

The Novel *ps* and *ps-2* Specific Markers for Selection of Functional Male Sterile Tomato Lines in Breeding Programs and Hybrids Seed Production

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Abstract

Functional male sterility in tomato (*Solanum lycopersicum* L.), controlled by the *ps-2* and *ps* genes can be utilized in the production of F₁ hybrid tomato seed. Two novel cleaved amplified polymorphic sequence (CAPS) markers linked to the *ps-2* and *ps* genes were found in tomato. The C4-30 and C2-21 markers were developed based on the conserved ortholog set II (COSII) sequences C2_At3g20020 and C2_At1g65900 located on tomato chromosomes 4 and 2, respectively. The *Hinf*I-derived PCR restriction product C4-30₈₀ was applicable in the detection of functional male sterile plants. In case of the C2-21 marker, a polymorphism was revealed after digestion of the amplicon with restriction enzyme *Mbo*I. Specificity of the DNA markers identified was verified by scoring the tomato parental lines, F₂ progeny and F₁ hybrids, in which maternal lines possessed the *ps* or *ps-2* gene. C4-30 and C2-21 can be used as the diagnostic tools in tomato breeding programs and in F₁ hybrid seed production.

Keywords: *Solanum lycopersicum* L., CAPS markers, functional male sterility, marker assisted selection (MAS)

1. Introduction

Traditional methods of F₁ hybrid tomato (*Solanum lycopersicum* L.) seed production require manual emasculating of the flowers in the early bud stage. Using female lines possessing the trait of functional male sterility controlled by recessive *ps* and *ps-2* genes can reduce the time and cost of such work (Potaczek & Kubicki, 1986; Atanassova, 1999 & 2000; Staniaszek et al., 2000). Several tomato F₁ hybrids exhibiting functional sterility controlled by the *ps* gene, have been developed at the Research Institute of Horticulture in Skierniewice, Poland. They have been introduced into practical applications and commercial production (Staniaszek et al., 2000 & 2002; Kozik & Nowakowska, 2007). In Bulgaria, thirteen F₁ hybrids were developed based on functional sterility controlled by the *ps-2* gene (Atanassova, 1999). Significant progress in breeding new tomato F₁ hybrids has been made in the Czech Republic and Moldova (Atanassova, 1999). Unfortunately, the occurrence of selfings can in practice limit the application *ps* and *ps-2* sterility. The percentage of selfings, which depends on the temperature and humidity, is highest under high temperature conditions (Simonov, 1967; Atanassova, 2000). The recessive gene *ps-2* (*positional sterility-2*), described in Czech cultivar Vrbicanske nizke, is responsible for functional male sterility in tomato (Tronickova, 1962; Atanassova 1991 & 1999 & 2000). Plants homozygous for the *ps-2* allele exhibit pollen maturation but are sterile for mechanical reasons. The recessive gene *ps* (*positional sterility*) confers another type of functional male sterility in tomato. Positional sterility – *ps* is manifested by flowers with non-splitting anthers, corolla petals accreting to androecium at 2/3 of their length (Potaczek & Kubicki, 1986). This form of sterility has been identified by Larson and Paur in 1940 in the tomato cultivar John Baer (Larson & Paur, 1948; Potaczek, 1984; Atanassova, 2000). Expression of these two forms of sterility strongly depends on environmental conditions (reviewed in Atanassova, 1999).

The *ps-2* gene was localized in map segment T0958 -T0635 on tomato chromosome 4 (Gorguet et al., 2006), whereas the *ps* gene is closely linked to *aw* (*anthocyanin without*) gene on chromosome 2 (Dorossiev, 1976; Tanksley et al., 1992; Atanassova, 2000). Based on single nucleotide polymorphism (SNP), Gorguet and co-workers (2009) developed the *ps-2* marker, which was applied towards in the screening of 176 tomato breeding lines, 8 of them were *ps-2/ps-2*. Using the AFLP method and F₂ segregated population derived from the Bulgarian lines CMC1ps2, Li et al. (2006) indicated three markers E37/M47, E38/M48 and E33/M50 - useful in the identification of fertile lines. Recently, two co-dominant markers: microsatellite SSR450 and CAPS marker developed from the sequence RFLP TG123, were found to be linked with the *ps-2* gene (Sha et al., 2010). These markers were successfully tested in 123 F₂ plants (Sha et al., 2010). Staniaszek et al. (2000) described three RAPD markers, OPH04-670, OPAX10-780 and OPW13-1230, linked to the *ps* locus. OPAX10-780 and OPW13-1230 were specific to the fertile male parent and can be used for distinguishing true hybrids from self-pollination of the female parents. OPH04-670 was of use in the identification of functionally male sterile lines possessing the *ps* gene. However, co-dominant molecular markers for the *ps* gene need to be identified.

Here, we report two novel co-dominant diagnostic PCR markers, C4-30 and C2-21, for selection of *ps-2* and *ps* functional male sterile tomato lines, respectively.

2. Materials and Methods

2.1 Plant Material

The *ps-2* male sterile line M 3089 obtained after inbreeding and selection of line Start 24, was kindly provided by B. Atanassova, Institute of Genetics "Acad. Doncho Kostoff", Bulgarian Academy of Science, Sofia, Bulgaria (Kozik & Nowakowska, 2007). M 3089 was crossed with a male fertile line M 3372, which was selected by eight generations of inbreeding of the F₁ hybrid 7-2-92 (Rijk Zwaan B.V., The Netherlands). The single F₁ plant mating was self-pollinated to produce the 131 F₂ progeny.

The second F₂ population of 119 plants was generated from F₁ derived from the highly inbred tomato *ps* line W 1.8a (*ps* line) and the fertile line M 4191 (selected from Pearly F₁ hybrid, DAPco B.V., The Netherlands). The *ps* sterile line W 1.8a was generated from a cross between line PH 1106 (*ps*) and *S. chilense* LA 1969 (Potaczek, 1999; Kozik & Nowakowska, 2007).

Two *ps-2* male sterile lines M 3090 and M 3091 (selected from line Start 24), 13 F₁ hybrids: E1231, E1233, E1235, E1236, E1237, E1240, E1241, E1242, E1243, E1244 (derived from Department of Genetics, Breeding and Biotechnology, Research Institute of Horticulture, Skierniewice, Poland), Elina, Odysseus, Prekoz (Institute of Genetics "Acad. Doncho Kostoff", Bulgarian Academy of Science, Sofia, Bulgaria) and two male fertile lines M 3586, M 3597 (selected by eight generations of inbreeding from Daniela F₁, Hazera Genetics, Israel and Monika F₁, Syngenta Seeds B.V., The Netherlands, respectively) were used for the presence of the *ps-2* linked marker C4-30 (Table 1).

Table 1. Distribution of the restriction fragments of the marker C4-30 in tomato breeding lines and F₁ hybrids

F ₁ hybrids/breeding lines	Origin	C4-30 restriction fragments	
		80 bp	190 bp
E1231 F ₁ , E1233 F ₁ E1235 F ₁ , E1236 F ₁ E1237 F ₁ , E1240 F ₁ , E1241 F ₁ , E1242 F ₁ , E1243 F ₁ , E1244 F ₁ ,	1	+	+
Odysseus F ₁ , Elina F ₁ , Prekoz F ₁	2	+	+
M 3090, M 3091	1	+	-
M 3586, M 3597	1	-	+

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+ presence of marker

- absence of marker

The *ps*-specific marker C2-21 was tested in six male sterile lines: W 1.5, W 1.11a (made from a cross between the line PH1106 and *S. chilense*, Department of Genetics, Breeding and Biotechnology, Research Institute of Horticulture Skierniewice, Poland), 16, 17, 18, 19 (Seeds & Breeding Company PNOS, Ożarów Maz, Reguły, Poland), 12 F₁ hybrids: E11.93, E12.09, E936, Remiz F₁, Perkoz F₁, Bekas F₁, Słonka F₁ (Department of Genetics, Breeding and Biotechnology, Research Institute of Horticulture, Skierniewice, Poland), 13/06, 17/06, 22/06,

31/06, 101/06 (Seeds & Breeding Company PlantiCo, Zielonki, Gołębiew, Poland) and two fertile lines M 4156 (selected from Colibri F₁, Clause Vegetable Seeds, France), M 4157 (selected from Fuensanta F₁, Seminis Vegetable Seeds, Inc., USA), (Table 2).

Table 2. Distribution of the restriction fragments of the marker C2-21 in tomato breeding lines and F₁ hybrids

F ₁ hybrids/ breeding lines	Origin	C2-21 restriction fragments	
		380 bp	420 bp
E11.93 F ₁ , E12.09F ₁ , E936 F ₁ , Remiz F ₁ , Perkoz F ₁ , Bekas F ₁ , Słonka F ₁	1	+	+
13/06 F ₁ , 17/06 F ₁ , 22/06 F ₁ , 31/06 F ₁ , 101/06 F ₁	2	+	+
16, 17, 18, 19	3	+	-
W 1.5, W 1.11a	1	+	-
M 4156, M 4157	1	-	+

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2 Seeds & Breeding Company PlantiCo, Zielonki, Gołębiew, Poland;

3 Seeds & Breeding Company PNOS, Ożarów Maz, Reguły, Poland.

+ presence of marker

- absence of marker

Plants were grown in a greenhouse. Young, fresh leaves from parental lines, breeding lines, F₁ hybrids (10 plants per each genotype) and two F₂ populations segregating for the *ps-2* and *ps* were harvested and stored at -70°C until DNA extraction.

2.2 Expression of *ps-2* and *ps*-Deriving Sterility

Expression of *ps-2* and *ps*-deriving sterility was observed on each F₂ plant. Plants were classified functionally male sterile when no selfed seeds were found in the fruits. Segregation data were analyzed by the Chi-square (χ^2) test.

2.3 DNA Extraction

Total DNA was isolated from frozen young tomato leaves of each plant using DNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany). DNA concentration and purity was determined spectrophotometrically and by visualization on a 0.8% agarose gel.

2.4 PCR Primers Design, PCR Amplification, Restriction Protocol and Electrophoresis

The CAPS marker C4-30 was developed based on a conserved ortholog set II (COSII) sequence C2_At3g20020 (Solanaceae Genome Network, SGN; www.solgenomics.net), which was positioned at 19.8cM on tomato chromosome 4 (Tomato-EXPEN 2000, SGN), (Wu et al., 2006). The CAPS marker C2-21 was obtained based on sequences C2_At1g65900 specific for tomato chromosome 2. Forward and reverse primers sequences were: f: 5'-ATGTTACAACCAACAGACGGCGG-3'; r: 5'-TGAAGTTTTGATGCTGAAAAATTGC-3' for C4-30 and f: 5' TGTGGTGCATTCAGAGTTTAGAC 3'; r: 5' GAGGCCACGTATGTTGATGT 3' for C2-21.

PCR was performed in 20 µl of 20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM MgCl₂, 0.1 mM of each deoxynucleotide, 0.2 µM of each primer, containing 1U *Taq* DNA Polymerase (Invitrogen, Carlsbad, CA, USA) and 30 ng genomic DNA as template. The PCR parameters were: initial denaturation at 94°C for 60 s followed by 40 cycles of denaturation at 93°C for 15 s, annealing at 55°C for 20 s and extension at 72°C for 60 s. A final extension was at 72°C for 5 min. The GeneAmp 9700 thermal cycler was used for DNA amplification.

Amplicon C4-30 was digested with restriction enzyme *Hinf*I (MBT Fermentas, Lithuania), whereas C2-21 product was treated with *Mbo*I (MBT Fermentas, Lithuania). Digestion of PCR products were carried out at 37°C for 3 h in a 20 µl mixture containing 5U of restriction enzyme, 18µl PCR product and 10x concentrated restriction enzyme buffer.

Restriction products were visualized by electrophoresis in 1.4% agarose (Sigma-Aldrich, USA) gel and 3.5% MetaPhor agarose (Lonza, Rockland, ME, USA) in 1xTBE (tris-borate-EDTA) buffer after staining with ethidium bromide.

3. Results and Discussion

3.1 Identification of the CAPS Marker C4-30 Linked to Locus *ps-2*

PCR product C4-30 of approximately 1000 bp was amplified in male sterile and fertile plants (not shown). The CAPS fragment of about 80 bp was found in the homozygous male sterile line M 3089 (Figure 1, lane 1), whereas the restriction product of about 190 bp was identified in the homozygous male fertile line M 3372 (Figure 1, lane 2) after *HinfI* digestion and electrophoresis in 3.5% MetaPhor agarose gel.

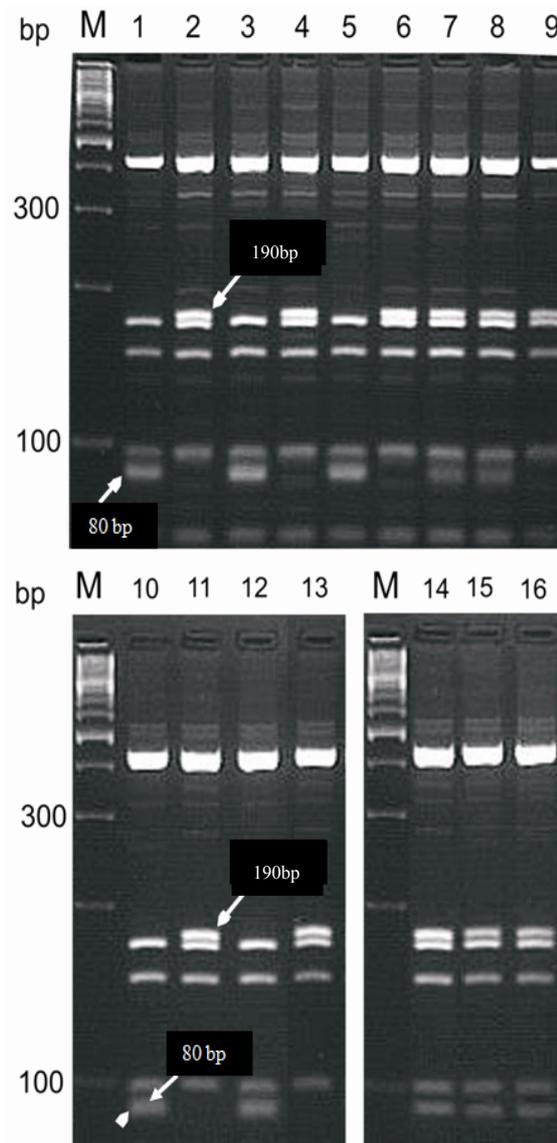


Figure 1. Electrophoretic patterns of the CAPS marker C4-30 linked to the tomato gene *ps-2* after digestion with *HinfI*. Lanes 1, 10 and 12 show *ps-2*-homozygous sterile lines M 3089, M 3090 and M 3091, respectively. Lanes 2, 11 and 13 contain *ps-2*-homozygous fertile lines M 3372, M 3586 and M 3597, respectively. Lanes 3 – 9 show restriction patterns for F₂ progeny. Lanes 14 – 16 are for F₁ hybrids Prekoz, Odysseus and Elina, respectively. M – 100 bp DNA ladder (MBT Fermentas, Lithuania).

The segregation of sterile and fertile plants was 35 : 96 in the F₂ progeny, which is in agreement with a ratio of 1 : 3 ($\chi^2 = 0.16$, $P = 0.69$). The marker C4-30₈₀ was detected in all 35 F₂ sterile plants. In the group of 96 F₂ fertile plants 35 were homozygous for C4-30₁₉₀, whereas both restriction products were observed in 61 plants. The restriction patterns of amplicon C4-30 for 7 F₂ progeny are shown in Figure 1, where lanes 3 and 5 are for homozygous sterile plants, lanes 4, 6 and 9 show homozygous fertile plants and lanes 7 and 8 are for *ps-2* heterozygous plants. The marker C4-30 was also of relevance for the detection of *ps-2* homozygosity in the sterile (M 3090 and M 3091) and fertile (M 3586 and M 3597) tomato lines (Figure 1, lanes 10, 12, 11 and 13, respectively), as well as for 3 F₁ hybrids: Prekoz, Odysseus and Elina provided by B. Atanassova (Figure 1, lanes 14, 15 and 16, respectively). C4-30₈₀ and C4-30₁₉₀ were present in 10 F₁ hybrids generated in a tomato breeding program at the Research Institute of Horticulture, Skierniewice, Poland. These results were summarized in Table 1.

The presented study indicates that the marker C4-30 shows a high *ps-2* - selection specificity. These results show that the marker C4-30 can simplify the screening of functional male sterile lines in tomato breeding programs and hybrid seed production.

3.2 Identification CAPS Marker Linked to Locus *ps*

A 1800 bp PCR marker C2-21, was amplified in all parental lines and F₁ plants (not shown). Polymorphism of this amplicon was revealed after digestion with restriction enzymes *Mbo*I and electrophoresis in 1.4% agarose gel. The restriction fragment of 380 bp was found in sterile lines with gene *ps* (Figure 2, lanes 1, 3, 5), whereas 420 bp long fragment was observed in fertile lines (Figure 2, lanes 2, 4, 6). The heterozygous genotypes F₁ plants were given three fragments, 380, 420 and 580 bp. The third fragment of 580 bp from the heterozygous plants was shown to be a heteroduplex between the two fragments 380 and 420 bp (Figure 2, lanes 7-9).

Restriction products were analyzed in 119 plants of the segregating F₂ population. In the F₂ population, segregation of sterile and fertile plants was 24:95. A chi square (χ^2) test confirmed a 1:3 segregation of sterile to fertile individuals ($\chi^2 = 1.48$, $P = 0.22$). The *ps* specific restriction fragment of 380 bp was detected in 24 functionally male sterile F₂ plants. Among the 95 F₂ fertile plants 37 were homozygous for C2-21₄₂₀, whereas three restriction products were observed in 58 plants (Figure 2, lines 10-23).

To confirm this marker as a good diagnostic tool for marker-assisted selection, 20 genetically diverse tomato lines and F₁ hybrids were tested (Table 2). C2-21 was applicable for the detection of *ps* homozygosity in the sterile tomato lines: W 1.5, W 1. 11a (Figure 2, lanes 3, 5), 16, 17, 18 and 19 (Table 2). C2-21₃₈₀, C2-21₄₂₀ and C2-21₅₈₀ were revealed in 12 F₁ hybrids generated in the tomato breeding programs, (Table 2, Figure 2, lanes 7-9), whereas the marker C2-21₃₈₀ was not observed in fertile lines M 4156 and M 4157 (Table 2, Figure 2, lanes 4, 6).

The functional male sterility in tomato controlled by recessive gene *ps* and *ps-2* is the important trait in F₁ hybrids seed production. Identification of functionally male sterile plants is labour – consuming, it is possible only during of flowering time or on based the number of seeds per fruit (Potaczek & Kubicki, 1986; Staniaszek et al., 2000, 2002). Molecular markers associated with genes *ps* and *ps-2* may be helpful for the identification functionally male sterility. The RAPD AFLP and CAPS markers were used to identify locus *ps* and *ps-2* (Staniaszek et al., 2000; Li et al., 2006; Gorguet et al., 2009). RAPD is relatively simple and easy molecular technique, highly discriminate for genetic polymorphism in tomato, but they are dominant and poor reproducibility (Jones et al., 1997). On the other hand the use of AFLP markers for marker-assisted selection (MAS) is very time consuming and expensive. Because of recombination events different molecular tools should be used for MAS in breeding practice.

The results of this study demonstrate that the new CAPS markers C4-30 and C2-21 may be helpful for the identification of the *ps-2* and *ps* genes, respectively. Both markers are co-dominant, the heterozygous genotype can be distinguished from the dominant or recessive homozygous individuals. They can be used in marker assisted selection of the functional male sterility in tomato plants.

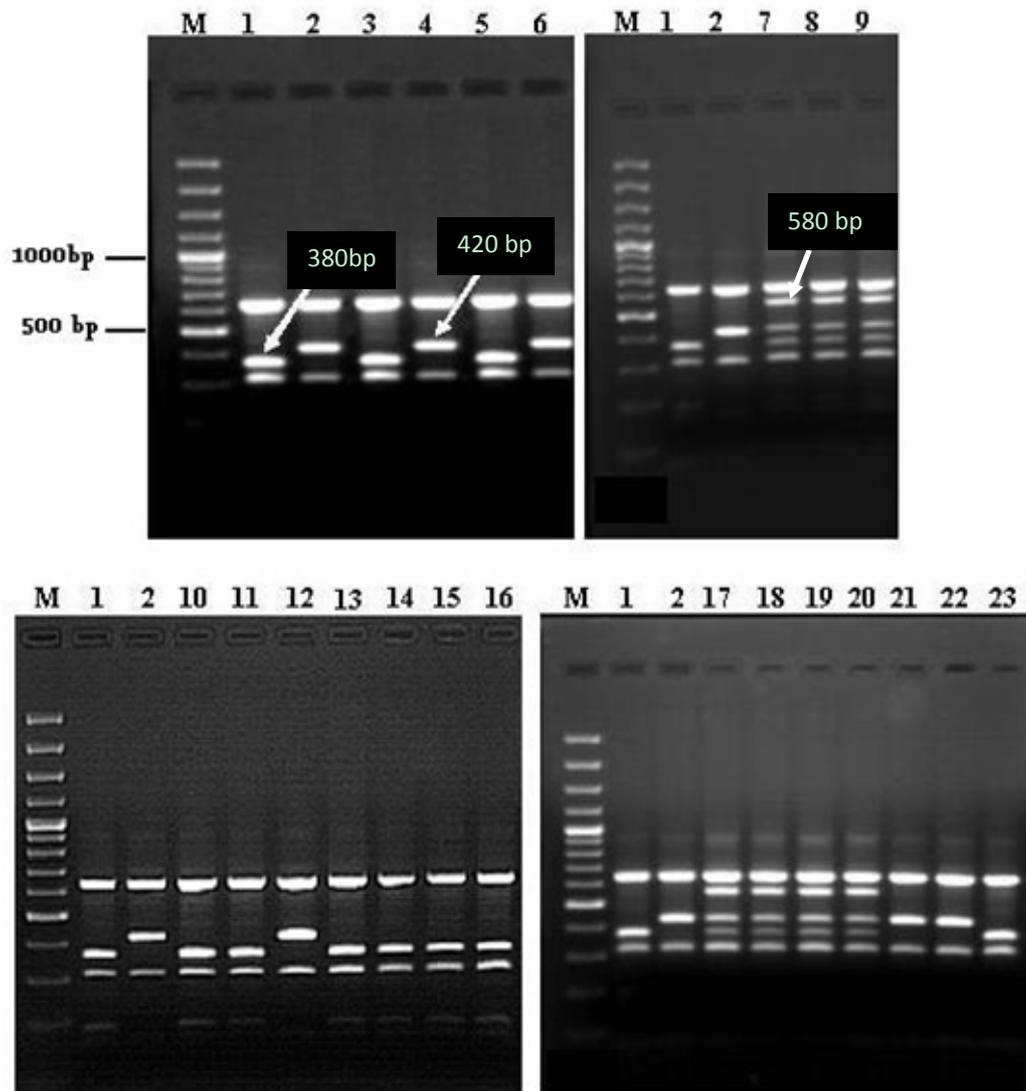


Figure 2. Electrophoretic patterns of the CAPS marker C2-21 linked to the tomato gene *ps* after digestion with *Mbo*I. Lanes 1, 3 and 5 show *ps* homozygous sterile lines W 1.8a, W 1.5 and W 1.11a, and respectively. Lanes 2, 4, and 6 contain *ps-2*-homozygous fertile lines M 4191 M 4156 and M 4157, respectively. Lanes 7, 8, 9 - are for F₁ hybrids E 11.93, E 12.09, E 936 respectively. Lines 10-23 show restriction patterns for F₂ progeny. M – 100 bp DNA ladder (MBT Fermentas, Lithuania).

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