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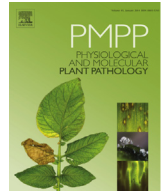

Identification of allele specific AFLP markers linked with bacterial wilt [*Ralstonia solanacearum* (Smith) Yabuuchi et al.] resistance in hot peppers (*Capsicum annuum* L.)

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ABSTRACT

Even though the bacterial wilt is identified as the most destructive disease in hot peppers world-wide, robust molecular markers that facilitate marker assisted selection are absent till date. Kerala Agricultural University (India) has released two hot peppers named Ujwala and Anugraha which show high level field resistance to this pathogen. The variety Anugraha was developed through backcross breeding between a high yielding but highly susceptible variety Pusa Jwala with the highly resistant Ujwala, using Pusa Jwala as a recurrent parent. Thus, Pusa Jwala and Anugraha are near isogenic lines (NILs) differing for the resistance to bacterial wilt only and the resistance is governed by a homozygous recessive (rr) gene action. The F_1s of Anugraha × Pusa Jwala were selfed to generate the segregating F_2 population. The F_2 population has been field screened, 10 highly susceptible and 10 most resistant plants were identified and DNA from these plants were bulked separately. Bulked segregant analysis with AFLP primer combination *Eco*ACT + *Mse*CAC was done using the DNA from donor parent Ujwala, susceptible parent Pusa Jwala, resistant parent Anugraha, bulked susceptible F_2 and bulked resistant F_2 plants. On resolution using capillary electrophoresis system in genetic analyzer, the AFLP products have yielded three polymorphic bands (103, 117, and 161 bp) which were linked with the resistant recessive allele and three polymorphic bands (183, 296, 319 bp) linked with the dominant susceptible allele of the bacterial wilt resistance gene. The results were confirmed through co-segregation analysis in most resistant and susceptible plants of F_2 segregating population.

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Introduction

Bacterial wilt (BW) incited by the soil-borne, vascular pathogen *Ralstonia solanacearum* (Smith, Yabuuchi et al., 1992) [1] is highly challenging and one of the most destructive diseases of Solanaceous crops worldwide [2]. Bacterial wilt of pepper is caused predominantly by biovars 1, 3, 3a, 3b and 4 of race 1 of this pathogen [3,4]. In all the Asian countries, the disease is widely prevalent in tomatoes, potatoes, eggplants and peppers [5]. In the management of bacterial wilt, many strategies including the grafting on a resistant root stock [6] are proposed. Even though the events on successful management of the disease by the application of fertilizers and cost-effective soil amendments such as compost, manure, urea and calcium oxide are reported [7,8], the chemical management of the disease remains almost impossible [9].

Initially, at Kerala Agricultural University, India, a hot pepper cultivar White Khandari was identified to have strong resistance to bacterial wilt. The line was used to breed the cultivar Pant C 1 that stably expressed high level resistance [10]. Using the resistance available in this source, Ujwala, another hot pepper cultivar with consistent high level resistance was developed [11]. Further, through four backcrosses, the resistance available in this variety was transferred to very high yielding and widely adapted but highly susceptible variety in India, Pusa Jwala, to develop a high yielding, widely adapted and highly resistant variety, christened Anugraha.

Abbreviations: AFLP, amplified fragment length polymorphism; BSA, bulked segregant analysis; BW, bacterial wilt; NIL, near isogenic lines; PAGE, polyacrylamide gel electrophoresis.

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Thus Anugraha and Pusa Jwala were near isogenic lines except for the bacterial wilt resistance [12].

The need for developing a reliable molecular marker for any desirable trait, that could be used in marker assisted selection for the development of improved varieties has been well recognized in all the crops. Amplified fragment length polymorphism (AFLP) [13] is a very powerful highly reproducible marker technique, which can be used in generating linkage map, mapping of plant genome, diversity analysis, variety identification and pedigree analysis [14–16]. AFLP can simultaneously screen many different DNA regions distributed randomly throughout the genome and generates many genome wide polymorphic markers with no prior sequence information. Previously, through cDNA-AFLP, we had shown the differential expression of the gene contributing to the bacterial wilt resistance in tomato [17]. The AFLP markers related to the bacterial wilt resistance are already reported in tomato [18–20], potato [21,22], eggplant [23] and tobacco [24]. Reports on the markers for bacterial wilt resistance in peppers are rare and further the highly contradictory reports on the gene action for resistance, ranging from recessive [25–27], polygenic [28] to incomplete dominance [19,29,30], points that the markers developed for the resistance with one gene action are unlikely to be successful in another line showing a different gene action [31]. Since the marker already reported in pepper is on lines showing polygenic resistance [28], the development of workable markers for the lines showing resistance at homozygous recessive allelic status, as in case of the resistant accessions under study [32], is highly relevant.

The initial use of microsatellite markers was based on agarose gel assays for construction of linkage maps and diversity analysis in plants. However, forensic genetic analysis requires higher resolution fragment separation and greater accuracy. Thus, agarose gel electrophoresis was soon replaced by sophisticated and semi-automated PAGE platforms and later, major upward shift in the accuracy of marker based analyses took place with the introduction of capillary electrophoresis technology [33,34]. Capillary electrophoresis contributes to consistent allele sizing with minimal manual intervention, thereby reducing the sizing error and is most recommended for high-density genetic mapping projects [35]. This study reports the development of allele specific AFLP markers for resistance to bacterial wilt in hot peppers, through Bulked Segregant Analysis (BSA) [36], using AFLP followed by capillary electrophoresis on the segregating F_2 population derived by crossing the NILs differing only for the bacterial wilt resistance.

Materials and methods

Plant materials

The breeder seeds of two pepper NILs differing in their resistance to BW, Anugraha (highly resistant) and Pusa Jwala (highly susceptible) were procured from their breeders at the Department of Olericulture, College of Horticulture, Kerala Agricultural University and Department of Vegetable Crops, Indian Agricultural Research Institute, New Delhi, respectively. The seeds of the resistance donor Ujwala were also collected from the Department of Olericulture, College of Horticulture, Kerala Agricultural University.

Development of mapping population for BSA

The segregating F_2 generation for BSA was developed by crossing the resistant Anugraha and susceptible Pusa Jwala cultivars. While selecting the parent plants for the initial crossing, extreme care was taken. In the usual breeding programmes, a population with less than 10% disease incidence is generally referred as resistant population [37,38], and hence if we pick the plants from this population without thorough field screening, there may be up to 10% chance to get a susceptible plant. So, all the plants in an Anugraha population may not be resistant and to avoid any chance of wrong selection of a susceptible plant from the resistant population in the crossing programme, even if the chance is less than 10%, a screening of the population of the resistant cultivar was done. Similarly, to avoid any chance of selection of a resistant plant from the susceptible population, screening of the susceptible population was also necessary. Since the severity of bacterial wilt will be more in summer [10], during 2012 summer (January–April), two hundred plants each of Anugraha and Pusa Jwala were initially field screened through artificial inoculation (Fig. 1). The screening was carried out in acidic soils with pH 5.8–6.0, at an average day temperature of 32–36 °C and 80–90% humidity. Under the acidic conditions, coupled with high humidity and temperature, the soils will be generally bacterial sick however, to avoid any further problems related to the artificial inoculation of pathogen, the field was selected in an uncropped area, away from the cropping plots of the campus. The crops of resistant and susceptible cultivars were raised under two random blocks with 3 replications for each. The freshly collected ooze (10^6 spores/mL) of bacteria *R. solanacearum* biovar 3 of race 1 [10,32] from the field wilted hot peppers was used in the artificial inoculation by two methods; leaf cutting with inoculum dipped scissors followed by pricking with inoculum dipped pins and soil drenching followed by stem wounding on five to six-leaved seedlings. Plants were monitored on daily basis and efficacy of inoculation methods and disease incidence in both the cultivars were recorded. Truly resistant Anugraha and highly susceptible Pusa Jwala plants were identified and one plant each from both the populations was used for the crossing.

Following the standard procedures of emasculation and bagging, the resistant plant of Anugraha was crossed with the pollen from the wilting plant of Pusa Jwala. The hybrid seeds were harvested and during October–January, F_1 crop consisting of 100 plants was raised in pots containing sterile potting mixture (1 plant/pot). Since the reported gene action for resistance in the lines under study is homozygous recessive [10,32], all F_1s are supposed to be susceptible. This was the basis for selection of pots rather than the open field, for raising the F_1 crop. The plants were selfed to produce the seeds for the F_2 generation and the F_2 generation with 200 plants was raised during 2013 summer (January–April). The screening of this generation for bacterial wilt resistance has been done as in case of parental generation, except that two rounds of artificial inoculation, the second at 21 days after the first inoculation was done. The second inoculation, though not necessary, was done to ensure the highest degree of resistance in the surviving plants.

In the F_2 generation, 10 plants which have wilted first, with the typing ooze symptoms, have been considered as the most susceptible and tender leaf samples from those plants were collected for the isolation of DNA through CTAB method [39] and subsequent bulking by the mixing of equal quantities of DNA, to generate the bulked susceptible sample for BSA. Similarly, 10 plants which have not shown any morphological symptoms or the oozing, even after the double inoculation, have been considered as the most resistant plants and were used in the preparation of bulked resistant sample. Genomic DNA of resistant and susceptible parent plants used in the crossing, bulked DNA of susceptible and resistant F_2 plants and resistance donor Ujwala were finally employed in BSA using AFLP.

BSA–AFLP

BSA using AFLP assay was used to identify the polymorphic markers linked to the alleles of the gene conferring resistance to BW. AFLP minikit (Chromous Biotech, Bengaluru, India) was used

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