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More than meets the eye: A multi-year expressivity analysis of tomato sterility in ps and ps-2 lines

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Abstract

Functional male sterility in tomato (*Solanum lycopersicum* L.) positional sterile *ps* and *ps-2* lines provides a good facultative sterility system for F_1 hybrid seed production. In order to elucidate the observed variability in the trait stability, expressivity was observed in a comparative multi-year study (2008 – 2012) across seven *ps* and two *ps-2* tomato lines. Tomato lines undergoing the study were cultivated from seeds obtained in the preceding year and grown in spring-summer season in greenhouse with constant temperature monitoring. Flowers (normally-shaped or *ps*-shaped), fruit number, and seed number per fruit were counted for the lowest first six branches of each plant, every year of the study. Lapses in sterility (expressed as percentage of seedless fruits or as number of seeds per seeded fruit produced) were attributed to dramatic greenhouse temperature fluctuations during the flowering period. Analyses of independent descendant F_2 hybrid populations of *ps* × fertile crosses indicated a repeated presence of hitherto unreported phenotypic classes, *ps*-flowered plants with low or normal seed counts, strongly suggesting the gene responsible for the *ps* phenotype is distinct from tomato sterility gene. Segregation analyses of all independent crosses showed that either of these traits (*ps* flower phenotype or fruit sterility) was conferred by a single recessive gene. The calculated genetic distance between these two genes was 13.4 cM, based on the calculation of occurrence of the segregants across the investigated independent F_2 populations. All F_2 segregants classes produced viable pollen, pointing that the occurrence of novel segregants classes was not due to changed pollen viability. The distinction of *ps* from tomato sterility gene was further confirmed with molecular analyses of F_2 sub-populations using a recently developed *ps*-linked DNA marker, resulting in a calculated genetic distance of 12 cM between it and the sterility gene.

Keywords: expression stability; facultative sterility; functional male sterility; high temperature; hybrid seed production; marker-assisted selection; *Solanum lycopersicum*; trait linkage.
Abbreviations: $C_{2\text{-}21}$_CAPS marker $C_{2\text{-}21}$ developed based on a conserved ortholog set II (COSII) sequence $C_{2\_At1g65900}$; cM_ centimorgan; *ex_exserted stigma*; *ms_male sterile*; *ps*(*-2*)*_positional sterile* (*-2*); *sl_stamenless*; T_{min}/T_{max}_minimal/maximal daily greenhouse temperature (recorded every minute).

Introduction

Tomato (*Solanum lycopersicum* L.) is widely considered a high value crop grown for both fresh market and processing (Nowicki et al., 2013). Since it is a self-pollinating inbred crop, tomatoes develop bisexual flowers, which require hand emasculation and pollination for producing hybrid seeds (Atanassova and Georgiev, 2007). While significant quantities of hybrid seeds are produced using a fertile seed parent, the benefit of incorporating male sterility into tomato hybrid breeding programs was recognized shortly after acknowledgement of heterosis-deriving advantages and discovery of male sterile genotypes of tomato (Rick, 1944; Cheema and Dhaliwal, 2005; Atanassova and Georgiev, 2007). Genetic male sterility occurs widely in tomato, while cytoplasmic male sterility does not occur naturally in the genus *Solanum*. Based on the classical anther development studies and the phenotype, the male sterile mutants in higher plants were classified into structural, sporogenous, and functional types (George et al.,1984; Atanassova, 2007; Gorguet, 2007). Georgiev (1991) described male sterility in tomato as auto-sterility and categorized the sterile tomatoes into *male sterile* (including the male sterile (*ms*) and *stamenless* (*sl*) series) and functional sterile (including *positional sterile* (*ps*), *positional sterile-2* (*ps-2*), and *exserted stigma* (*ex*)). Despite the majority of identified mutants belonging to the *ms* or *sl* series, functional sterility is much more frequently employed in tomato hybrid seed breeding programs, regardless of the lower frequency of such mutations (Atanassova, 2007). Indeed, the only type of male sterility applied in the production of hybrid seeds of tomato is the positional sterility. Sterility of *ps*-type is characterized by connate petals, normal viable pollen grains, but non-dehiscent anthers because of persistent stomium (Larson and Paur, 1948), and pronounced changes in cuticular waxes and, thus, increased water permeance due to suggested effective blocking of the decarboxylation pathway of wax biosynthesis in epidermal cells of tomato fruits (Leide et al., 2011). Comparatively, *ps-2* type of tomato sterility produces normal flowers with viable pollen grains but non-dehiscent anthers due to alternative splicing in a polygalacturonase gene product expressed in the zone of anther dehiscence; (Gorguet et al., 2006; Gorguet et al., 2009).

Several tomato F_1 hybrids exhibiting functional sterility described as the *ps*-type have been produced and released by the Research Institute of Horticulture (Skierniewice, Poland) and successfully released into commercial production (Kozik and Nowakowska, 2007; Staniaszek et al., 2012). The *ps-2* is commonly used in Bulgaria, Czech Republic, and Moldova owing to the high yield of hybrid seeds using this as a female parent and up to 100% *ps/ps* progeny obtained by facultative selfing despite significantly reduced seed number obtained in this manner (Kozik and Nowakowska, 2007; Roy et al., 2012; Staniaszek et al., 2012). It is a common observation that depending on extreme environmental conditions (in particular, low and high temperatures, as well as low and high relative humidity) changes in expressivity, and thus the occurrence of selfings, can limit the application of both, *ps* and *ps-2* sterility (Atanassova, 1999; Atanassova, 2000; Roy et al., 2012; Staniaszek et al., 2012). High-resolution, multi-year studies are lacking to support these assertions. Considering the importance of functional male sterility in basic and applied research, the present investigation covers five years of comparative analyses of (i) sterility expressivity in relation to *ps* and *ps-2* traits in several tomato lines, (ii) the genetic analyses of the sterility trait in the respective segregating F_2 generations of *ps* lines confirming a monogenous character of this trait, and (iii) occurrence of never before reported segregants in the descendants of *ps*: In order to broaden the genetic variability pool regarding the most important functional traits of chosen *ps* germplasms studied, crosses with fertile lines were conducted. Upon selection for *ps*/*ps* phenotypes within newly obtained F_2 populations, several groups of plants were observed. In addition to previously reported normal-flowered functional sterile plants in the F_2 descendants from crosses of *ps*-sterile × fertile lines (Dorossiev, 1976), we also obtained *ps*-flowered F_2 plants producing normal seeds at a low but significant number, and at normal level. Therefore, (iv) subsequent studies focused on clarification of new recombinants' background by means of a recently developed DNA marker linked to *ps* (Staniaszek et al., 2012). Finally, (v) morphological observations of the flowers and pollen viability analyses of the segregating populations (including the newly occurring *ps* segregants) provided mechanistic insights into the sterility mechanisms.

Results

Expressivity of ps *and* ps-2 *sterility*

In accordance with the suggested multi-year observations towards more reliable than hitherto scoring of *ps* and *ps-2* expressivity (Atanassova, 1999; Atanassova, 2000; Atanassova and Georgiev, 2007), trends for sterility were observed in a multi-year (2008 – 2012) experiment during spring-summer growth. Seven greenhouse-grown *ps* tomato lines and two *ps-2* tomato lines were analyzed for comparative purposes. Due to the high volume of data obtained over the course of this study, sterility level was characterized quantitatively as percentage of seedless fruits produced in six first clusters on a respective plant (Fig. 1, B), and as seed number per seeded fruit produced on a given plant (Fig. 1, C), similar to reported *ps-2* studies (Gorguet, 2007; Gorguet et al., 2008). Distribution of the expressed sterility level ranged from 0 to 100% overall, while the range observed in a given year never exceeded seven classes (max.70% in sterility difference recorded for one tomato line in one year). The two-way ANOVA (Analysis of Variance) indicated highly significant differences among genotypes, from one year to another, as well as in the interaction of both factors (Table 1). Variation in fruit sterility levels were observed between progeny, regardless of the fact that descendants from a given accession were used for observations in the subsequent year. To substantiate the postulated environmental influence on expressivity of tomato sterility and parthenocarpy (Atanassova and Georgiev, 2007; Gorguet, 2007; Gorguet et al., 2008; Hazra and Dutta, 2010a), the daily minimal and maximal greenhouse temperatures (T_{min} and T_{max}, respectively) were plotted (Fig. 1, A). Based on the collected data, the T_{min} set at 15 °C was kept almost constant during the observed period (T_{min}; Fig. 1, A). Only miniscule fluctuations were observed in the months of April and May in the years 2008 - 2012, and the T_{min} never dropped below 14 °C throughout that time (Fig. 1, A). Conversely, the T_{max} set at 25 °C posed a challenge to maintain during this same period and varied greatly over the course of the study (Fig. 1, A). Changes in the sterility levels of the analyzed tomato lines (Fig. 1, B-C) could have been influenced mainly by the greenhouse T_{max}, owing to only minimal fluctuations in T_{min}. Indeed, drops in fruit sterility levels (Fig. 1, B-C) correlated with sharp changes in T_{max} over short periods, particularly in 2008, 2009, and 2011 (Fig. 1, A). Further evidence of T_{max} influencing expressivity of the tomato lines sterility was provided by seed production data, recalculated as seed number per seeded fruit produced on respective plant (Fig. 1, C), to better reflect the extent of sterility trait lapses. Recorded seed data is in line with the sterility levels, i.e., increased seed production was observed in years conducive to lower tomato sterility, such as 2008, 2009, or 2011 (Fig. 1, C). Lapses in seed production occurring in 2010 can also be attributed to highly variable greenhouse daily temperature during the tomato flowering stage that year (Fig. 1, A). It needs to be stressed, that the highest levels of sterility, regarded as either seedless fruits frequency or seed number produced per seeded fruit during the course of this study, was recorded in the *ps* line W1.20 (Fig. 1, B-C). Indeed, this line exclusively produced seedless fruits in a previous study (Kozik and Nowakowska, 2007), two years prior to this study (2006-2007; data not shown), and over the entire course of this study.

Genetic background underlying the tomato ps *trait*

In order to broaden the genetic pool variability regarding the most important functional traits of chosen *ps* germplasms undergoing this study, crosses with fertile tomato lines were procured (Table 2). Consequently, the hybrid progeny derived from several biologically diverse tomato *ps* lines containing various agro-botanical traits provided a rich opportunity to test the genetic background of the *ps* trait. Observations of F_2 populations from crosses of *ps* × fertile (*ps* lines: W1.5 and W1.8; fertile lines: M 4155, M 4156, M 4157, and M 4192) were carried out in spring or fall in a way similar to testing the expressivity of *ps* (Table 2). The seven independently developed F_2 populations undergoing this study included in total five phenotypical classes: (i) normal-flowered plants typical for fertile plants, (ii) *ps*-flowered plants bearing sterile fruits, but also (iii) plants with normal (fertile-type) flowers producing fruits with low or (iv) no seed, as previously described elsewhere (Dorossiev, 1976, Atanassova, 2000), and (v) novel *ps*-flowered plants producing seeded fruits. This finding has been confirmed across all the investigated F_2 populations , regardless of the spring-summer or summer-fall cultivation period (Table 2). These results indicate the *ps* (abnormal flower phenotype) and fruit sterility are separate traits, hence, gene

Table 1. Analysis of Variance (ANOVA) on fruit sterility levels (expressed as percentage of seedless fruits in total fruits produced over six first clusters on respective plant) for seven *ps* and two *ps-2* tomato genotypes, as observed on five consecutive years (2008-2012; 20 plants per genotype each year). Due to observed low *P*-values (below assumed α=0.05) and observed variation (*F*) greater than the critical variation (F_{crit}) for all three hypothesis, the nine tomato genotypes tested for 5 years show different behavior regarding the here analyzed sterility levels. Statistically significant variance is observed when comparing the genotypes, when comparing the years with one another, and when analyzing the interaction of both factors and marked with (*).

Analysis of Variance

Source of variance	*SS*	*df*	*MS*	*F*	*p-value*	F_{crit}
Genotype	109601.5	8	13700.19	74.88386*	2.4E-93*	1.949215
Years	258268.6	4	64567.15	352.9176*	2.6E-179*	2.382344
Interaction	147583.1	32	4611.971	25.20857*	2.6E-101*	1.457219
Within	156424.4	855	182.9525			
Total	671877.6	899				

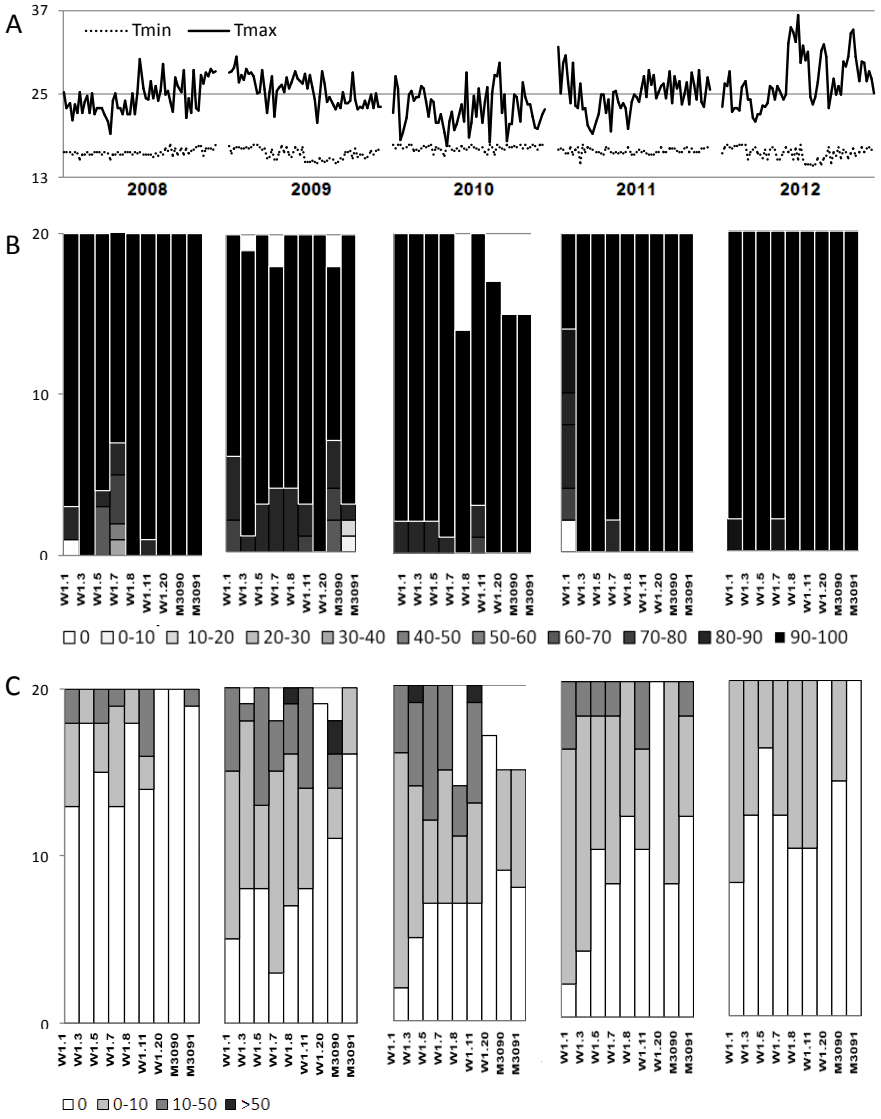

Fig 1. Sterility level of the tested tomato lines as influenced by growth conditions. (**A**) Greenhouse temperatures were recorded and daily extremes (T_{min} and T_{max}) are represented for each year (2008 – 2012). Only the months (April, May) during which flowering on all six first branches took place, are shown here for clarity of presentation. (**B**) Tomato lines sterility performance expressed as frequency distribution of the sterility levels (percentages) over the six first branches for the eight tested lines during each year. "0" refers to plants without seedless fruits; "0-10" indicates plants with at least 1 seedless fruit to less than 10% of seedless fruits produced by this plant, similar to previous representations (Gorguet, 2007; Gorguet et al., 2008). (**C**) Tomato lines sterility levels expressed as frequency distribution of seed production per seeded fruit over the six first branches for the eight tested lines during each year."0" refers to plants without seeded fruits; "0-10" indicates plants with at least 1 seed but less than 10 seeds in at least 1 seeded fruit produced by this plant.

action underlying the tomato sterility in these populations was calculated irrespective of *ps* plant phenotype. For all seven independently developed F_2 populations, a single recessive gene responsible for the sterility trait was suggested (Table 2). The calculated average genetic distance between *ps* and sterility traits based on the F_2 crosses segregation was 13.4 cM (spread: 8.0 – 18.0 cM).

Pollen viability analysis

To ensure the occurrence of new F_2 recombinant classes was not due to possible variation in pollen viability, cytological analyses of pollen germination were conducted. Despite the observed variation within and among the F_2 classes regarding the pollen germination *in vitro*, all tested plants belonging to the different phenotypic classes described above produced viable pollen (Fig. 2). Moreover, seed production in tomato fruit regardless of the segregant class coincided with the higher pollen germination rates; however, this result needs a more systematic validation. Pollen viability was further confirmed by production of seeded fruits in the tested selfed plants (above the sixth cluster; seeds produced for reproduction purposes; data not shown), irrespective of variation in the pollen viability levels within a given segregants class.

Molecular analyses of ps × fertile F_2 populations

In contrast to broad expressivity of the *ps* and *ps-2* sterility traits in the examined tomato lines (Fig. 1, Table 1), the *ps* × fertile populations displayed rather stable 1:3 (*ps* : normal-flowered) segregation ratio, regardless of the growth season tested, or the parental lines they were derived of (Tables 2-3). This prompted the molecular analyses of two of these populations (W1.8 × M 4192 grown in 2007 and W1.8 × M 4155 grown in fall of 2009) with CAPS marker $C_{2\text{-}21}$, developed based on a conserved ortholog set II (COSII) sequence $C_{2\_At1g65900}$, located on tomato chromosome 2, linked with the *ps* trait, and suggested for the breeding purposes (Staniaszek et al., 2012). Results of the molecular analyses are in line with the phenotypical observations of the F_2 hybrid populations: Despite separate analyses of both traits (sterility and *ps*) yielding a clear 1:3 segregation ratios, typical for monogenic recessive traits (Table 2), detailed molecular studies confirm the existence of novel recombinants. In both tested F_2 populations, DNA marker analyses (Table 3) show, in addition to each of the previously described plant classes (*ps*-flowered plants with seedless fruits; normal-flowered plants with seedless fruits; normal-flowered plants with high number of seeds), novel classes of recombinants: (i) *ps*-flowered plants with seedless or low-seeded fruits but genotypically fertile (heterozygous) and (ii) normally-flowered plants with high number of seeds but genotypically sterile (homozygous) (Table 4). The calculated genetic distance between the $C_{2\text{-}21}$ locus and fruit sterility locus for the analyzed F_2 sub-populations was 12.0 and 11.9 cM (W1.8 × M 4192 grown in 2007 and W1.8 × M 4155 grown in fall 2009, respectively).

Altogether, the data collected in this multi-year study indicates that high temperatures have an influence on *ps* and *ps-2* tomato sterility traits. Moreover, the analyses provide the first evidence that the floral malformation trait (*ps*) is linked but distinct from the tomato sterility gene. Finally, phenotypical and molecular observations of the segregating hybrid progeny of *ps* × fertile indicate the monogenic recessive character of both traits (*ps* and sterility), while highlighting their distinctness.

Discussion

Sterility, as a particular type of parthenocarpy (George et al., 1984; Lukyanenko, 1991; Atanassova, 2007; Atanassova and Georgiev, 2007; Gorguet, 2007), has attracted considerable attention as a desirable tool for breeding and improving tomato fruits (Lukyanenko, 1991; Cheema and Dhaliwal, 2005; Atanassova, 2007; Atanassova and Georgiev, 2007). Despite many tomato sterility systems' existence (George et al., 1984; Atanassova and Georgiev, 2007; Gorguet, 2007), facultative sterility such as *ps* and *ps-2* types is of particular interest because of easy maintenance by artificial selfing (Lukyanenko, 1991; Atanassova, 2007; Atanassova and Georgiev, 2007; Kozik and Nowakowska, 2007). These lines, however, were considered less promising for hybrid seed production, particularly owing to occasional lapses in expressivity of this trait (Atanassova and Georgiev, 2007; Kozik and Nowakowska, 2007; Roy et al., 2012). The lack of a comprehensive understanding regarding the long-term relationship between sterility levels and plant growth conditions (Atanassova, 2000; Atanassova and Georgiev, 2007; Roy et al., 2012) precipitated the current several-years study on changes in stability of the *ps* and *ps-2* traits in several greenhouse-grown tomato lines. It needs to be stressed, that due to the difficulties in maintaining a consistent T_{max} in the current set of experiments, the investigation regarding the influence of only T_{max} and not T_{min} on sterility is justified. Our studies investigated stability of and high temperature influences on the levels of sterility in tomato are in line with previous observations of this phenomenon. Higher percentage of selfings in *ps* and *ps-2* tomato lines occurred under fluctuating T_{max} over the duration of this study; this factor was previously reported as strongly influencing the trait expressivity (Simonov, 1967; Atanassova, 1999). Indeed, several short-term studies indicated that high temperatures affected fruit production and seed production in both fertile and functionally-sterile lines (Sato et al., 2000; Masuda et al., 2007; Hazra and Dutta, 2010a; Roy et al., 2012). General conclusion drawn from these studies suggests that cultivar differences, including pollen release and germination changed under heat stress, were the most important factors determining the plant's ability to set (seeded) fruit (Sato et al., 2000; Comlekcioglu and Soylu, 2010; Hazra and Dutta, 2010a; Roy et al., 2012). The novel element this study focused on, was the observation and comparison of stability of the sterility trait over several years in response to temperature. The results obtained, in terms of fruit sterility percentage and seed production (Fig. 1, B-C), constitute conclusive proof of a causative relationship between growth temperature and observed sterility lapses. The next logical step in this line of research is the identification of transcription regulatory elements responsible for the redirection to the sterility/fertility phenotype of produced fruits after high temperature stimulus is received, through the plant hormonal network (Anwar and Mehdi, 2009; Pandolfini, 2009). Genetic analyses of the F_2 populations from different *ps* × fertile crosses indicated a stable ratio of the sterility trait, as well as the *ps* trait, independent of each other. These results suggest single recessive character for either trait, irrespective of parental reproductive phenotype or the growing season (spring / fall). The tomato *ps* trait is a well-documented single recessive gene trait (Potaczek and Kubicki, 1986; Atanassova, 2000); however, occurrence of new classes of segregants in each of the F_2 populations observed here made it imperative to re-visit the *ps* trait with regards to its independence from the fruit sterility trait. Finally, to our knowledge, this is a first

Table 2. Characterization of F_2 populations from tomato crosses of *ps* × fertile. Both parental lines and respective time of testing (season, year) are listed for each independent experiment. The number of plants obtained from each segregant class derived either from *ps* flowered- or plants with typical fertile flowers given for each experiment. Statistical analyses for each experiment show statistically significant fit (marked with *) to the assumed 1:3 segregation ratios (sterile : fertile or *ps* : typical fertile, respectively; traits regarded separately) using the Fisher's test (F-test), and Chi-square (χ^2) with the corresponding *P* value (at α=0.05).

F_2 of cross (Season, Year)	*ps*		Typical flower		Sum sterility		Sum *ps*		Sterility			*ps*		
	no seeds	seeds	no seeds	low seeds	sterile	fertile	*ps*	fertile	Test F	χ2	P	Test F	χ2	P
W1.8 × M 4192 (2007)	30	14	3	1	48	176	44	180	0.92*	1.52	0.22*	0.88*	3.43	0.06*
W1.8 × M 4192 (Fall 2008)	19	11	0	1	31	106	30	107	0.94*	0.41	0.52*	0.93*	0.70	0.40*
W1.8 × M 4156 (Spring 2009)	23	4	1	10	38	82	27	93	0.81*	2.84	0.09*	0.94*	0.40	0.53*
W1.8 × M 4155 (Fall 2009)	17	4	11	4	36	93	21	108	0.92*	0.58	0.45*	0.81*	5.23	0.02
W1.8 × M 4155 (Spring 2010)	12	13	4	1	30	70	25	75	0.86*	1.33	0.25*	1.00*	0.00	1.00*
W1.5 × M 4157 (Spring 2011)	21	18	0	4	43	103	39	107	0.88*	1.54	0.21*	0.95*	0.23	0.63*
W1.8 × M 4155 (Spring 2012)	13	17	2	5	37	105	30	112	0.97*	0.08	0.78*	0.91*	1.14	0.29*

Table 3. Summary of two sub-populations (W1.8 × M 4192 grown in 2007 and W1.8 × M 4155 grown in Fall 2009) undergoing molecular analyses (DNA marker $C_{2\text{-}21}$).This analysis underlines the distinctness between the observed phenotype(sterile; fertile) and the genotypic status of *ps*-linked DNA marker (**……***ps*/*ps*, assumed sterile; **– –***Ps*/*ps* or *Ps*/*Ps*, assumed fertile; Staniaszek et al., 2012). Plants from phenotypically distinct classes (*ps*-flowered seedless or seeded fruits; normally-flowered but seedless or with low seed count or fertile) segregate with regards to both, observed *ps*/sterility phenotype and the status of the DNA marker $C_{2\text{-}21}$. Not all plants listed in Table 2 were subjected to these analyses due to unsatisfactory quality of isolated gDNA from some samples or ambiguous PCR results. Statistical analyses show a fit to the assumed 1:3 segregation ratios (*ps*/*ps* : fertile or *ps* : normal fertile, respectively; α=0.05; marked with [*]).

F_2 of cross (Season, Year)	*ps*-flowers; no seed		*ps*-flowers; low seed		normal flowers; fertile		Sum *ps* flowers	Sum normal flowers	Test F	χ^2	P	Sum *ps*/*ps*	Sum fertile	Test F	χ2	P
	ps/*ps*	fertile	*ps*/*ps*	fertile	*ps*/*ps*	fertile										
W1.8 × M 4192 (2007)	**23**	**7**	**11**	**3**	**7**	**90**	44	97	0.82*	2.90	0.09*	41	100	0.89*	1.25	0.26*
W1.8 × M 4155 (Fall 2009)	**12**	**5**	**3**	**1**	**8**	**89**	21	97	0.84*	3.27	0.07*	23	95	0.87*	1.91	0.17*

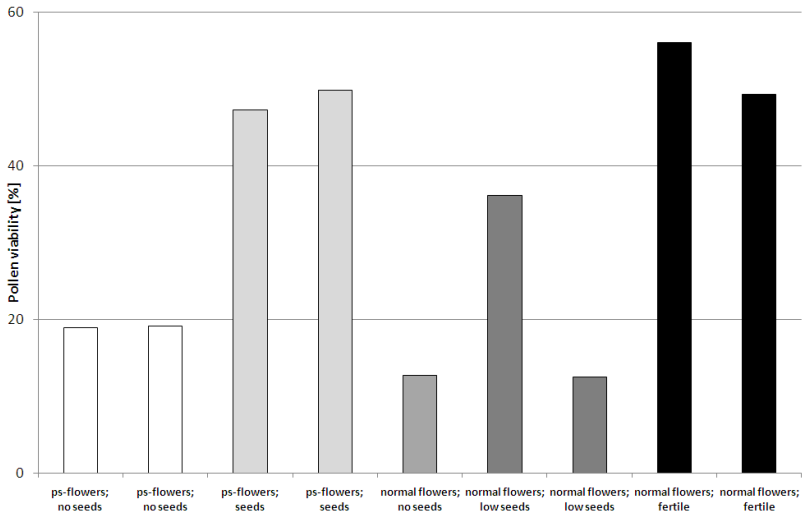

Fig 2. Pollen viability analysis as germination rate. Pollen collected from plants of the *ps* × fertile cross W1.8 × M 4155 (Fall 2009) F_2 generation was subject to standard germination analysis. Percentage of germinated pollen grains was used to indicate the pollen viability and was based on the microscopic counting.

report of novel segregants classes in such tomato crosses; the classical find of normal-flowered *ps*-functional sterile plants in the F_2 progeny of *ps* × fertile crosses (Dorossiev, 1976; Atanassova, 2000; Hazra and Dutta, 2010b) is herewith complemented by the reverse phenotype of *ps*-flowered plants producing seeded fruits. Such plants presented in all *ps* × fertile crosses (Table 2) complete the classical two-trait cross. Depending on the number of seeds produced in the fruits, they could either be regarded as sterile when containing only negligible seed number (Hazra and Dutta, 2010a, b), or true segregants, when substantial numbers of seeds are present.

The influence of pollen viability in relation to tomato fruit production is a well-documented phenomenon (Abdul-Baki and Stommel, 1995; Peet and Bartholemew, 1996). Observations of pollen viability in response to heat treatments is generally agreed on as genotype-dependent predictor of fruit set (Abdul-Baki and Stommel, 1995; Peet and Bartholemew, 1996) and is again supported by this study's findings (Fig. 2). Moreover, we observed that, despite different levels of viable pollen, the F_2 tomato lines developed (seeded) fruits with relatively stable efficiency, further validating the feasibility of *ps* and *ps-2* tomato use for hybrid production (George et al., 1984; Peet and Bartholemew, 1996; Masuda et al., 2007; Hazra and Dutta, 2010a, b).

Recent cloning of the *ps-2* gene provided a mechanistic insight into the background of this trait, indicating anther-expressed single-nucleotide mutated polygalacturonase as main reason for the sterile line's stomium remaining closed (Gorguet et al., 2006; Gorguet, 2007; Gorguet et al., 2009). mere first steps towards identification of the gene responsible for *ps* sterility culminated in mapping it to the distal arm of tomato chromosome 2 (Staniaszek et al., 2012). This is in accordance with the related phenotypic markers *anthocyaninless of Hoffman* and *ex* located on the same chromosome (Dorossiev, 1976, Atanassova et al., 1997). Despite the genetic linkage between the DNA marker $C_{2\text{-}21}$ and the sterility trait of approx. 12 cM (Table 3), this marker needs to be regarded as the first step towards identification of the gene underlying the *ps*/sterility traits in tomato. Genomic analyses confirmed the existence of a putative polygalacturonase gene within the distance of the *ps*/sterility and $C_{2\text{-}21}$ linkage (Mueller et al., 2009; Sato et al., 2012; www.solgenomics.net), but it remains to be determined, whether the sterility background in these lines is due to a mechanism similar to the one present in the *ps-2* tomato lines. Finally, comprehensive analysis is required with regard to the *ps*/sterility background, because the *ms1035* (characterized by male sterility with abnormal flower development including exertion of stigma), is also located on the tomato chromosome 2, within the calculated genetic distance from the $C_{2\text{-}21}$ locus (Jeong et al., 2009).

Materials and Methods

Plant materials

Seven *ps* male sterile lines (W1.1, W1.3, W1.5, W1.7, W1.8, W1.11, and W1.20) and two *ps-2* male sterile lines (M3090 and M3091) were used in this study. The *ps* lines were derived from a cross of PH 1106 (*ps*) and *S. chillense* LA 1969 (Potaczek, 1999). Both parents exhibit high levels of inbreeding, as well as differences in morphology, growth habit, inflorescence length, number of flowers per inflorescence, and fruit size. Four of the *ps* lines undergoing the analyses possess the *anthocyaninless of Hoffman* (Atanassova et al., 1997). Both *ps-2* lines carry *potato leaf* (*c*), show a short style, and were obtained after inbreeding and selection of line Start 24 (a gift from B. Atanassova, Institute of Genetics, Sofia, Bulgaria). This study has been carried out over a five-year period (2008-2012) as a continuation of a previously reported five-year study (Kozik and Nowakowska, 2007), performed in a glasshouse at the Research Institute of Horticulture (RIH, Skierniewice, Poland). Seeds were sown in February for the spring observations and at the end of June for the fall observations. Plants were transplanted at the first true leaf stage into 15 cm diameter plastic pots containing Kronen Mix. After four weeks, up to 20 plants per line were transplanted into 8 L plastic pots, and placed in the greenhouse with density of 2.5 plants × m^{-2} with temperature under constant recording. Hybrid F_2 crosses for genetic and molecular investigations of the *ps* trait were performed using the tomato sterile *ps* lines W1.5 and W1.8, and highly inbred fertile lines, M 4155,

M 4156, M 4157, M 4191, and M 4192 (selected from Pearly F_1 hybrid, DAPco B.V., the Netherlands).

Expressivity of* ps *and* ps-2 *sterility

Phenotypic observations of the flowers (*ps* or normal) for each plant were performed in order to investigate the uniformity of the *ps* trait expressivity within a given line. Each year, the first six branches (clusters) were isolated independently using mesh before the green buds stage and left untouched (were not vibrated) to minimize the induction of pollination. To classify the level of sterility upon fruit set, these clusters were analyzed for number and type (*ps* or normal) of flowers, fruit number (seeded and seedless), and number of seeds produced (normally seeded, low-seeded with up to 10 seeds/seeded fruit or seedless). Fruits were scored at the mature stage. The sterility level was expressed quantitatively, as frequency of seedless fruits set over the six clusters on each plant, and is reported as the number of plants per line in sterility level increments of 10%, similar to reports on *ps-2* and parthenocarpy studies (Gorguet, 2007; Gorguet et al., 2008). In addition, seed count was expressed as number of seeds per seeded fruit. Subsequently, two or three inflorescences of each plant were selfed by hand pollination to maintain the line for the next generation. Only plants exhibiting the highest level of sterility (*i.e*., lowest percentage of self-pollination over the lowest six clusters) within each line were used as single-plant selections for production of the next-generation inbreeds.

Molecular investigations

The recently reported *ps*-linked DNA marker $C_{2\text{-}21}$ (Staniaszek et al., 2012); (Fw: 5'-tgtggtgcattcagagtttagac-3' and Rv: 5'-gaggccacgtatgttgatgt-3') was employed essentially as reported therein. In short, the PCR mix of 20 µL consisted of 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM each dNTP, 0.2 µM of each primer, 1 U of REDAllegro Taq DNA Polymerase (Novazym, Poland) or 1 U Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA), and 30 ng of tomato genomic DNA as template (Plant DNA Mini Kit, Qiagen, Venlo, Netherlands). The polymerase chain reaction (PCR) thermoprofile used followed initial denaturation at 94 °C for 1 min, 40 cycles of denaturation (94 °C for 25 s), annealing (55 °C for 35 s), and extension (72 °C for 1.5 min), with a final extension step of 72 °C for 5 min. Amplified product (~1800 bp) has been digested with *Mbo*I (Blirt, Poland or MBT Fermentas, Lithuania; 2 U per reaction) in final volume of 20 µL in appropriate buffer and at 37 °C for 2 h, and the resulting products were separated and visualized in ethidium-bromide stained 1.7% agarose gel (Genoplast, Poland or Sigma-Aldrich, USA).

Morphological pollen observations

Flowers belonging to each segregation classes: (i) plants with normal fertile flowers and producing seeded, low-seeded, or seedless fruits; (ii) *ps*-flowered plants producing seedless fruits; (iii) *ps*-flowered plants with fruits of low but significant number of seeds, were collected at consecutive stages of plant development. Pollen viability was assessed by germinating the squeezed-out pollen grains in 10% sucrose (w/v, aq) for 24 h, and ascertaining the phenotype after 48 h incubation at RT (Kozik and Dyki, 2001). After completion of germination, samples were stained with 1% (w/v, aq) acetocarmine (Kozik and Dyki, 2001). Pollen viability was calculated as a mean of % from at least 10 views per plant.

Statistical analysis

All statistical analyses performed in this study (ANOVA, χ^2, Fisher test) have been carried out with MS Excel. Statistical significance has been marked.

Conclusion

As a first multi-year analysis of tomato sterility in relation to growth conditions, data of this study link the high temperature fluctuations with sterility lapses in several *ps* and *ps-2* tomato lines. One line (W1.20) seems of particular interest for possible future commercial F_1 hybrid production due to high and stable sterility level. This is also a first comprehensive study reporting the distinctness of tomato sterility trait from the *ps* phenotype. Uniqueness of these two traits is concluded from the detailed analyses of independent F_2 populations descendant from *ps* × fertile crosses with first observations of new segregants (*ps*-flowered plants developing seeded fruits), and further confirmed by means of molecular analyses with a DNA marker.

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