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Full Length Article

Development of SNP based Markers Associated with Bacterial Wilt Resistance in Pepper (*Capsicum annuum* L.)

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Abstract

Bacterial wilt (BW) caused by *Ralstonia solanacearum* is one of the most prevalent vascular disease of diverse plant species from dicots to monocots. Because of its complex genetics mediating BW resistance, the genomic factors have been poorly understood. Based on previous studies, we selected 28 candidate genes related to BW resistance and identified significant differences in the expression levels of five genes between two pepper cultivars, YCM334 (BW resistant) and Taean (BW susceptible). Cleaved amplified polymorphic sequences (CAPS) markers were developed on two genes, CA03g23820 and CA04g02500 and BW susceptible/moderate resistant/resistant genotypes can be successfully determined. CA03g23820 is annotated as beta-galactosidase that may play an important role in pathogen invasion through vascular tissue and CA04g02500 is annotated as detected protein of unknown function. The CAPS markers for CA04g2500 showed significant association with BW resistance among a recombinant inbred line population (YCM334 x Taean). Although different markers can be developed depending on genotypes, the results in this study will help account for BW resistance in pepper, and facilitate breeding process using marker-assisted selection.

Keywords: *Ralstonia solanacearum*; Hot pepper; CAPS marker; qRT-PCR

Introduction

Chili pepper (*Capsicum annuum* L.) belongs to the Solanaceae family, which is one of the largest families in the plant kingdom containing more than 3,000 species, including pepper, tomato, eggplant and tobacco (Knapp, 2002). Peppers had been cultivated in Mexico since at least 6000 B.C (Kraft *et al*., 2014). After the Columbian Exchange, many cultivars of chili pepper have been spread across the world and pepper fruits have become important vegetable foods and the spice for human diet because of its nutritional value and hot flavor, particularly in Korea and China. Peppers are great sources of vitamins C and E and carotenoid compounds and provitamin A with antioxidant properties (Tripodi *et al*., 2018; Farhoudi *et al*., 2019). Pepper cultivars are rich in capsaicinoids, and capsaicin and dihydrocapsaicin are the major capsaicinoids found in spicy cultivars, with pharmacological properties and antioxidant activities, providing the specific taste to pepper fruits (González-Zamora *et al*., 2015).

Ralstonia solanacearum causes bacterial wilt (BW), one of the most prevalent soil-borne vascular disease of diverse plant species from dicots to monocots. BW is distributed over tropical and subtropical countries and is the causal agent of potato brown rot, bacterial wilt of tomato, tobacco, eggplant and some ornamentals as well as Moko disease of banana (Mansfield *et al*., 2012). Infected host shows discoloration of the vascular tissue, foliar epinasty and collapse by wilting, resulting in complete necrosis (Yuliar *et al*., 2015).

Because of its economic and scientific consequences, BW resistance has been much studied in various plant species, especially in the Solanaceae family (Mansfield *et al*., 2012). Although most donor candidates' resistance to BW is controlled by polygenes in tobacco, one major QTL explaining more than 30% of resistance has been reported (Nishi *et al*., 2003). Genetic analysis in tomato revealed several major QTLs for BW resistance and significant roles of each QTL in the control of strain-specific resistance (Wang *et al*., 2013; Kim *et al*., 2016). In pepper, although a few amplified fragment length polymorphism (AFLP) (Thakur *et al*., 2014), simple sequence repeat (SSR) and cleaved amplified polymorphic sequences (CAPS) (Mimura *et al*., 2012) markers associated with BW resistance have

been developed, different inheritance patterns have been observed depending on different materials. Therefore, the genomic loci for BW resistance in pepper have been poorly understood because of the complicated inheritance of BW resistance.

Current reference genome sequence and transcriptomic data in pepper have facilitated genetic studies for BW resistance (Kim *et al*., 2014). Through transcriptomic analysis using the two genotypes, BW resistant YCM334 and BW susceptible Taean, putative single nucleotide polymorphism (SNP) and SSR marker positions were searched (Lu *et al*., 2011). To identify putative disease resistance genes, YCM334 and Taean were re-sequenced and putative SNP markers were investigated (Kang *et al*., 2016). Microarray analysis was performed with KC350, a BW resistant genotype, and Chilbok, a BW susceptible genotype and identified resistance/susceptibility-specific genes (Hwang *et al*., 2011). Although candidate genes possibly responsible for BW resistance and SNPs on these candidate genes have been reported, SNP based markers have not been developed or validated using commercially grown cultivars in these previous studies.

To prevent BW, various chemical agents have been applied, because BW leads to heavy yield loss in pepper production. However, the molecular breeding technique to import BW resistance to the susceptible cultivars by means of genomic and transcriptomic information can be the feasible, effective and eco-friendly approach to circumvent the problem of BW in pepper. Although genomic and transcriptomic information have been published for BW resistance in pepper, SNP-based markers associated with BW resistance have not been developed. The goal of this study was to validate the relative expression levels of candidate genes for BW resistance identified from the previous studies and to develop CAPS markers that can characterize susceptible and resistant cultivars in pepper. Determination of CAPS markers linked to BW resistance will help develop novel cultivars with enhanced resistance to BW.

Materials and Methods

Plant Materials

To validate phenotypic variation between BW resistant YCM334 and BW susceptible Taean under BW disease (Lu *et al*., 2011), *R. solanacearum* (10^7-10^8 CFU·mL^{-1}) was applied to soil when they have 6-8 leaves, and phenotypic variation was evaluated visually 25 days after inoculation (Fig. 1). For RNA extraction, YCM334 and Taean were grown in Murashige and Skoog (MS) media until they had 6-8 leaves (MS 4.4 g/L, Sucrose 30 g/L, Agar 7 g/L in ddH_2O 1L, pH 5.8) (Murashige and Skoog, 1962). *R. solanacearum* (10^7-10^8 colony-forming units (CFU)·mL^{-1}) was inoculated into slightly wounded roots and re-planted in new solid media.

R. solanacearum was provided by National Institute of Horticultural & Herbal Science, Rural Development Administration, in the Republic of Korea. The CAPS markers we developed were applied on six cultivars commercially grown in South Korea, including YCM334, Taean, Saengryeg211, Wonkwang3, 99.9 and Tantan.

RNA Extraction and cDNA Synthesis

RNA samples were extracted from young leaf tissues collected at 0, 6 and 12 h after inoculation using Trizol reagent (Thermo Fisher, USA) according to manufacturer's instruction and the quality was checked using RNA 600 Nano Kit (Agilent, USA) and 2100 Bioanalyzer (Agilent, USA). cDNA library was constructed using QuantiTect Reverse Transcription Kit (Qiagen, USA).

Primer Design and Real Time PCR Analysis

Primer sets targeting 28 candidate genes were designed for quantitative reverse transcription-polymerase chain reaction (qRT-PCR) using Primer3Plus (https://primer3plus.com/) (Supplementary Table 1) (Rozen and Skaletsky, 2000 p. 3; Wan *et al*., 2011). The specificity of primer sets was confirmed by PCR using genomic DNA. PCR mixture was prepared in total volume of 20 μL (60 ng DNA template, 2 μL 10× PCR buffer, 2 μL dNTP (10 m*M*), 0.5 μL forward primer (5 μM), 0.5 μL reverse primer (5 μM) and 0.2 μL I-Taq polymerase (Intron biotechnology, Korea)). Denaturation at 94°C for 4 min was followed by 34 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s, then a final extension at 72°C for 10 min. qRT-PCR was performed with Roter-gene Q (Qiagen, USA) using SYBR Green-based PCR kit (QuantiNova SYBR Green PCR kit (Qiagen, USA)). Amplification efficiency (E) and correlation coefficient (R^2) were calculated using the standard curve method (Svec *et al*., 2015).

$$E = 10^{-\left(\frac{1}{slope}\right)} - 1$$

The primer sets with E value between 90 and 110 and R^2 over 0.99 were used for further analysis (Table 1) (Kubista *et al*., 2006). β-TUB was used as control to quantify relative gene expression (Livak and Schmittgen, 2001; Wan *et al*., 2011). Statistical differences were determined by Duncan's Multiple range test ($p < 0.05$) using agricolae and laercio R packages (Fig. 2).

Development and Validation of CAPS Markers

Primer sets for CA04g00830, CA04g02500 and CA10g15480 were designed based on possible CAPS marker sites identified between YCM334 and Taean by Kang *et al*. (Kang *et al*., 2016). Primer sets for CA03g23820 and CA10g10490 were designed using Primer3plus based on SNP information identified by Kang *et al*. (Table 2).

PCR was performed in the same way as described above. PCR products were cleaved by adding 1 μL of restriction enzymes (BstUI (NEB), MluCI (NEB), MseI (Enzynomics) and TaqI (Enzynomics)), 2 μL of buffer and 7 μL of distilled water to 10 μL of PCR product (Table 2). After incubation for two hours, purification was performed using the MEGAquick-spin™ Plus Total Fragment DNA Purification Kit (Intron biotechnology, Korea) and electrophoresis was analyzed using Gel Doc ™ XR + (BIO-RAD, USA) and Owl ™ A3-1 Large-Gel Electrophoresis System (Thermo Fisher, USA).

The CAPS markers were applied on a RIL population consisting of 155 lines derived from a cross between YCM334 and Taean provided by National Institute of Horticultural & Herbal Science, Rural Development Administration, in the Republic of Korea. For statistical analysis, generalized linear model was used for single-marker analysis using GLM procedure in SAS.

Results

Phenotypic Variation between YCM334 and Taean

Hot pepper cultivars, YCM334 and Taean, have been known to be resistant and susceptible, respectively, to pepper diseases. We infected YCM334 and Taean with *R. solanacearum* and confirmed YCM334 and Taean were resistant and susceptible to *R. solanacearum* (Fig. 1). BW susceptible genotype, Taean, were wilted and completely dead while BW resistant genotype, YCM334, were healthy after inoculation.

Identification of Candidate Genes

In total, four groups consisting of 28 candidate genes related to disease resistance were selected based on previously reported studies (Supplementary Table 1). The first group of 10 genes are the most polymorphic genes between YCM334 and Taean by non-synonymous SNPs (Kang *et al*., 2016). The second group of eight genes are the polymorphic genes between YCM334 and Taean by non-synonymous SNPs among differentially expressed genes (DEGs) between KC350 and Chilbok (Hwang *et al*., 2011; Kang *et al*., 2016). The third group of seven genes are pepper homologous genes polymorphic between YCM334 and Taean by non-synonymous SNPs where disease related QTLs have been mapped in tomato (Kang *et al*., 2016). The last group of three genes are candidate DEGs for BW resistance identified between KC350 and Chilbok (Hwang *et al*., 2011). Amplification efficiency (E) and correlation coefficient (R^2) were tested for the primer sets targeting these 28 genes.

Relative Expression Levels of Candidate Genes

The expression levels of β–TUB, GAPDH and UEP-3, suggested as reference genes in pepper by were tested and

Fig. 1: Phenotypic variation between Taean (BW susceptible) and YCM334 (BW resistance) after inoculation of *R. solanacearum*. The picture was taken in 25 days after inoculation

β–TUB showed the most stable expression in YCM334 and Taean under BW condition (E: 109%, R^2: 0.9995) (Wan *et al*., 2011). Therefore, β–TUB was used to normalize quantitative reverse transcription-polymerase chain reaction (qRT-PCR) results (Table 1). Among 28 candidate genes, eight genes were successfully amplified and five genes were selected for further analysis based on amplification efficiency ($90 < E < 110$) and correlation coefficient ($R^2 > 0.99$) (Table 1) (Kubista *et al*., 2006). All five genes showed statistically significant differences in the expression levels between YCM334 and Taean. The expression level of CA03g23820 was higher in Taean at 6 h after inoculation but decreased at 12 h in Taean (Fig. 2). The expression levels of the other four genes, CA04g00830, CA04g02500, CA07g10490 and CA10g15480 were higher in YCM334 and gradually increased after inoculation (Table 1).

Development and Validation of CAPS Marker

To develop CAPS markers for the five genes, CA03g23820, CA04g00830, CA04g02500, CA07g10490 and CA10g15480, which showed significant differences in the relative expression levels between YCM334 and Taean (Fig. 2), the genomic regions containing SNPs between YCM334 and Taean were amplified by PCR with six cultivars, Taean (susceptible), Saengryeg211 (susceptible), YCM334 (resistant), Wonkwang3 (resistant), 99.9 (moderate resistant) and TanTan (moderate resistant).

Table 1: Primer sequences for five candidate genes and the reference gene (β-TUB) with E value between 90 and 110 and R^2 over 0.99

Gene name	Annotation	Primer sequence (5' to 3')	Amplicon length (bp)	E	R^2
CA03g23820	beta-galactosidase 4	For TCGCTGGTCGAAAAGGTTTG Rev GCTGCAATTCACTTCAACAGC	123	100	0.997
CA04g00830	Phosphatidylinositol 4-kinase, putative	For AACGTGAAGCCGCCAATTAC Rev TTTCGATACCCTGCTGCATG	105	104	0.9974
CA04g02500	Detected protein of unknown function	For ATGCGCGGAGATACTTGATG Rev TGTTTCTTCAACCTCCCCTTCC	144	110	0.9964
CA07g10490	lipid transfer protein 1	For AGGCAATGGTGGAACTAGAAGG Rev GATGTTGGGCCGACGAATTG	121	106	0.9965
CA10g15480	Putative disease resistance protein	For TGGGATATTCTGCTTTTGAGGTC Rev ACAATGGCACGTTTAGAGTTCAG	125	100	0.9941
EF495259.1	Beta-tubulin	For GAGGGTGAGTGAGCAGTTC Rev CTTCATCGTCATCTGCTGTC	167	109	0.9995

E: amplification efficiency, R^2: correlation coefficient

Table 2: Primer sequences for CAPS marker development

Gene	5' primer sequence	3' primer sequence	Product size	Restriction enzyme	Incubation	Inactivation
CA03g23820	GGCAGTCTGGCAAAATGCTAA	AGCTATCCTGAGCATGTCCTG	300	BstUI	60°C, 2hrs	N/A
CA04g02500	GGTGAAGATGTCGCGGAGAT	GAATCGAGCAGGGAGAGAGC	311	MluCI	37°C, 2hrs	N/A
CA04g00830	CAACAGGCCTCAGATCCTAA	AGGATCCTTTTCAGGCGGTG	309	MseI	37°C, 2hrs	65°C, 20min
CA07g10490	TGCACCAGACAAACAAGCAG	AGGAACTGTTTTGTGATTTTCCCT	302	MluCI	37°C, 2hrs	N/A
CA10g15480	GCAAAACCTCAAAGATGAAC	AACTTCAACCCCTCTAAAGC	357	Taq.I	65°C, 2hrs	80°C, 20min

N/A: Not applicable

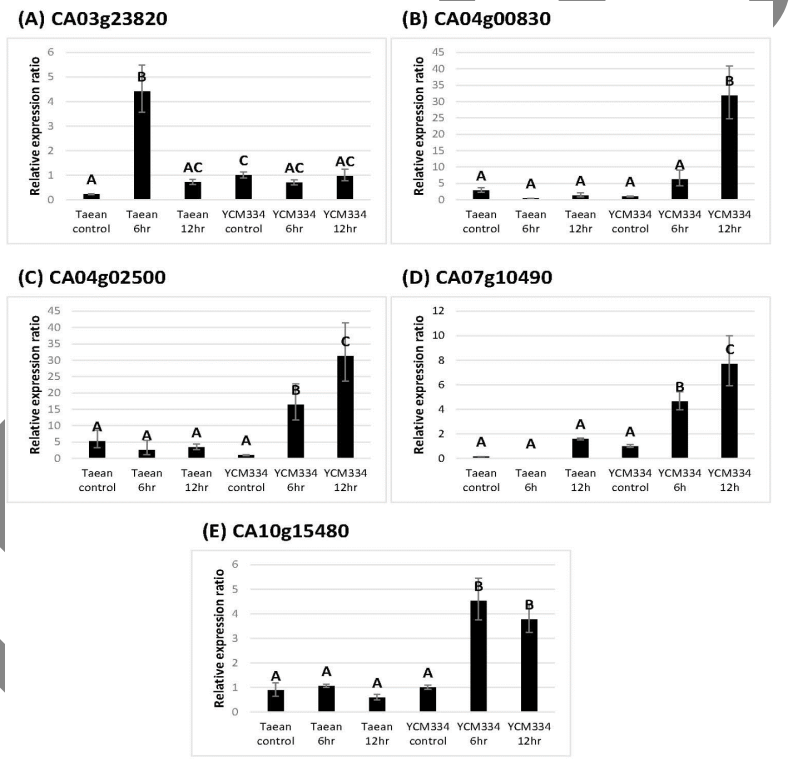

Fig. 2: Relative expression levels of five candidate genes related to bacterial wilt of two pepper cultivars. Different letters above each bar indicate statistical difference determined by the Duncan's Multiple Range Test ($p < 0.05$). (A) CA03g23820 (B) CA04g00830 (C) CA04g02500 (D) CA07g10490 (E) CA10g15480

The PCR products targeting CA04g00830 and CA07g10490 were cleaved by MluCI and CA03g23820, CA04g02500 and CA10g15480 were restricted by BstUI, MSeI and Taq.I, respectively (Table 3). The CAPS marker for CA04g02500 showed band polymorphisms between resistant and susceptible cultivars (Fig. 3). The CAPS marker for CA03g23820 could determine susceptible and moderate resistant cultivars.

These two CAPS markers for CA04g02500 and CA03g23820 were applied to a RIL population derived from a cross between YCM334 (BW resistance) and Taean (BW susceptible) (Supplementary Table 4).

Table 3: Non-synonymous SNPs of five candidate genes for CAPS marker development

Gene name	SNP position	Ref.	YCM334	Taean	Restriction enzyme	Recognition site	expected fragment of Ref. (bp)	expected fragments with SNP (bp)
CA03g23820	intron	G	A	G	BstUI	CGCG	218 and 82	300
CA04g02500	exon	T	T	C	MluCI	AATT	78 and 233	311
CA04g00830	exon	C	C	A	MseI	TTAA	309	256 and 53
CA07g10490	exon	T	C	T	MluCI	AATT	213 and 89	302
CA10g15480	exon	C	T	C	Taq.I	TCGA	184 and 173	357

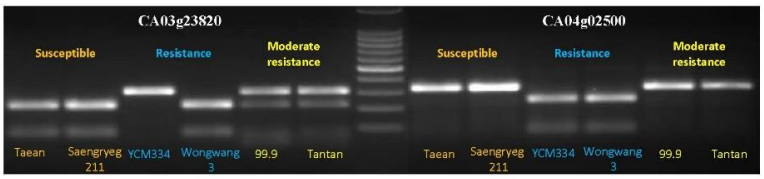

Fig. 3: Band polymorphisms among BW susceptible, resistant and moderate resistant genotypes for two CAPS markers

The CAPS marker for CA04g02500 had band polymorphisms among the RIL population significantly associated with BW resistance ($p < 0.05$) (Supplementary Fig. 1).

Discussion

The general goal of crop breeding is the accumulation of useful alleles into a single plant variety. The marker assisted selection can facilitate the breeding process, therefore it is necessary to develop genetic markers to identify donor accessions containing useful alleles for a target trait. *R. solanacearum* resulting in BW is ranked second out of top 10 pathogens because of its destructive symptoms and economic impact on crop production (Mansfield *et al*., 2012). The resistance to *R. solanacearum* has been reported in various crop species to be controlled by different genetic mechanisms depending on resistance genitor and testing conditions (Lebeau *et al*., 2011). In pepper, partial resistance has been reported to be oligogenic and the expression of resistance is quantitative (Lafortune *et al*., 2005; Lebeau *et al*., 2011).

A RIL population derived from a cross between YCM334 and Taean has been constructed, to study diverse agronomic traits in pepper, including pathogen resistance, yield, leaf length, stem diameter, etc. (Lu *et al*., 2012). Resequencing, transcriptome and microarray analysis have been performed on these two cultivars to identify candidate genes related to BW resistance (Hwang *et al*., 2011; Lu *et al*., 2011; Kang *et al*., 2016). Based on these previous studies, we selected 28 candidate genes related to BW resistance and developed SNP based genetic markers on the candidate genes which showed significantly different expression levels between YCM334 and Taean (Supplementary Table 1).

The relative expression levels of five candidate genes related to BW resistance showed statistically difference between the two cultivars. CA04g00830, CA04g02500, CA07g10490 and CA10g15480 had higher expression level in YCM334 than Taean, while CA03g23820 had higher expression level in Taean (Fig. 2). CA04g00830 is annotated as phosphatidylinositol (PI) 4-kinase which has been identified to play important roles in salicylic acid (SA)-mediated defense signaling in *Arabidopsis thaliana* (Table 1) (Antignani *et al*., 2015). SA is one of molecules plants synthesize in response to biotic stresses. PI 4-kinase is also associated with the formation of plant cytoskeleton which is involved in plant defense against pathogen attack (Schmidt and Panstruga, 2007). CA07g10490 is annotated as lipid transfer protein 1, which has been reported to contribute to plant defense against bacterial pathogens and fungi in various plant species (Jayaraj and Punja, 2007; Sarowar *et al*., 2009). CA10g15480 is annotated as a putative disease resistance protein which belongs to late blight resistance protein R1 gene family (IPR021929). Late blight is caused by *Phytophthora infestans*, which is known as the cause of the Irish potato famine of 1840s (Martin *et al*., 2013). A nucleotide binding domain and a leucine-rich repeat domain (NB-LRR, PF12061) were detected in CA10g15480 by interproscan and pfam domain search (Supplementary Fig. 2). Plant NB-LRR proteins are one of the largest and the most studied gene families, and have been reported to play a key role in plant resistance to bacterial disease (Takken and Goverse, 2012). CA04g2500 was annotated as "detected protein of unknown function" and no domains with particular functions were detected. In contrast to the expression patterns of these four genes, the expression of CA03g23820, annotated as beta-galactosidase 4, was much higher in BW susceptible Taean than BW resistant YCM334 (Fig. 2). This enzyme has been reported to degrade cell wall components during fruit ripening (Shi *et al*., 2014). High expression of beta-galactosidase may affect *R. solanacearum* to degrade the cell wall structure of the vascular tissues and to facilitate its invasion to the host plants (Hwang *et al*., 2011).

Out of the five candidate genes, CAPS markers were successfully developed on two genes, CA03g23820 and CA04g02500 (Fig. 3). The CAPS marker on CA04g02500 had band polymorphism between susceptible and resistant cultivars and was verified using the 155 RIL

population ($p < 0.05$) (Supplementary Fig. 1). The CAPS marker on CA03g23820 showed band polymorphism between susceptible and moderate resistant cultivars. Because diverse levels of resistance exist in the population, its association with phenotype was not significant among the population. Considering the complexity of genetics underlying BW resistance in pepper, different genes may be involved in BW resistance depending on genotypes (Lebeau *et al.*, 2011). Therefore, it might be difficult to develop a single marker that can determine susceptible/resistant genotypes over whole pepper cultivars.

Conclusion

Functional markers were developed on CA03g23820 and CA04g02500, that can reduce the risk of linkage drag compared to neutral markers based on linkage maps. Because the success of crop breeding depends on identifying markers closely linked to desirable phenotypes, these two CAPS markers we developed will be useful to screen large germplasms to identify breeding materials for BW resistance. Although sequence variations on two genes out of five genes had significant association with BW resistance, the validation of expression levels of five genes will help elucidate the genetic factors controlling BW resistance in pepper.

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