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Full Length Research Paper

Genetic characterization of flood tolerance in a segregating population of tomato using RAPD Markers

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Abstract

Due to the heterogeneity of soils and the effects of changes in climate, the roots, although well anchored in the soil, are subject to environmental conditions. Flooding causes the absence of oxygen which negatively affects crop production and yields, resulting in decline of yields up to 10% or 40% decreasing in food supply for a rising human population. Therefore, the aim of the present work is to identify genes related to chlorophyll content, yield and yield component in tomato subjected to flooding, and to determine markers associated with these traits. The experiment was carried out in the greenhouse and a total of 122 F_2 plants obtained by crossing flood-tolerant cultivar (CLN2498E) with flood-sensible wild species (LA1579) and 4 plants for each parental line. Bulked segregant analysis was used as a method for identifying markers linked to genes responsible for flooding tolerance in tomato. Two pooled DNA sampled were obtained from F2 populations. Three RAPD markers were found tightly linked to the genes responsible for flood tolerance in tomato.

Keywords: Flooding, BSA, RAPD primers, genes responsible for flood tolerance in tomato.

INTRODUCTION

Climate change in the world is related to abiotic stress including waterlogging factor. Flooding causes the absence of oxygen which negatively affects crop production and yields, resulting in decline of yields up to 10% or 40% decreasing in food supply for a rising human population (Safavi-Rizi1 et al., 2020; Nakayama et al., 2017; Ezin et al., 2010; Patel et al., 2014). Most crop plants anchor themselves steadfastly in the ground through their roots and draw water and mineral nutrients from it for their growth and development. Due to the heterogeneity of soils and the effects of changes in climate, the roots, although well anchored in the soil, are subject to environmental conditions. Drought, flooding, nutrient deficits or pathogen attacks can represent severe stresses for the roots and the plant (Kulichikhin et al., 2014; Mustroph, 2018; Safavi-Rizi et al., 2020). One of flood factor influence is oxygen absence, or anoxia or hypoxia, owing to the ca. 10^4-fold lower diffusion rate of oxygen in water than in air (Kozlowski, 1997; Bailey-Serres et al., 2012). Unlike animals, plants cannot quickly escape from environmental stresses. They must therefore be able to perceive them and respond quickly and efficiently. In natural conditions or in the field, plants are repeatedly challenged by more complex situations, combining multiple environmental stresses. Their response modes to these complex contexts are still not clearly apprehended.

For the continuous development and growth and the up taking of essential elements and water, young roots show a strong metabolic activity and intensively breathe oxygen

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(O2) which diffuses into the soil cavities. This process is slowed down in case of flooding or excessive irrigation, resulting in an O2 deficit (hypoxia) and a deep metabolic stress of the root cells (Ponnamperuma, 1972). Flooding inhibits aerobic respiration which cause restriction of energy metabolism and plant growth at all developmental stages from emergence to reproductive stage (Jackson and Drew, 1984; Pan et al., 2021). These stress conditions have important repercussions on world agricultural production (Douglas, 2009; Hirabayashi et al., 2013). According to some estimates, Europe loses 5 billion euros of agricultural crops each year, simply due to river overflows. These losses could double by 2080 due to increasing changes in climate (Hirabayashi et al., 2013). The understanding of plant response to flooding is thus a major agronomic challenge (Voesenek LA, Bailey-Serres, 2015).

The development of improved flooding-tolerant tomato cultivars would be speeded up if the genes in charge of diverse underlying processes could be identified and tagged with molecular markers (Qiu et al., 2007). Many molecular markers such as SSR, RFLP, AFLP, RAPD, CAPS, and SCAR have been of great use in localizing genes of interest, gene expression technique such as RNAseq has been used to identify both gene sequence and expression discrepancy amongst wild tomato cultivars and wild relative species (Koenig et al., 2013). RAPD is one of the easiest and hastiest molecular marking technologies (Gabriel et al. 2005, Zhang et al. 2008). Williams et al. (1990) reported that RAPD technique possesses great benefit over other approaches of genetic records for the fact that it has a universal set of primers, no preliminary work such as probe isolation, filter preparation, or nucleotide sequencing is necessary (Williams et al. 1990). RAPD technique owing to its easiness and simplicity make it ideal for genetic mapping, plant breeding programs. RAPD markers have been used successfully in the construction of genetic map (Singh et al., 2011). The use of DNA primers in plant breeding programs might greatly make ease the manipulation of genes among genotypes (He et al., 1992). Quarrie et al. (1999) reported that quantitative trait locus analysis is generally associated with both genotyping and phenotyping (a) for the genotyping, the selected markers cover the genome, and (b) the phenotyping evaluates the traits of interest. Genotyping a large mapping population is wearisome and quite expensive and consumables. By pooling plants in accordance to either high or low expression of a particular trait and extracting DNA from these two pools, the method of genotyping the plants is trimmed down to only two DNA samples to be analyzed instead of having to analyze DNA separately from each plant. Two alternatives of the bulk segregant analysis (BSA) method are possible according to whether these plants are derived from a cross between two contrasting parents or from a population of plants with various genetic backgrounds. Bulk segregant analysis was used for the first time by Michelmore (1991) and it is an efficient approach to rapidly identify molecule markers linked to a specific gene using DNA bulk from F2 plants. This technique along RAPD primers has been used in many research studies to identify genes of interest.

The objectives of the current study were to (1) determine the morphological and physiological characteristics of F2 population, and (2) identify the genes responsible for flood tolerance by using a segregating F_2 populations derived from a cross between a flooding tolerant accession (‘CLN2498E’) and a susceptible accession (‘LA1579’).

MATERIALS AND METHODS

Plant material: 122 F2 plants and 2 parental lines ofCLN2498E and LA1579 genotypes were used.

DNA extraction

Young leaf tissues were collected in eppendorfs from individual F_2 plants and parental lines and placed into a box of ice. In the laboratory young tomato leaves were immediately frozen in liquid nitrogen and ground with a sterilized pestle 4-5 times while keeping them frozen with liquid nitrogen to break cells and homogenize tissue. Extraction buffer containing 10ml of 1M Tris-cl (pH=7.5), 12.5ml of 1M NaCl, 2.5 of 0.5M EDTA, and 0.25g SDS was added to each tube and shake sticks in the tube to dissolve macerated tissues in buffer completely and vortex tubes for 5 min. Mixed tubes was centrifuge at 12000rpm for 2 min. 300 µl of supernatant was taken out in a separate properly labeled tube and 300 µl isopropanol was added in them. Tubes were carefully mixed by slowly inverting them 5 times and allowing them to rest for 2 min. The mixed tubes were centrifuge at 12000rpm for 5 min. The supernatant was discarded by gently inverting tubes in the sink. Tubes were put in open and inverted position onto tissue paper for 15 min. Next, 100 µl of TE buffer (10mM Tris-Cl and 1 mM EDTA was added to each tube.

RAPD analysis

Screening RAPD Markers

Ten random 10-mer primers were used to screen between the two parents to determine which primers could be used in this study. Markers that segregated between parents during the initial primer screening were screened with the ten most resistant F_2 plants and ten most sensible F_2 plants.

Polymerase chain reaction

The PCR reaction mixture was 5µl of 5x Buffer, 2 µl of 25mM MgCl2, 0.5µl of 10mM dNTPs, 1µl of 125ng/ µl

primer, 0.125µlTaq DNA Polymerase, 2µl of genomic DNA, and 14.5 µl of ddH_2O. A drop of mineral oil was added to the mixture. An automatic thermal was programmed for 5 min at 94^0C, 45 cycles of 1 min at 94^0C, 2 min at 44^0C and 2 min at 72^0C for each cycle, and a final extension step of 10 min at 72^0C. Reaction product was resolved on a 1% agarose gel.

Bulked Segregant Analysis

Bulked segregant analysis (BSA) was performed following the method of Michelmore et al. (1991). Two DNA bulks, called resistant bulk (RB) and susceptible bulk (SB) were prepared from F2 individuals. The RB consisted of 10 individuals with high value of total yield, high and SB contained 10 individuals with low value of total yield. These two DNA bulks were prepared by pooling equal amounts of DNA (20 µl) from each individual. PCR was run with polymorphic primers between parents on the bulks and parental DNA samples using the same reaction conditions as described above. PCR was repeated for at least two times for those primers that were polymorphic between bulks.

Gel electrophoresis

1% W/V agarose gels for electrophoresis were made from a solution consisting of 2.5 g agarose and 250 ml of 1x TBE buffer. The solution was mixed by swirling in a 1000 mL bottle and then placed in a microwave on high power for about 2and 30 minutes. The solution was mixed again and returned to the microwave for an additional 30 seconds on high power. Upon removal from the microwave the bottle was placed under a running tap water and stirred until the agarose solution had cooled. While cooling the gel, 20 µl of ethidium bromide was added. Once cooled the solution was poured into a prepared electrophoresis gel tray. 42 well combs were placed into the gel at even distances from each other. The gel was allowed to solidify for 30 minutes, then the combs were carefully removed and the resulting gel and plate were placed into the electrophoresis box. 1x TBE buffer was used to fill the electrophoresis box; additional buffer was added as needed to just cover the top of the agarose gel.

Gels were filled by pipeting the primer samples directly into the wells. Once the gels were loaded the covers were replaced and connected to 20 the power source. The voltage was set at 250 and the current (I= 3A) was allowed to run for 1.5 hours.

After the current was turned off, the gel was removed from the electrophoresis box. The gel was placed onto an ultraviolet illuminator with light box and digital camera setup attached and photographed. The gels were disposed of appropriately after photographing.

Data Analysis

The length of the marker was estimated, in base pairs. A molecular weight base pair ladder was used to measure the size of the RAPD marker bands on the agarose gel.

Linkage Analysis

Band presence and absence was scored within the individual F_2 of the bulk and parents for marker analysis. In the initial analysis, RAPD bands was scored for presence 1 and absence 0 for each population to perform a data matrix. In subsequent analysis, the markers were scored as absent (0), polymorphic (1).

RESULTS

Bulk Segregant Analysis from the response of F2 plants to flooding

Ten plants taken from the most tolerant formed the tolerant bulk (TB) and 10 plants from the most sensitive represent sensitive bulk (SB).

Screening of RAPD markers for flood tolerance

In this study 38 RAPD markers (Table 1) were screened with the tolerant parent and susceptible parental line. Of the 38 primers only 9 (Table 2) were found polymorphic between the two parental lines which represent 21.05%. Percentage of polymorphism between parents ranged from 20 to 50. The high values of polymorphism were recorded with RAPD markers OPK-19 and OPX-17 with 50% each followed by OPB-17 (43%)while the lowest was recorded with RAPD marker 17OPE-07 (20%). The following two markers namelyOPX-17 and OPK-19were identified to be associated with genes of interest. Thus, these two RAPD primers could be considered as candidate markers for further molecular analysis with the F2 population using bulk segregant analysis.

Figure 1A shows the amplification pattern using RAPD primer to identify the 15OPC-08 marker linked to the tolerant allele. Lane M is 100 bp molecule-weight ladders, lane SP was the susceptible parent, and lane TP was the resistant parent. The arrow in Figure 1 shows polymorphic banding pattern indicating the tolerant allele. The resistant gene associated with the tolerant parent corresponds to 600 bp. Figure 1B represents the electrophoresis pattern of DNA fragments generated by RAPD marker OPA-09. The polymorphic band linked to resistant gene between tolerant parent (TP) and susceptible parent is indicated by an arrow. The resistant gene associated with the tolerant parent was 1200 bp while it was absent in the sensitive parent.

Figure 2C is the amplification pattern of DNA fragments

Table 1. Sequence of the 38 RAPD primers used in this study.

Name of the primer	Sequence of primer (5►3)	Name of the primer	Sequence of primer (5►3)
OPX-02	TTCCGCCACC	OPA-09	GGGTAACGCC
17OPE-07	AGATGCAGCC	OPC-08	TGGACCGGTG
OPA-19	CAAACGTCGG	OPX-14	ACAGGTGCTG
OPAK-19	TCGCAGCGAG	OPB-06	TGCTCTGCCC
OPB-15	GGAGGGTGTT	15OPC-08	TGGACCGGTG
OPC-18	TGAGTGGGTG	9OPA-19	GACGCTGGGC
OPC-06	GAACGGACTC	OPB-17	AGGGAACGAG
OPAK-05	GATGGCAGTC	19OPI-09	TCGAGAGCAG
OPA-04	AATCGGTGTT	OPC-19	GTTGCCAGCC
OPA-13	CAGCACCCAC	4OPA-07	GAAACGGGTG
OPX-08	CAGGGGTGGA	OPB-05	TGCGCCCTTC
OPX-17	GACACGGACC	2OPA-02	TGCCGAGCTG
8OPA-16	AGCCAGCGAA	OPA-08	GTGACGTAGG
7OPA-11	GGACTGGAG	MRTOMR-027	ACCTGATGCA
MRTOMR-046	CCATGCGCTA	Z13	GACTAAGCCC
OPE 1	CCCAAGGTCC	OPD 6	ACCTGAACGG
OPU-03	CTATGCCGAC	OPV-19	GGGTGTGCAG
J20	AAGCGGCCTC	MRTOMR-117	CCG AAC AAT C
OPD 3	GTCGCCGTCA	OPA 15	TTCCGAACCC

Table 2. RAPD primers polymorphic between resistant and susceptible parents of flood tolerant in tomato.

Primer	Amplified bands	Band size(bp) Max min	Polymorphic bands	% polymorphic	Tm
OPX-17	6	900 300	3	50	36
MRTOMR-022	7	1517 300	2	29	41
17OPE-07	5	700 500	1	20	32
OPB-17	7	1100 400	3	43	32
OPX-08	3	1200 400	1	33	32
OPA-09	7	1000 300	2	29	32
15OPC-08	3	1000 600	1	33	32
OPK-19	6	1100 200	3	50	32
19OPI-08	5	800 400	1	20	32

generated by RAPD marker OPX-17. The polymorphic bands linked to resistance gene between resistant parent (RP) and resistant bulk is indicated by arrows. RAPD marker OPX-17 exhibited two positive polymorphic bands which were found only in the tolerant parent and tolerant F2 bulk with molecule size of 900 bp while there were absent in the sensitive parent and susceptible F2 bulk. Figure 2D shows that RAPD primer MRTOMR-022 was identified as being tightly linked to the gene of flood tolerance. Several bands are evident in each lane but the 1000 bp product (arrowed) has the important property of being present in the tolerant bulk lane but absent from the susceptible bulk.

The amplification pattern using RAPD primer 17OPE-07 is showed in figure 3. RAPD primer 17OPE-07 produced amplification present in the resistant parent and tolerant F2 bulk. This polymorphic band was not observed in the susceptible parent or in the susceptible F2 bulk. This band was generated by RAPD primer 17OPE-07 possesses 700 bp.

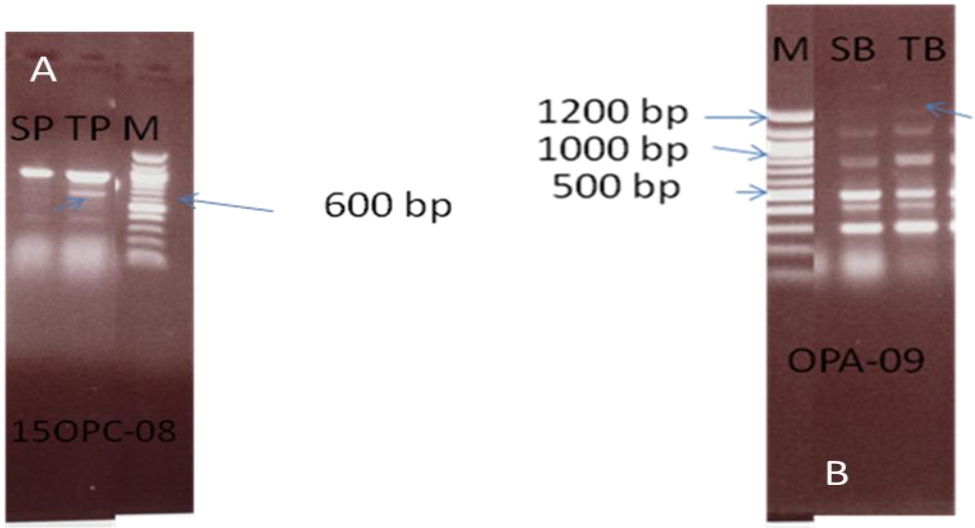

Figure 1:A: RAPD marker 15OPC-08 expressing polymorphism between susceptible parent (SP), tolerant parent (TP), and molecular marker (M). **B:** RAPD marker OPA-09 expressing polymorphism between susceptible bulk and Tolerant bulk.

Figure 2. C. RAPD marker OPX-17 expressing polymorphism between susceptible parent (SP), tolerant parent (TP), susceptible bulk (SB), and tolerant bulk (TB), M is molecular marker (ladder). D: Bulk analysis with RAPD primer MRTOMR-022.

DISCUSSION

When breeders need to improve plants, they have to find a source of germoplasm that would supply the genes needed to undertake the breeding project (Acquaah 2007). Base on the screening of RAPD markers, three RAPD primers OPX-17, MRTOMR-022 and OPA-09 were identified to be linked to the putative tolerance genes and one RAPD marker 17OPE-07 linked to the susceptible gene. Through the bulking of two extreme phenotypes of F2 populations we were able to rapidly tag the markers associated with the chromosomal segment that has a role on reaction to the flooding stress. With the bulk segregant analysis technique consisting of 10 individual plants in each bulk, three RAPD markers gave different band sizes that were found to be linked to flooding resistance.

RAPD primer OPX-17 exhibited a positive molecule marker which was found only in the resistant parent (CLN2498E) and resistance F_2 bulk with molecular size of 900 bp while it was absent in the sensitive parent (LA1579)

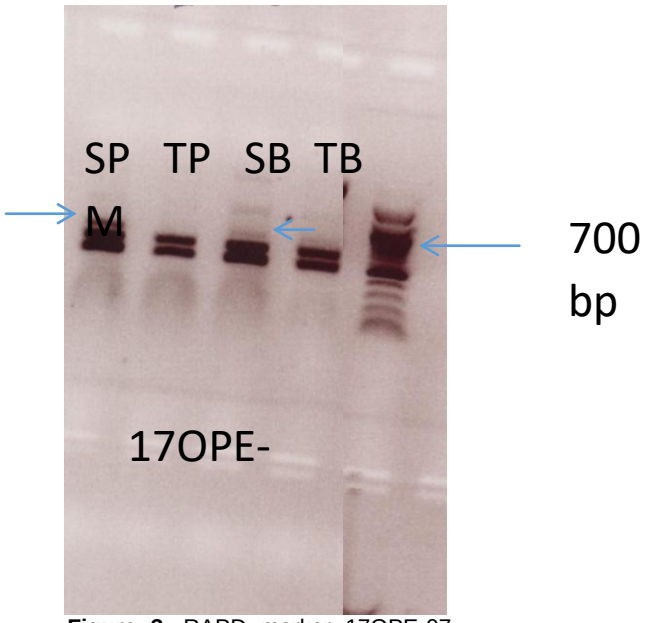

Figure 3. RAPD marker 17OPE-07 expressing polymorphism between susceptible parent (SP), tolerant parent (TP), susceptible bulk (SB), and tolerant bulk (TB), M is molecular marker (ladder).

and susceptible F_2 bulk. RAPD marker OPA-09 and MRTOMR-022 exhibited 2 positive molecule markers which were found only in the tolerant F_2 bulk with molecular size of 1200 bp for RAPD marker OPA-09 and 1000 bp for RAPD marker MRTOMR-022, while there were absent in the susceptible F_2 bulk. Our results are consistent with those of Smiech et al. (2000) who used bulk segregant analysis in an F2 segregating population and found five markers that distinguished resistant and susceptible bulks. On the other hand, RAPD marker 17OPE-07 exhibited a negative molecule marker which was found only in the sensitive parent (LA1579) and sensitive F_2 Bulk with molecule size of 700 bp. We identified three potential and positive RAPD markers associated with flood tolerance and could be regarded as reliable markers for flood tolerance in tomato. Similar results were observed by Koenig et al. (2013), Ahmed et al., (2013) and Lin et al. (2010). Safavi-Rizi et al. (2020), who worked on novel genes and routes related to flood tolerance in tomato using RNA-Seq, reported differentially regulated genes associated with carbon and amino acid metabolism and redox homeostasis. They also found that differentially regulated transcription factors are involved in tomato plants to short and long period of oxygen depletion.

Bulk segregant analysis allowed us to quickly find RAPD markers linked to gene responsible for flood tolerance in tomato using a segregating population. A total of 38 RAPD primers were screened but only three of the markers showed polymorphism and were linked to resistance gene and only one was linked to susceptible allele. Therefore, BSA allowed us to directly target the gene responsible for flood tolerance as illustrated by Michelmore et al. (1991). They furthered stated that bulk segregant analysis successfully identified markers linked to Dm5/8 and demonstrated that it required fewer than 300 PCR reactions to identify and map three new markers. Quantitative trait loci related to tolerance to flooding or waterlogging has been studied in other crops such as wheat (Burgos et al., 2001), soybean (Reyna et al., 2003), maize (Mano et al., 2007; Qiu et al., 2007), rice (Xu et al., 2000a; Toojinda et al., 2003; Jiang et al., 2006; and Angaji et al., 2010), Echinocloa (Fukao et al., 2007) and Loliumperenne (Pearson et al., 2011). Safavi-Rizi et al. (2020) in their study found 267 and 1421 genes under short- and long-term hypoxia in tomato.

The bulking of a large number of individuals increases the probability that the two pools will not differ for alleles other than those adjacent to the target trait (Chagué et al. 1996). Giovanni et al. (1991) also suggested that increasing the pool size could also reduce the effect of false positives due to non-specific annealing. Michelmore et al. (1991) demonstrated that for a dominant RAPD marker segregating in an F_2generation, the likelihood of a pool of n individuals showing a band and a second pool equal size not having a band is $2(1/4)^n[1-(1/4)^n]$ when the

locus is unlinked to the target gene. From this, they concluded that a few individuals per bulk are enough to identify the linked markers. In the present study, we used 10 individuals out of a 122 segregating F_2 population studied and based on Michelmore et al. formula, the proportion of false positive is 2×10^{-6} which is very low. Michelmore et al. (1991) showed that even when many loci are screened, the chances of detecting an unlinked locus are very small; however, when smaller pools or bulks are employed, the frequency of false positives will increase. Therefore, as the linkage of all polymorphisms is confirmed by analysis of a segregating population, bulked segregant analysis with only reasonable numbers of individuals in both pools will offer great enrichment for markers linked to target alleles.

CONCLUSION

It is important to understand the genetic basis of flood tolerance in tomato in order to develop new approach for selection. RAPD markers OPX-17 and MRTOMR-022 and OPA-09 were identified as being tightly linked to flood tolerance in tomato. Figure 10 shows the amplification products for the two parental lines and the two bulks with this primer OPX-17.

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