set of 9 primers, two of them being codominant, based on the sequence information for the *CS(Pun1)* gene, and showed that the *CS* gene is cosegregated perfectly with the *Pun1* locus. The segregation ratio was 3:1 (pungent to non-pungent) for 121 F_2 populations.

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* Option 1(a): Use of molecular characteristics which are directly linked to traditional characteristics (gene specific markers) – see documents TC/38/14-CAJ/45/4 and TC/38/14 Add.-CAJ/45/5 Add.)

Our preliminary work screened 8 F_1 hybrids as a variety subset for further selection procedures of markers. When we initially applied the original six SCAR markers (marker 1 to 6, Table 1) to the variety subset, the dominant type markers showed simple segregation in accordance with the pungency type except marker 3, while the codominant type markers produced some escapes or unstable amplifications, from no detection to pseudo-amplicons (Table 1). The resulting pattern indicated some different approaches, marker-by-marker, are necessary to develop a reliable marker system.

Because codominant type markers are generally preferred, first of all, we designed four more markers (marker 7 to 10, Table 1), three of them being codominant type, based on possible different primer combinations. Among those, two codominant type markers showed stable amplification and DNA banding patterns as expected. Among those, marker 8(5+9) produced 4,900 and 2,000 bp fragments, respectively for pungent type and non-pungent type varieties, and was selected as a candidate to test all the varieties (Table 1).

The LDS 59 [marker 8 (5+9)] marker was applied to all the 136 varieties. The resulting amplicons generated three band patterns of homozygous dominant, homozygous recessive, and heterozygous. The genotyping by LDS 59 marker showed full matches with phenotyping for pungency by HPLC analysis or breeders' data based on catalogues. Unexpectedly, however, the heterozygous type patterns detected from five hybrids and one inbred line apparently did not correspond to the known pedigree information. That indicates that heterozygotes should be assessed on parental lines at the given locus. In any case, the present marker system shows perfect predictability for the pungency characteristic.

Current Status

The presentation will deliver more information on the results concerning another dominant type marker. We are planning to test the real situation of heterozygosity for the varieties showing heterozygotic pattern by LDS 59. The potential impact accompanying the conversion of examination method for pungency trait will be discussed. We are also considering a segregation analysis on F_2 populations for the developed markers in the future.

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Table 1. Marker selections and prediction of pungency trait in pepper varieties.

Variety code and Primer combinations	1 (1+4)	2 (1+2)	3 (3+4)	4 (5+8)	5 (6+8)	6 (7+9)	7 (7+8)	8 (5+9)	9 (6+9)	10 (6+4)	Pungency type[1)]
001	1,670	434	615	5,500	2,900	896	1,100	4,900	2,600	3,500	P
046	1,670	434	615	5,500	2,900	896	1,100	4,900	2,600	3,500	P
057	1,670	434	615	2,900	271	896	1,100	4,900 /2,000	2,600 /100	1,100	P
059	1,670	434	615	2,900	271	896	1,100	4,900 /2,000	2,600 /100	1,100	P
072	1,670	434	615	5,500 /2,900	2,900 /271	896	1,100	4,900 /2,000	2,600 /100	1,100	P
074	1,670	434	615	5,500 /2,900	2,900 /271	896	1,100	4,900 /2,000	2,600 /100	1,100	P
111	*	*	615	2,900	271	*	*(?)	2,000	100	1,100	NP
113	*	*	615	2,900	271	*	*(?)	2,000	100	1,100	NP

1) P = pungent; NP = non-pungent.

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