agronomy

MDPI

Article

Multi-Parental Advances Generation Inter-Cross Population, to Develop Organic Tomato Genotypes by Participatory Plant Breeding

Gabriele Campanelli [1,*], Sara Sestili [1], Nazzareno Acciarri [1], Francesco Montemurro [1], Daniela Palma [1], Fabrizio Leteo [1] and Massimiliano Beretta [2]

[1] Research Centre for Vegetable and Ornamental Crops, Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria (CREA), Via Salaria 1, 63077 Monsampolo del Tronto (AP), Italy; sara.sestili@crea.gov.it (S.S.); nazzareno.acciarri@crea.gov.it (N.A.); francesco.montemurro@crea.gov.it (F.M.); danielapalma82@gmail.com (D.P.); fabrizio.leteo@crea.gov.it (F.L.)

[2] ISI Sementi SpA, 43036 Fidenza (PR), Italy; m.beretta@isisementi.com

* Correspondence: gabriele.campanelli@crea.gov.it; Tel.: +39-0735-701706

Received: 6 February 2019; Accepted: 26 February 2019; Published: 2 March 2019

Abstract: A Multi-parent Advanced Generation Intercross (MAGIC) tomato population was developed by crossing eight founder lines chosen to include a wide range of variability. The lines were previously genotyped by a genotyping by sequencing approach. The MAGIC population was used to develop genotypes with important agronomic traits and to perform the Participatory Plant Breeding (PPB). Among the 400 plants of generation 4 (G4) of the MAGIC population cultivated in an organic field experiment, 22 individuals were phenotypically selected and a molecular analysis was done for both presence of resistance genes and fruit shape (marker assisted selection) on G5 seedlings. Three selected plants showed both the pyramiding gene of resistance to the main diseases and the *ovate* gene for pear shape typology. The 400 G10 stable lines that obtained from single seed descent will represent an important genetic resource for the tomato scientific community. The MAGIC population G4 was also cultivated in three organic farms located in North, Central and South Italy to carry out the PPB. The plants showed significant phenotypic differences in development, productivity and fruit color. This variability was used to select families of tomato adapted to low input crop management, different environments, agricultural practices and market conditions.

Keywords: MAGIC development; organic agriculture; participatory plant breeding; tomato selection and seed production; biotic stress tolerance; marker assisted selection

1. Introduction

In the last years, the application both of intensive agronomical practices and cropping systems contributed to the progressive worsening of environmental conditions, induced by large application of deep soil tillage, inappropriate utilization of inorganic fertilizers and single-crop farming, especially of horticultural plants. Consequently, phenomena of soil fertility deterioration, nitrate losses, water pollution and soil compaction have often been observed. On this matter, there are many studies assessing the environmental sustainability of different cropping systems [1–3]. To overcome these problems a combination of good agricultural practices (GAP) and innovative plant breeding is needed. Particularly, the organic agriculture production, which is increasing both in term of cultivation area and economic importance, is an example of GAP. It is based on conservative techniques that increase the soil organic matter and environmental sustainability and reduces the ecological risks, due to a lower use of synthetic products [2]. On the other hand, the plant breeding, that essentially relies on the utilization of genetic variation within the breeding material, is also a crucial tool to manage environmental

impacts on cultivation systems by providing improved varieties. Different Authors [4,5] highlighted that organic production, especially in horticultural agriculture, reached less yield compared to the conventional one, even if this statement should be not generalized. This outcome could be mainly due to the genotypes cultivated in organic farming. There is a lack of varieties specifically adapted to organic agriculture since most of the available varieties have been obtained under high input conditions. As a consequence, there is an increasing need to improve performances and competitiveness of organic seeds and breeding program useful to develop varieties adapted to organic systems, that is a key point for realizing the full potential of organic agriculture [5]. This issue is important for all vegetable crops and particularly for species, like the tomato (*Solanum lycopersicum* L.) one, that in the past was subjected to high genetic improvement for conventional farming. Tomato crop, both for processing and fresh market, is one of the most important vegetables in Italy and worldwide and, therefore, it is cultivated in different environmental conditions as well as both in open field and greenhouses. As a consequence, in the last decades, a large number of hybrids were developed by seed companies. This selection process reduces the number of traditional local varieties, which are appreciated by consumers, and usually adapted to low agronomical inputs.

The tomato breeding programs developed within organic agronomic systems in combination with wild germplasms and the advanced breeding populations can represent an important resource to map agronomical high-value quantitative trait loci (QTLs) and to produce suitable germoplasm for sustainable tomato cultivation. In this framework, new crossing schemes have been suggested to create powerful breeding materials as the multi-parent advanced generation intercross (MAGIC) populations. They are developed by intercrossing multiple parental lines and self-crossing the progeny, for several generations, to generate recombinant inbreed lines (RILs) [6]. The combination of different genomes from more founders increases the allelic diversity (including rare alleles) allowing to overcome the main constraints of classical bi-parental crosses, where only two alleles can be investigated. In this way, it is possible to develop new phenotypes that represent highly valuable pre-breeding resource [7]. The MAGIC populations have been developed in tomato [8], cowpea [9], chickpea [10], rice [11], barley [12], and wheat [13]. These highly variable populations could represent an important genetic material for the participatory plant breeding (PPB) approach, which is focused to develop new cultivars adapted to local and organic production systems. The PPB originated in developing countries for marginal lands, has been proposed as a breeding methodology to increase and optimize the development and the adoption of several new varieties adapted to specific microclimates [5]. Even if tomato PPB has been carried out in different countries and environment [14], there is a lack of knowledge about how to joint PPB and innovation in plant breeding (MAGIC) in order to develop new cultivars adapted to organic cropping systems.

In light of these considerations, the objectives of this research were: (i) to develop a tomato MAGIC population; (ii) to perform an organic PPB program, in order to obtain new varieties adapted to low agronomical inputs, appreciated by farmers and consumers and resilient at both environmental conditions and organic agronomical procedures; (iii) to evaluate the selected seedling by the Marker Assisted Selection (MAS), for the pyramiding of interesting genes that are present in the eight founders; (iv) to obtain the RILs through SSD that will be genotyped to find QTLs and develop new molecular markers associated with important agronomic traits.

2. Materials and Methods

2.1. The MAGIC Population Construction

Eight tomato lines were selected on the basis on their breeding relevance to develop the MAGIC population: seven *Solanum lycopersicum* lines obtained by the breeding program of ISI Sementi SpA and one wild accession of *Solanum cheesmaniae* that belongs to the TGRC (http://tgrc.ucdavis.edu) coded as LA1407 (Table 1). The *Solanum lycopersicum* lines were selected for the resistance to fungi, bacteria and viruses. Other important phenotypic traits related to plant resilience and fruits segregated

in the collection. The *Solanum cheesmaniae* is a wild relative accession and it was selected for a very large dataset of traits as biotic and abiotic stress tolerance, yield and resiliency [15].

Table 1. Summary of the main plant and fruit phenotypic traits present in the eight founders.

Founders	Shape	Weight	DTF [1]	Sp [2]	H3 [3]	Fruit Color	Peduncle	Green Shoulder	Hypocotil	OBV [4]
ISI 1	Blocky	70	50	sp/sp	Present	Red	Jointless	Absent	Light green	Not transparent
ISI 2	Round	160	50	sp/sp	Absent	Red	Jointed	Absent	Violet	Not transparent
ISI 3	Oval	90	47	sp/sp	Absent	Red	Jointed	Absent	Violet	Not transparent
ISI 4	Blocky	80	43	sp/sp	Absent	Red	Jointless	Absent	Violet	Transparent
LA1407	Round	5	73	SP/SP	Absent	Orange	Jointed	Present	Green	Not transparent
ISI 6	Blocky	80	36	sp/sp	Absent	Red	Jointless	Absent	Violet	Transparent
ISI 7	Elongated	75	46	sp/sp	Absent	Red	Jointless	Absent	Violet	Not transparent
ISI 8	Round	50	55	sp/sp	Absent	Red	Jointless	Present	Violet	Not transparent

[1] Days to flowering; [2] sp = self-pruning gene; [3] H3 = high holding-ability hybrid; [4] OBV = obscura venosa.

The eight stable founder lines were chosen to maximize the phenotypic diversity and were genotyped using the genotyping by sequencing (GBS) approach, following the RAD technology. The genotyping was performed by the external service provider PTP–Parco Tecnologico Padano. The SNPs dataset was filtered for MAF < 0.05 using the R software version 3.4.4. The four initial crosses were performed using the cross plan proposed by Pasqual et al. [8]. Each G1 (Generation one) was then crossed in pair to obtain two different sets of double cross G1 hybrid (G2). The two offspring obtained from each set were sowed and intercrossed producing 100 plants, each containing parts of the eight founder genomes. Some plants from these eight-way families were selfed through single seed descent (SSD) to monitor phenotypic variation and to increase seed availability for the next SSD steps. The presence of alleles for tomato spotted wilt virus resistance, *Beta (B) locus* for beta-carotene and self-pruning trait (*sp locus*) were tested on the segregant population genotypes to demonstrate the successful crossing.

2.2. Cultivation and Selection of MAGIC Population

A core population of 400 plants (four plants for each cross—G3) of the developed tomato MAGIC population, were cultivated at the Research Centre for Vegetable and Ornamental Crops (CREA) on the Monsampolo organic vegetable long-term field experiment—MOVE LTE (AP, 42°53′ N, 13°48′ E) [16]. The MOVE LTE reproduces a typical cropping system of an organic vegetable farm (four-year crop rotation with four cash crops and three different agro-ecological service crops), including the most important crops in the area. Agronomic management follows an agro-ecological approach based on both conservation tillage and crop diversification strategies. In the spring–summer 2017 growing season, a first evaluation made by researchers of the MAGIC population and the selection of the best 30 G3 plants was carried out by detecting several traits. In particular, this evaluation took into account plant *habitus*, time of flowering, ripening time, leaf and fruit shape, firmness, shelf-life, color, °Brix and pH. In the spring–summer 2018 following year, the 400 G4 plants of the MAGIC were cultivated and a further phenotypical selection was carried out by researchers selecting the best 22 G4 plants. In each generation cycle, tomato seeds were collected from all 400 cultivated plants to continue the Single Seed Descent (SSD).

2.3. Participatory Plant Breeding Approach and Description

The 400 G4 tomato plants obtained in summer 2017 on a field trial conducted in the MOVE LTE at CREA represented a living gene bank and it was used in participatory breeding programs (PBB). This activity was carried out during the spring–summer 2018 growing season on three Italian organic farms: North (Po valley, Padua province, Italy), Central (hills near the Adriatic coast, Fermo province, Italy) and South (along the Jonian coast, Matera province, Italy). The farmers, in collaboration with

the scientists, selected the most desirable plants of the core collection in their own fields. In particular, to conduct participatory selection in each location it was adopted a partially replicated (p-rep) experimental design [17]. It was used the optimized randomization [18] to allocate the following 480 plants: 370 G4 MAGIC tomato single plant obtained by the 2017 field experiment on MOVE LTE; the best 30 G4 MAGIC selected plants at MOVE LTE in 2017; 25 tomato local varieties coming from Italy and Spain. These latter selected and local varieties plants were replicated twice.

The field experiments carried out in three farms were conducted by applying the local technique for tomato organic production, following the agronomic practices indicated in the European legislation on organic farming [19]. In each trial, the single plant evaluation was visually performed by farmers, researchers and technicians, who individually indicated a score ranging from 1 (= bad value) to 4 (= very good value). On the basis of this score, the fruits were collected from the selected plants and were evaluated for the quality parameters. The farmers cultivated the G4 as unexpected and potential genetic materials in terms of adaptation to environmental conditions and specific agricultural management techniques (e.g., sustainable soil management, organic agriculture, etc.).

2.4. Molecular Analysis of the Selected G4 Plants

The seedlings of the eight founders and the 22 plants (G5) selected during the 2018 summer season at CREA (total of 30 plants) were grown in a greenhouse at 25 ± 2 °C with 80–90% of relative humidity in plastic pots filled with sterilized soil. Total DNA was extracted from young leaves using a slightly modified version of the CTAB method [20]. DNA was quantified with a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA) spectrophotometer and diluted in sterile water to a final concentration of 20 ng μL^{-1}. The quality of DNA was checked by electrophoresis on 0.8% agarose gel. The amplification was performed in a PTC-100 MJ thermal cycler (Bio-Rad Inc., Hercules, CA, USA) in a total volume of 20 µL containing 40 ng of genomic DNA, 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.8 mM primer and 0.8 U Taq (Promega, Madison, WI, USA). The amplified products were separated on 1.2% agarose gel for 2 h at 80 volts and stained with 0.5 g mL^{-1} of ethidium bromide. Gels were visualized using Kodak 1D 3.6 software. A 1 Kb ladder (SIGMA) was used. The PCR cycling profiles followed those reported in the literature [21–26] for each gene detected. The molecular markers used were related to resistance genes and fruit shape characters (Table 2).

Table 2. List of characters analyzed and primes used.

Gene	Marker	Sequences Primer (5′–3′)		References
		Forward "F"	Reverse "R"	
Resistance				
I-2 (FOL)	TAO1	GGGCTCCTAATCCGTGCTTCA	GGTGGAGGATCGGGTTTGTTTC	[21]
Frl (FORL)	T1212	AAGTGCTCTAGACAAAAAGACTCC	CCAATGTACAATGGAACTCGTTGATG	[22]
Mi-1 (M)	Mi23	TGGAAAAATGTTGAATTTCTTTTG	GCATACTATATGGCTTGTTTACCC	[23]
Sw-5 (TSWV)	Sw5	CGGAACCTGTAACTTGACTG	GAGCTCTCATCCATTTTCCG	[24]
Pto	Pto	ATCTACCCACAATGACCATGAGCTG	GTGCATACTCCAGTTTCCAC	[25]
Fruit Shape				
Ovate (Pear shape)	OvaNest	AATGCCAACACCAAGAGGAG	TCTCCCAAATGTCTGAGAACG	[26]

FOL = *Fusarium oxysporum* f.sp. *Lycopersici* (race 2); FORL = *Fusarium oxysporum* f.sp. *radicis-lycopersici*; Mi = Meloidogyne spp.; TSWV = Tomato spotted wilt virus; Pto = *Pseudomonas syringae* pv. *tomato*.

3. Results and Discussion

3.1. MAGIC Development and Tomato Selection of G3 Population

In the MAGIC population development, the multiple parents were intercrossed to form a broad genetic base following a prescribed order for each line. In this way, we obtained a set of lines in which the genomes included the contributions from each of the founders. According to Huong et al. [27], the design and selection scheme used in the cross will impact on the eventual genetic makeup of the

population. The founder lines selection is extremely important to develop a new population since there is a need to achieve an optimal level of genetic diversity [7]. The founders must be chosen based on genetic and/or phenotypic diversity, either in a constrained set of materials (e.g., elite cultivars, geographical adaptation) or using genotypes of different origins (worldwide germplasm collections, distant relatives) [27]. An average amount of 500,000 raw data points (DP) were identified by the GBS analysis. A total of 7567 new useful SNPs was identified, and SNPs were located in the tomato genome (v 2.50) as reported in Table 3. The distribution of SNPs varied among the chromosomes and, in particular, chromosome 6 showed the highest density of SNPs (1509) while chromosome 7 the lowest (157) (Table 3).

Table 3. Chromosome-wide distribution of polymorphic founder alleles.

Chromosome	Number of Founder Alleles
Chr 1	224
Chr 2	340
Chr 3	475
Chr 4	1221
Chr 5	1309
Chr 6	1509
Chr 7	157
Chr 8	249
Chr 9	325
Chr 10	205
Chr 11	1161
Chr 12	392
Total	7567

These data, along with those obtained from the genotyping of the stable lines produced at the end of the selection cycles, will be used to estimate the proportion of founder alleles segregating within the RILs and to follow the allelic frequencies evolution in the different environmental conditions. The cluster analysis of the founders has grouped the genotypes in two main groups, which did not seem to fit with any traits related to the plant structure nor fruit related traits (Figure 1). The dendrogram is the results of the genotyping of the founders and it presents the genetic similarity among the founders used to develop the MAGIC population. In particular, the line ISI 4 was obtained from the segregated population of ISI 7 crossed with an elongated parental line not included in this study. The ISI 7 and ISI 6 were obtained from a segregating population including old fresh market indeterminate lines and selecting for the self-pruning trait. The ISI 1, ISI 2 and ISI 3 are determinate parental lines obtained by the selection of processing segregating varieties. The ISI 8 is a determinate line with the genetic background of Ailsa Craig (indeterminate), containing spontaneous mutations involved during the flowering stage. The LA1407 (*Solanum cheesmaniae*) is an indeterminate wild relative with semi-prostrate habits, which was expected to belong to the cluster of the lines having indeterminate pedigree. This different clustering, nearest to the processing determinate lines, suggested that intercross among *Solanum lycopersicum*, *Solanum cheesmaniae* and indeterminate tomato lines has occurred in recent times to introduce valuable agronomic traits.

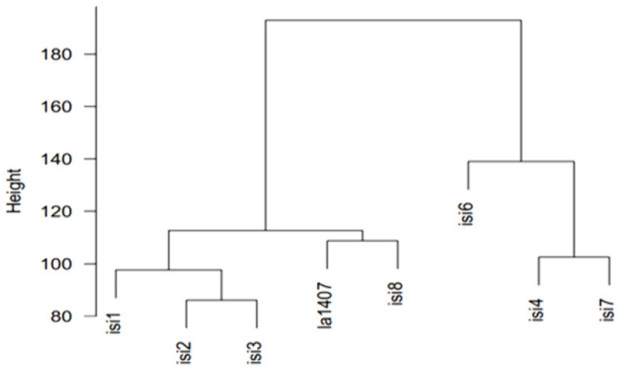

Figure 1. Dendrogram of the founders obtained by Genotyping By Sequencing approach.

The large number of new molecular markers obtained by the GBS of the founders and associated to important traits will be used in all lines developed from the MAGIC populations, allowing us to follow the allelic frequencies evolution in different environmental conditions.

The stable lines that will be obtained at the end of this work will represent a new and permanent resource for the tomato scientific community and farmers, allowing the constitution of varieties adapted to different environments, agricultural practices and market conditions. The potential of MAGIC populations to include a wide range of variation highlighted in a publication of the tomato genome [28] opened new avenues for the exploitation of this variation [8]. The crop domestication leads to a lack of genetic variation in the cultivated species and, in particular, for both the fruit aspect and adaptation to a wide range of environmental conditions. This lack of variation induced a strong reduction of molecular diversity, that limited the exploitation of intraspecific variation [29–31]. Currently, most of the scientific papers on tomato [27,29] are based on genetic material developed during the 90s. As a consequence, scouting for new agronomical interesting traits, as well as new gene and alleles, are needed for academic research, breeding and markets.

Figure 2 presents the MAGIC population scheme. The first stage of our innovative breeding program was carried out during the autumn 2015–winter 2016 season and the eight founder lines (G0) were pairwise crossed. The lines were separated in two sets: one containing four determinate *Solanum lycopersicum* parental lines, and one containing three determinate *Solanum lycopersicum* and the wild indeterminate *Solanum cheesmaniae* accession. In Figure 2 the flow chart of the research is reported, according to the suggestion of Bandillo et al. [11]. The four resulting G1s were pairwise crossed (4-way crosses performed in spring–summer 2016). The seeds (G2) of each offspring were sowed and the plants were used to perform 100 single crosses to obtain the G3 segregating population (autumn–winter 2016). The flowers obtained by the pedigree containing the *Solanum cheesmaniae* accession were used as pollen donor in order to avoid fertility problems due to the wild incompatibility. All obtained descendants segregating crosses were sowed during spring–summer 2017. The wide phenotypical segregation obtained confirmed the success of crosses.

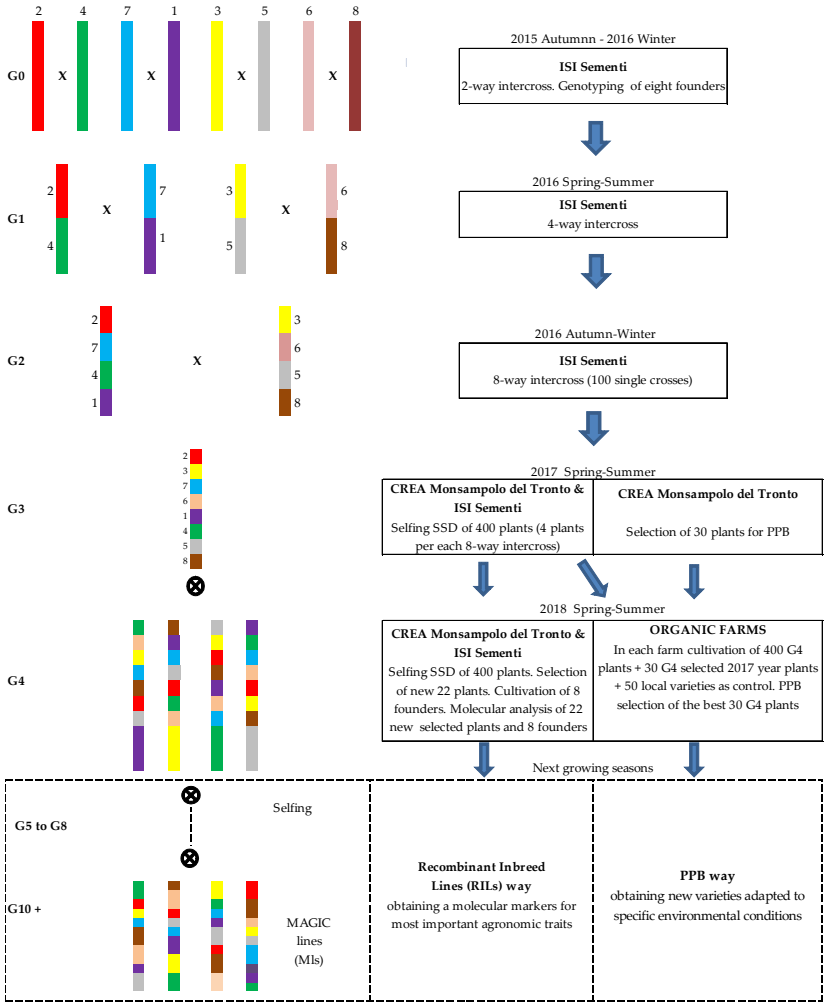

Figure 2. Multi-parent Advanced Generation Intercross population development scheme and the flow chart of the research; plants 1, 2, 3, 4, 6, 7, 8 = ISI lines; 5 = LA1407 (*S. cheesmaniae).*

3.2. Evaluation and Selection within the MAGIC Population

Figure 2 also shows the evaluation of the MAGIC population. In particular, the 400 G3 plants (4 plants for 100 crosses) of the MAGIC population were cultivated in spring–summer 2017 on MOVE LTE at CREA Monsampolo del Tronto and 30 G3 plants, coming from different crosses, were selected. In the following growing season (spring–summer 2018), among the 400 G4 plants of the MAGIC population, 22 plants were selected and only five individuals were the ones derived from the 30 G3 plants previously selected. The plants resulting from the advanced intercrossing stage will be progressed to create homozygous individuals in the next growing seasons (Figure 2). Even if the RILs in plants can be created in different ways [32,33], in our study we adopted the SSD approach with the aim to introduce additional recombination, albeit less than during the MAGIC development. The final collection of tomato pure lines (G10) will be phenotyped with classical tomato breeding tools through the field book app and the tomato analyzer software. They will be also genotyped by GBS approach, in order to develop association studies and speed up the selection of new breeding varieties. The GBS is a widely used range of methods for detecting SNPs using high-throughput sequencing technology and combined with phenotypic data. It provides a powerful basis for rapid mapping and identification of genes underlying agronomic traits which can then be introduced into germplasm [34]. The collection could represent a milestone in tomato genomics, since it will be a resource for both scientific and farmers community, in order to face the new challenges of worldwide sustainable tomato cultivation in the near future.

3.3. Molecular Characterization of the G4 Selected Plants

A total of 30 seedlings (G5) were molecularly characterized and they were represented by the 22 selected G4 tomato plants from the MAGIC population plus the eight founders. The molecular characterization was aimed at detecting genes toward the main tomato biotic stress and fruit shape. The resistance, susceptible and heterozygous genotypes were detected for all genes amplified (Figures 3 and 4). In Figure 3, the presence of the Pto resistance gene is showed in two parental lines (single band of 552 bp) and one of four selected lines, the line numbered eight showed the three bands indicating the heterozygosity. In Figure 4 two parental lines and 12 selected lines showed the presence of FOL race 2 (two bands of 390 and 410 bp). Among these 12 selected lines, three were heterozygotes.

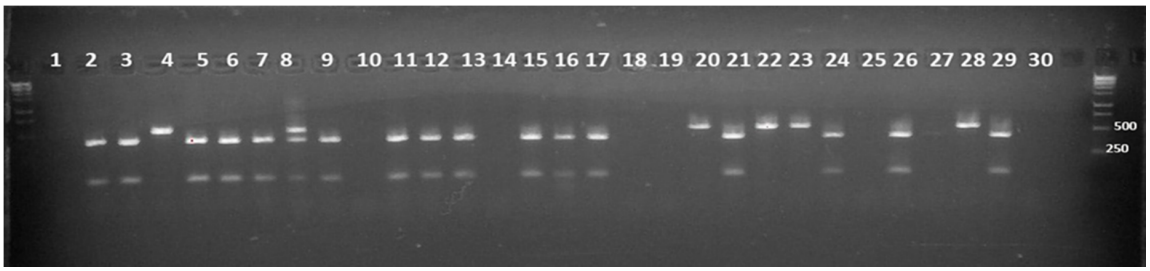

Figure 3. PCR amplification of the selected plants and parent lines with Pto marker for resistance towards *Pseudomonas syringae* pv. *Tomato*. Lanes 1–22: selected plants; lanes 23–30: parental lines; 1 Kb ladder.

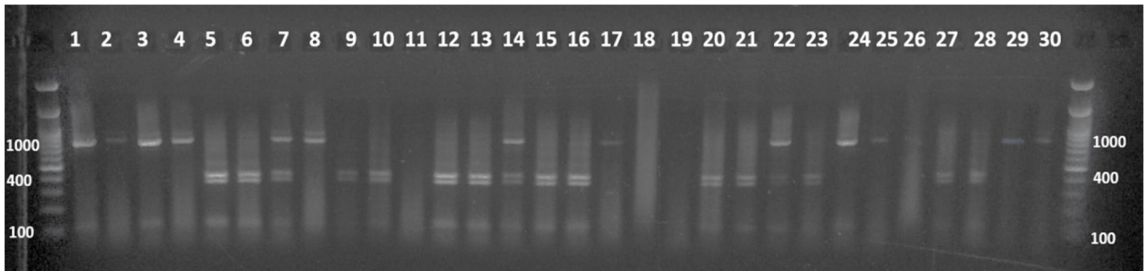

Figure 4. PCR amplification of the selected plants and parent lines with TAO marker for resistance towards *Fusarium oxysporum* f.sp. *Lycopersici*, race 2 (FOL). Lanes 1–22: selected plants; lanes 23–30: parental lines; 100 bp ladder.

In Table 4 is reported the molecular characterization of both 22 selected lines and eight parents. All the results obtained were in accordance with cited literature [21–26]. The wild type *S. chesmaniae* resulted susceptible to all diseases except for Meloidogyne, *P. syringae* and TSWV. In five plants, among the 22 selected, the markers amplified all genes for biotic stress, suggesting the positive pyramiding of the resistance. The *ovate* gene was present in 13 of the 22 selected plants that showed the "pear shape" character [26].

Table 4. Molecular markers of the selected and parental plants.

Plant		Resistance	Fruit Shape
Selected	**1**	Meloidogyne; FORL; TSWV	
	2	Meloidogyne; TSWV	
	3	Meloidogyne; FORL; TSWV	
	4	Meloidogyne; *P. syringae*; TSWV	
	5	Meloidogyne; FOL; FORL; TSWV	Pear shape
	6	Meloidogyne; FOL; FORL; TSWV	Pear shape
	7	Meloidogyne; FOL; FORL; TSWV	Pear shape
	8	Meloidogyne; FORL; *P. syringae*; TSWV	Pear shape
	9	Meloidogyne; FOL; *P. syringae*; TSWV	
	10	Meloidogyne; FOL; FORL; TSWV	Pear shape
	11	Meloidogyne; *P. syringae*; TSWV	Pear shape
	12	Meloidogyne; FOL; FORL; *P. syringae*; TSWV	
	13	Meloidogyne; FOL; FORL; *P. syringae*; TSWV	Pear shape
	14	Meloidogyne; FOL; TSWV	Pear shape
	15	Meloidogyne; FOL; FORL; *P. syringae*; TSWV	Pear shape
	16	Meloidogyne; FOL; FORL; *P. syringae*; TSWV	Pear shape
	17	Meloidogyne; *P. syringae*; TSWV	Pear shape
	18	Meloidogyne; TSWV	
	19	Meloidogyne; TSWV	
	20	Meloidogyne; FOL; FORL; TSWV	Pear shape
	21	Meloidogyne; FOL; FORL; *P. syringae*; TSWV	
	22	Meloidogyne; FOL; FORL; TSWV	Pear shape
Parents	**23**	Meloidogyne; FOL; FORL; *P. syringae*; TSWV	
	24	Meloidogyne; FORL; TSWV	
	25	TSWV	Pear shape
	26	Meloidogyne; TSWV	
	27	Meloidogyne; FOL; FORL; TSWV	
	28	Meloidogyne; FOL; FORL; *P. syringae*; TSWV	Pear shape
	29	Meloidogyne; TSWV	Pear shape
	30	Meloidogyne; FORL; TSWV	Pear shape

The phenotypic performed selection in 2017 identified 30 G3 plants among the 400 original MAGIC population. The next 2018 selection identified further new 22 G4 plants. Among these 22 G4 plants only five derived from the 30 G3 plants previously selected in 2017. The MAS performed in 2018 on the 22 G5 seedlings (derived from G4) identified three genotypes that showed both all resistance genes and the *ovate* gene for the pear shape (Table 4). The molecular analysis, therefore, confirmed the visual selection carried out during the two years of phenotypic selection. The molecular analyses are still undergoing for fruit color, habitus and consistency characters, in order to identify the best genotypes. This activity will be important to develop stable tomato varieties. The molecular characterization will also proceed on the seedlings obtained by seeds collected from the G4 plants selected in each organic farm to provide only the more genetic and agronomic interesting plants. Furthermore, the selected tomato plants will be analyzed with new functional molecular markers developed from the 20,000 new

SNPs obtained by the genotyping of the eight founders. The GBS approach has been successfully used to implement both genome-wide association (GWAS) and genomic diversity study, genetic linkage analysis, molecular marker discovery and genomic selection under a large scale of plant breeding programs [35].

3.4. Tomato Genotype Selection by Participatory Plant Breeding Program

The selection of offspring is an important step in this innovative breeding program, since it may not be practical to phenotype the whole population. In fact, it is difficult and expensive to measure the most important agronomical traits, particularly the ones related to the adaptation of environmental and agricultural systems. Furthermore, we have to take into account the high variability of the segregant population, which induces to select the plants for their adaptability to specific environmental cultivation. To accomplish this aim, in each location a team of farmers and researchers/technicians performed a visual selection of the best tomato plants, following a scheme arranged in a rows-columns (40 rows and 12 columns) pattern (Table 5).

Table 5. Evaluation of a single plant (three to five researchers and/or technicians and three to five farmers).

Plant	Column	Row	Researcher and/or Technicians Evaluation	Farmer Evaluation
1 to 480	1 to 12	1 to 40	1 to 4 [1] 1 to 4 [1]	1 to 4 [1] 1 to 4 [1]

[1] 1 = minimum value; 4 = maximum value.

During the visual selection on each individual plant: the mean score and its standard deviation were ranked to identify the plants with the highest mean score and the lowest standard deviation as an estimate of unanimous opinion of the team. The evaluation of tomato plants produced a selection of 30 plants in each organic field trial, which were considered desirable to select for the following cycles of selection. Finally, the fruits of these plants were collected, and the selection by the researchers testing other measuring parameters followed in the laboratory. The total number of parameters evaluated during the selection is reported in Table 6.

Table 6. Parameters choose to evaluate the single selected genotype.

Plant Develop	Plant Vigor	Plant Health	Production Rate	Fruit Shape	Fruit Size	Fruit Color	Homogeneity Ripe Fruit	Fruit Solidity	Internal Fruit Solidity	Fruit Taste	°Brix
VIS [1]	VIS	VIS	VIS	VIS	VIS	VIS	VIS	MEA	VIS	TAS	MEA

[1] VIS = visual assignment (1 = min; 5 = max); MEA = measured parameter; TAS = tested parameter.

In our study, the selection conducted in the three organic farms located throughout Italy, produced different genotypes particularly characterized by plant develop and fruit color (Table 7), confirming the findings of a previous study [4]. The parameters with a higher variability among the three locations were plant vigor, health and production rate. In particular, the plants selected in the North showed a higher vigor, healthy and production rate, while the ones selected in South Italy showed a higher solid soluble (°Brix) of fruits. Furthermore, the plant selected in the three Italian regions showed a significant difference both on fruit shape and fruit color. In particular, in the north, the plants had higher plant vigor, fruiting abundance and the majority of red fruits, with respect to the ones cultivated in the Center and South Italy. Even if the first selection years are not enough to draw a general conclusion on the production of adapted tomato materials, the findings of this research pointed out that the Participatory Plant Breeding program, assisted by the molecular analysis, is not only economically and agronomically feasible, but it also ensures beneficial environmental effects. Therefore, this study, together with other researches on organic tomato agronomic sustainability [36,37]

can be considered a key tool to produce plants adapted to low input crop managements especially in organic production.

Table 7. Mean values (range from one to four except for fruit firmness, which was expressed as kg cm^{-2} and solid soluble as °Brix) of plant parameters (mean ± standard deviation).

Organic Farm	Plant Vigor	Plant Health	Abundance Fruiting	Fruit Size	Homogeneity Fruit Ripe	Fruit Firmness	Puffiness	Solid Soluble
North	4.13 ± 0.57	3.53 ± 0.68	4.20 ± 0.85	2.97 ± 0.81	3.37 ± 0.89	6.20 ± 0.93	2.83 ± 0.75	5.22 ± 0.75
Center	3.10 ± 0.71	2.80 ± 0.85	3.50 ± 0.63	2.80 ± 0.61	3.20 ± 0.85	6.17 ± 1.61	2.97 ± 0.89	5.47 ± 0.99
South	3.03 ± 0.85	2.83 ± 0.79	3.30 ± 0.75	3.10 ± 0.85	3.73 ± 0.74	6.04 ± 1.77	3.23 ± 0.77	5.71 ± 0.86

4. Conclusions

The MAGIC population developed in tomato, that is one of the most important vegetables consumed worldwide, together with the whole genome sequences of the founder lines and the collection obtained from the PPB are extremely important to develop genotypes adapted to organic production and low input crop management. The results of this research highlighted the importance of this breeding program to select families of tomato under organic management techniques. The production of these materials could represent a stable, long-lasting collection and an important genetic resource both for scientists and farmers communities in order to face the new challenges of worldwide sustainable tomato cultivation in the near future. Even if we have already carried out the MAGIC population and the first adapted genotypes, the PPB research activity will proceed in the following years in each organic farm. In particular, this innovative breeding program is scheduled to cultivate the families of the selected plants (20 plants for each typology selected) and the varieties that will be obtained could be used both for fresh market and transformation process. The findings of our research also highlighted that the molecular characterization of the selected plants could be helpful for marker-assisted selection of the MAGIC population. Finally, the genotyping of the founders and the stable lines developed from the MAGIC populations will allow to identify the proportion of polymorphic alleles segregating within the MAGIC lines and to identify new molecular markers associated to important agronomic traits. This research will further allow us to follow the allelic frequencies evolution in the different environmental conditions.

Author Contributions: Conceptualization, G.C., S.S., N.A., F.M., D.P., F.L., M.B.; Methodology, G.C., S.S., N.A., F.M., D.P., F.L., M.B.; Writing-Original Draft Preparation, G.C., S.S. and F.M.; Project Administration, G.C.

Funding: This research was funded by LIVESEED Project Horizon 2020.

Conflicts of Interest: The authors declare no conflict of interest.

References and Notes

1. Gafsi, M.; Legagneux, B.; Nguyen, G.; Robin, P. Towards sustainable farming systems: Effectiveness and deficiency of the French procedure of sustainable agriculture. *Agric. Syst.* **2006**, *90*, 226–242. [CrossRef]
2. Montemurro, F.; Persiani, A.; Diacono, M. Environmental sustainability assessment of horticultural systems: A multi-criteria evaluation approach applied in a case study in Mediterranean conditions. *Agronomy* **2018**, *8*, 98. [CrossRef]
3. De Benedetto, D.; Montemurro, F.; Diacono, M. Impacts of Agro-Ecological Practices on Soil Losses and Cash Crop Yield. *Agriculture* **2017**, *7*, 103. [CrossRef]
4. Campanelli, G.; Acciarri, N.; Campion, B.; Delvecchio, S.; Leteo, F.; Fusari, F.; Angelini, P.; Ceccarelli, S. Participatory tomato breeding for organic conditions in Italy. *Euphytica* **2015**, *204*, 179–197. [CrossRef]
5. Ceccarelli, S. Efficiency of plant breeding. *Crop Sci.* **2015**, *55*, 87–97. [CrossRef]
6. Barabaschi, D.; Tondelli, A.; Desiderio, F.; Volante, A.; Vaccino, P.; Valè, G.; Cattivelli, L. Next generation breeding. *Plant Sci.* **2016**, *242*, 3–13. [CrossRef] [PubMed]

7. D'Agostino, N.; Tripodi, P. NGS-Based Genotyping, High-Throughput Phenotyping and Genome-Wide Association Studies Laid the Foundations for Next-Generation Breeding in Horticultural Crops. *Diversity* **2017**, *9*, 38. [CrossRef]
8. Pascual, L.; Desplat, N.; Huang, B.E.; Desgroux, A.; Bruguier, L.; Bouchet, J.P.; Le, Q.H.; Chauchard, B.; Verschave, P.; Causse, M. Potential of a tomato magic population to decipher the genetic control of quantitative traits and detect causal variants in the resequencing era. *Plant Biotechnol. J.* **2015**, *13*, 565–577. [CrossRef] [PubMed]
9. Huynh, B.L.; Ehlers, J.D.; Huang, B.E.; Muñoz-Amatriaín, M.; Lonardi, S.; Santos, J.R.P.; Ndeve, A.; Batieno, B.J.; Boukar, O.; Cisse, N.; et al. A multi-parent advanced generation inter-cross (MAGIC) population for genetic analysis and improvement of cowpea (*Vigna unguiculata* L. Walp.). *Plant J.* **2018**, *93*, 1129–1142. [CrossRef] [PubMed]
10. Gaur, P.M.; Thudi, M.; Srinivasan, S.; Varshney, R.K. Advances in chickpea genomics. In *Legumes in the Omic Era*; Nadarajan, N., Gupta, D.S., Eds.; Springer: New York, NY, USA, 2013; pp. 73–94.
11. Bandillo, N.; Raghavan, C.; Muyco, P.A.; Sevilla, M.A.; Lobina, I.T.; Dilla-Ermita, C.J.; Tung, C.W.; McCouch, S.; Thomson, M.; Mauleon, R.; et al. Multi-parent advanced generation inter-cross (MAGIC) populations in rice: Progress and potential for genetics research and breeding. *Rice* **2013**, *6*, 11. [CrossRef] [PubMed]
12. Sannemann, W.; Huang, B.E.; Mathew, B.; Leon, J. Multi-parent advanced generation inter-cross in barley: High-resolution quantitative trait locus mapping for flowering time as a proof of concept. *Mol. Breed.* **2015**, *35*, 86. [CrossRef]
13. Mackay, I.J.; Bansept-Basler, P.; Barber, T.; Bentley, A.R.; Cockram, J.; Gosman, N.; Greenland, A.J.; Horsnell, R.; Howells, R.; O'sullivan, D.M.; et al. An eight-parent multiparent advanced generation inter-cross population for winter-sown wheat: Creation, properties, and validation. *G3* **2014**, *4*, 1603–1610. [CrossRef] [PubMed]
14. Hoagland, L.; Navazio, J.; Zystro, J.; Vargas, J.G.; Gibson, K. Key Traits and Promising Germplasm for an Organic Participatory Tomato Breeding Program in the U.S. Midwest. *Hortscience* **2015**, *50*, 1301–1308. [CrossRef]
15. Nesbitt, T.C.; Tanksley, S.D. Comparative sequencing in the genus lycopersicon: Implications for the evolution of fruit size in the domestication of cultivated tomatoes. *Genetics* **2002**, *162*, 365–379. [PubMed]
16. Campanelli, G.; Canali, S. Crop production and environmental effects in conventional and organic vegetable farming systems: The case of a long-term experiment in Mediterranean conditions (Central Italy). *J. Sustain. Agric.* **2012**, *36*, 599–619. [CrossRef]
17. Cullis, B.R.; Smith, A.B.; Coombes, N.E. On the Design of Early Generation Variety Trials with Correlated Data. *J. Agric. Biol. Environ. Stat.* **2006**, *11*, 381–393. [CrossRef]
18. Coombes, N.E. DiGGer, a Design Generator. 2006. Available online: http://www.austatgen.org/files/software/downloads/ (accessed on 14 February 2019).
19. Commission Regulation (EC) No 889/2008. Official Journal of The European Union 18 September 2008.
20. Doyle, J.J.; Doyle, J.L. Isolation of plant DNA from fresh tissue. *Focus* **1990**, *12*, 13–15.
21. Staniaszek, M.; Kozic, E.U.; Marczewski, W. A CAPS marker TAO1902 diagnostic for the I-2 gene conferring resistance to Fusarium *Oxysporum* f. sp. Lycopersici race 2 in tomato. *Plant Breed.* **2007**, *126*, 331–333. [CrossRef]
22. Vakalounakis, D.J.; Laterrot, H.; Moretti, A.; Ligoxigaki, E.K.; Smardas, K. Linkage between Frl (*Fusarium oxysporum* f. sp. radicislycopersici resistance) and Tm-2 (tobacco mosaic virus resistance-2) loci in tomato (*Lycopersicon esculentum*). *Ann. Appl. Biol.* **1997**, *130*, 319–323. [CrossRef]
23. Pérez de Castro, A.; Blanca, J.M.; Dìez, M.J.; Nuez Viñals, F. Identification of a CAPS marker tightly linked to the Tomato yellow leaf curl disease resistance gene Ty-1 in tomato. *Eur. J. Plant Pathol.* **2007**, *117*, 347–356. [CrossRef]
24. Shi, A.; Vierling, R.; Grazzini, R.; Chen, P.; Caton, H.; Panthee, D. Identification of molecular markers for Sw-5 gene of tomato spotted wilt virus resistance. *Am. J. Biotechnol. Mol. Sci.* **2011**, *1*, 8–16. [CrossRef]
25. Yang, W.; Francis, D.M. Marker-assisted Selection for Combining Resistance to Bacterial Spot and Bacterial Speck in Tomato. *J. Am. Soc. Hortic. Sci.* **2005**, *130*, 716–721. [CrossRef]

26. Sabatini, E.; Rotino, G.; Voltattorni, S.; Nazzareno, A. A novel CAPS marker derived from the Ovate gene in tomato (L. esculentum Mill.) is useful to distinguish two Italian ecotypes and to recover pear shape in marker assisted selection. *Eur. J. Hortic. Sci.* **2006**, *71*, 193–198.
27. Huang, B.E.; Verbyla, K.L.; Verbyla, A.P.; Raghavan, C.; Singh, V.K. MAGIC populations in crops: Current status and future prospects. *Appl. Genet.* **2015**, *128*, 999–1017. [CrossRef] [PubMed]
28. The Tomato Genome Consortium. The tomato genome sequence provides insights into fleshy fruit evolution. *Nature* **2012**, *485*, 635–641. [CrossRef] [PubMed]
29. Miller, J.C.; Tanksley, S.D. RFLP analysis of phylogenetic relationships and genetic variation in the genus. Lycopersicon. *Theor. Appl. Genet.* **1990**, *80*, 437–448. [CrossRef] [PubMed]
30. Zamir, D. Improving plant breeding with exotic genetic libraries. *Nat. Rev. Genet.* **2001**, *2*, 983–989. [CrossRef] [PubMed]
31. Blanca, J.; Montero-Pau, J.; Sauvage, C.; Bauchet, G.; Illa, E.; Díez, M.J. Genomic variation in tomato, from wild ancestors to contemporary breeding accessions. *BMC Genom.* **2015**, *16*, 257. [CrossRef] [PubMed]
32. Bailey, D.W. Recombinant-inbred strains. An aid to finding identity, linkage, and function of histocompatibility and other genes. *Transplantation* **1991**, *11*, 325–327. [CrossRef]
33. Maluszynski, M.; Kasha, K.J.; Forster, B.P.; Szarejko, I. *Doubled Haploid Production in Crop Plants: A Manual*; Kluwer Academic Publishers: Dordrecht, The Netherlands, 2013.
34. Scheben, A.; Batley, J.; Edwards, D. Genotype-by-sequencing approaches to characterize crop genomes: Choosing the right tool for the right application. *Plant Biotechnol. J.* **2017**, *15*, 149–161. [CrossRef] [PubMed]
35. He, J.; Zhao, X.; Laroche, A.; Lu, Z.X.; Liu, H.; Li, Z. Genotyping-by-sequencing (GBS), An ultimate marker-assisted selection (MAS) tool to accelerate plant breeding. *Front. Plant Sci.* **2014**, *5*, 484. [CrossRef] [PubMed]
36. Diacono, M.; Persiani, A.; Fiore, A.; Montemurro, F.; Canali, S. Agro-ecology for adaptation of horticultural systems to climate change: Agronomic and energetic performance evaluation. *Agronomy* **2017**, *7*, 35. [CrossRef]
37. Diacono, M.; Persiani, A.; Canali, S.; Montemurro, F. Agronomic performance and sustainability indicators in organic tomato combining different agro-ecological practices. *Nutr. Cycl. Agroecosyst.* **2018**, *112*, 101–117. [CrossRef]