

# Distribution of DArT Markers in a Genetic Linkage Map of Tomato

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**Abstract.** A genetic linkage map was constructed using 188 F<sub>9</sub> RILs derived from a cross between *Solanum lycopersicum* H7996 (resistant to bacterial wilt) and *S. pimpinellifolium* WVa700 (highly susceptible to bacterial wilt). The map consisted of 361 markers including 260 DArTs, 74 AFLPs, 4 RFLPs, 1 SNP, and 22 SSRs. The resulting linkage map was comprised of 13 linkage groups covering 2042.7 cM. The genetic linkage map had an average map distance between markers of 5.7 cM, with an average DArT marker density of 1/7.9 cM. Based on the distribution of anchor SSR markers, 11 linkage groups were assigned to 10 chromosomes of tomato except chromosomes 5 and 12. The DArT markers were distributed across the genome in a similar way as other markers and showed the highest frequency of clustering (38.8%) at  $\leq 0.5$  cM intervals between adjacent markers, which is 3 times higher than AFLPs (13.5%). The present study is the first utilization of DArT markers in tomato linkage map construction.

**Additional key words:** diversity arrays technology, Hawaii 7996, marker distribution, recombinant inbred line (RIL)

## Introduction

The cultivated tomato, *Solanum lycopersicum* Mill., is one of the world's most important vegetable crops. It belongs to the Solanaceae family, which is a diverse family consisting of 96 genera and over 2800 species (Knapp et al., 2004). All tomato species are diploid ( $2n = 2x = 24$ ) and are similar in chromosome number and structure. The 12 chromosomes are highly differentiated and can be distinguished at pachytene. The genome size of tomato is estimated to be 950 Mb and smallest in the *Solanaceae* family (Lindhout, 2005).

The tomato genome is one of the most investigated plant genomes. A large number of various molecular markers has already been obtained (Saliba-Colombani et al., 2001; Tanksley et al., 1992). However, the search for new, highly polymorphic molecular markers is essential. Denser linkage maps will make map based cloning more feasible and will facilitate marker assisted plant breeding. Development and use of PCR-based markers in tomato has recently increased as they are generally cheaper, faster and less labor intensive than RFLP markers. However, limitation of polymorphisms among

closely related genotypes is an issue in marker development. Since the first high-density molecular map of tomato was published in 1992 (Tanksley et al., 1992), several other molecular maps have been constructed using different mapping populations (Foolad, 2007). However, most of these maps were developed based on RFLP markers from the high-density map, though some other markers, such as RAPDs, ESTs, AFLPs, SSRs and RGAs also have been utilized. Identification of polymorphic markers for interspecific crosses between cultivated tomatoes and its closely-related wild species such as *S. pimpinellifolium* and *S. cheesmannii* is very challenging (Chen and Foolad, 1999; Grandillo and Tanksley, 1996; Labate and Baldo, 2005; Thoquet et al., 1996). Diversity array technology (DArT) marker can overcome this challenge. DArT has potential for increasing marker density within a short time (Wenzl et al., 2004). A single DArT assay simultaneously types hundreds to thousands of SNPs and insertion/deletion polymorphisms spread across the genome. The cost of DArT per data point had been reported to be 10-fold lower than the cost of SSR (Xia et al., 2005). DArT has recently been used in genetic mapping and fingerprinting

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※ Received 21 December 2009; Accepted 28 April 2010. This paper is based on a Ph.D. dissertation by Truong Thi Hong Hai, who received a scholarship from the German Government through Project GTZ81070160. We thank S.M. Huang for her laboratory assistance.

studies in *Arabidopsis* (Wittenberg et al., 2005), barley (Wenzl et al., 2004), cassava (Xia et al., 2005), sugarcane (Lakshmanan et al., 2005), wheat (Akbari et al., 2006), pigeon pea (Yang et al., 2006) and sorghum (Mace et al., 2008). However, this marker has not been utilized in tomato yet. The objective of this study was, therefore, to construct a genetic linkage map in a recombinant inbred line (RIL) population using DArT, AFLP, and SSR markers, and to understand the distribution of new tomato DArT markers in tomato genome.

## Materials and methods

### Plant materials and DNA extraction

A population of 188 F<sub>9</sub> recombinant inbred lines (RILs), series number: 1-200 (except RIL number 7, 19, 34, 61, 99, 110, 123, 133, 174, 180, 181, 190) derived from a cross between *S. lycopersicum* 'Hawaii 7996' (H7996) (resistant to bacterial wilt) and *S. pimpinellifolium* 'West Virginia 700' (WVa700) (highly susceptible to bacterial wilt) (Thoquet et al., 1996) provided by Bacteriology Unit, AVRDC-The World Vegetable Center (AVRDC), was used in this study. Genomic DNA of 188 F<sub>9</sub> RILs and the parental lines was extracted from young leaves using the method as described by Diversity Arrays Technology (DArT P/L, Yarralumla, ACT 2600, Australia).

### AFLP analysis

The AFLP assay was performed as described by Vos et al. (1995) with minor modifications. Genomic DNA (250 ng) was digested with 8 U of *EcoRI* and 6 U of *MseI* (New England Biolabs, UK) and incubated at 37°C for 4 hours. Digestion solution was ligated to the two adaptors for *EcoRI* and *MseI* cutting sites and then preamplified with a pair of preselective primers for *EcoRI* and *MseI*. The selective amplifications were performed using various combinations of E and M primers with 3 selective nucleotides (Balatero, 2000). The amplification products were analyzed in parallel in a 5% denaturing polyacrylamide gel (19:1 acrylamide-bisacrylamide, 7.5 M urea) in 0.5 × TBE buffer (25 mM Tris, 25 M boric acid, 0.5 mM EDTA, pH 8.0) using a S3S T-Rex™ Aluminum Backed Sequencer and visualized by silver staining. Silver staining and developing was carried out according to Promega's DNA Silver Staining System.

### Microsatellite or SSR analysis

Eighty-five SSRs selected from the Tomato-EXPEN 2000 map (Fulton et al., 2002) and four unmapped SSRs from a reference map published by Smulders et al. (1997) were surveyed for polymorphism using the two parental lines, H7996 and WVa700, on 5% polyacrylamide gels. Each PCR reaction (25 µL final volume) contained 15-20 ng of genomic

DNA, 10X PCR buffer (10 mM Tris-HCl, pH 9.0; 50 mM KCl; 15 mM MgCl<sub>2</sub>), 20 mM dNTPs, and 20 µM of each forward and reverse primer and 2 U of Taq DNA polymerase (Violet, Taiwan). PCR reactions were performed in a MJ PT-200 thermocycler (MJ Research, GMI, Inc., Minnesota, USA). The amplification profile consisted of an initial denaturation for 5 minutes at 94°C followed by 35 cycles of 30 seconds at 94°C, 45 seconds at the annealing temperature 50-60°C (depending on the T<sub>m</sub> of the primers), 45 seconds elongation at 72°C, and a final extension step of 7 minutes at 72°C. Silver staining and developing was carried out according to Promega's DNA Silver Staining System.

### SNP analysis

Eleven SNP markers selected from Tomato Mapping Resource Database (<http://www.tomatomap.net/>) were screened on H7996 and WVa700. PCR amplification reactions were prepared in a total volume of 25 µL containing 10X PCR buffer (100 mM Tris-HCl, pH 9.0; 500 mM KCl; 15 mM MgCl<sub>2</sub>), 20 mM dNTPs, 20 µM of each forward and reverse primer and 2 U of Taq DNA polymerase (Violet, Taiwan), and 20 ng genomic DNA as template for PCR. The amplification procedure consisted of an initial denaturation for 5 minutes at 94°C and 35 cycles of 30 seconds denaturation at 94°C, 1 minute primer annealing at 50°C or 55°C depending on the primers used, 2 minutes extension at 72°C, followed by a final extension at 72°C for 5 minutes. After amplification, 5 µL of PCR product was digested in a 10 µL cocktail including 10X buffer 2 (500 mM NaCl, 100 mM Tris-HCl, 100 mM MgCl<sub>2</sub>, 10 mM dithiothreitol, pH 7.9) and 4 U restriction enzyme (New England Biolabs, UK) by using a MJ PT-200 thermocycler (MJ Research, GMI, Inc., Minnesota, USA). The digested products were separated in 1% agarose gels and 1X TBE buffer for 1.5 to 2 hours at 96 V. A 100 bp ladder was used as molecular weight marker. After electrophoresis, gels were stained with ethidium bromide (1.5 µg·mL<sup>-1</sup>) for 10 minutes, de-stained in distilled water for 15 minutes and photographed under UV light.

### DArT analysis

DArT markers were produced by Diversity Arrays Technology Pty Ltd (DArT P/L) (<http://www.diversityarrays.com/>), which is a whole-genome profiling service laboratory, as described by Wenzl et al. (2004), under a contract with AVRDC.

### Providing of RFLPs

The F<sub>8</sub> RFLP marker genotype data were kindly provided by Dr. Pascale Besse, Centre International de Reseaux Agriculture and Development (CIRAD), a collaborator of Dr.

Marker scoring and nomenclature

Polymorphic markers were scored visually. AFLPs were scored as dominant markers and SSRs and SNP were scored as codominant markers. Band presence or absence associated with the H9776 allele was coded as H; band presence or absence associated with the WVa700 allele was coded as W, and those bands with both parents were coded as HW for heterozygote. Ambiguous bands were considered as missing data for map construction purposes. Each AFLP marker was assigned a three-part name consisting of 3 letters as “afh” and the primer combination number followed by the letter. The locus designations used by DARt P/L were adopted in this paper. DARt markers consisted of the prefix “D”, followed by numbers corresponding to a particular clone in the genomic representation on the 96-well plates.

Segregation analysis and map construction

For each segregating marker, a  $\chi^2$  goodness-of-fit analysis was performed to test for deviation from the 1:1 expected segregation ratio at 1% level of significance. Linkage analysis was performed with MultiPoint mapping software package (<http://www.multiqtl.com>). The approach of multilocus ordering implemented in MultiPoint employs evolutionary algorithms of discrete optimization, which uses the minimization of the total map length as the mapping criterion (Mester et al., 2003,

2004). The population type “RIL-selfing” was used and the initial clustering of all markers into 37 linkage groups (LGs) was based on a preset threshold recombination rate (RR) of 0.27. Initial linkage groups could be further merged into 13 linkage groups based on information of the nearest cluster suggested by MultiPoint where markers were reordered. Map distances were calculated using the Kosambi mapping function, which assumes positive interference between crossovers. Linkage groups were drawn with the MAPCHART 2.2 program (Voorrips, 2002).

Results

Construction of the linkage map

Seventy-six polymorphic bands were yielded from 21 EcoRI/MseI selective primers with an average of 3.6 bands per primer pair. The number of polymorphic bands coming from H7996 was 40, whereas the remaining 36 bands came from WVa700. Eighty-nine SSR loci (Fulton et al., 2002; Smulders et al., 1997) revealed twenty-five polymorphic loci in the present mapping population. A total of 421 markers (313 DARts, 76 AFLPs, 25 SSRs, 1 SNP, and 6 RFLPs) were mapped into 37 linkage groups at a recombination rate (RR) of 0.27, each with 1-53 loci. Final mapping was performed by combining 2 or more linkage groups. Fifty-nine non-informative loci (14%) were excluded from mapping for the following reasons: (i) they did not meet the threshold of the selected

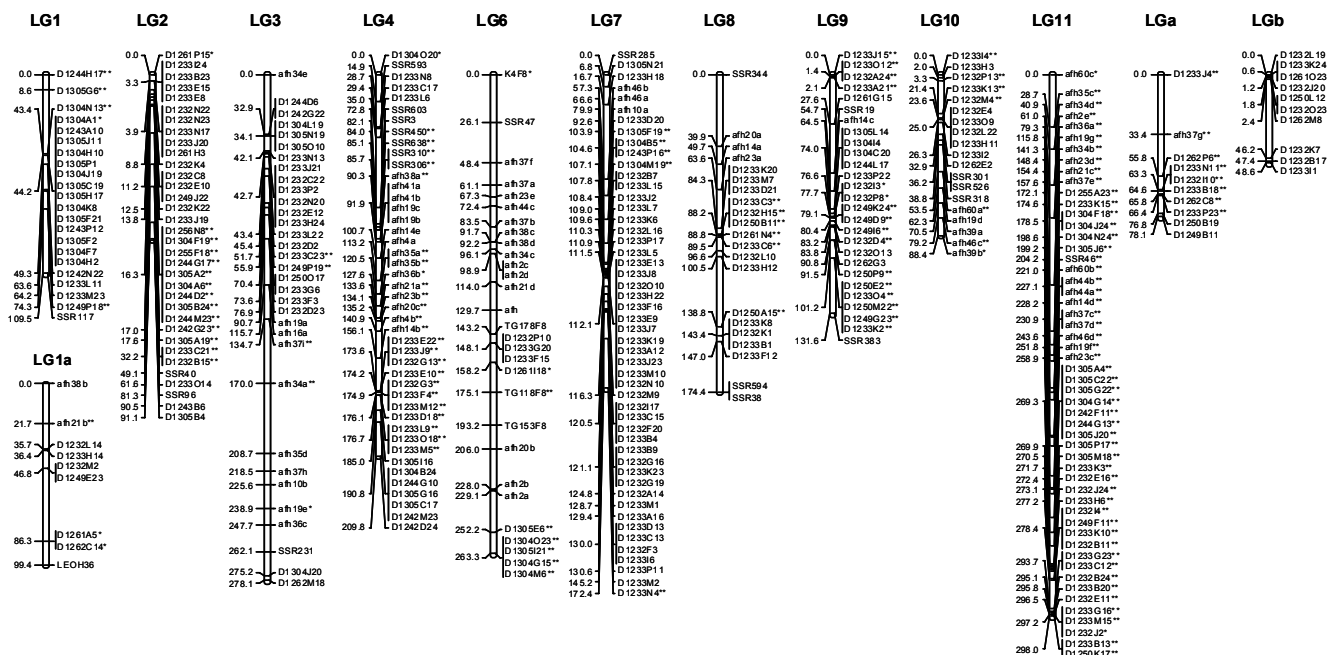


Fig. 1. Genetic linkage map of 362 loci in 188 F<sub>9</sub> recombinant inbred lines derived from a cross between *S. lycopersicum* H7996 and *S. pimpinellifolium* WVa700. Map distances are shown in centimorgans (cM) on the left side of the linkage groups and were calculated using the Kosambi mapping function. Loci marked with \* and \*\* deviate significantly from 1:1 ratio at P < 0.01 and P < 0.001, respectively.

recombination rate from MultiPoint; (ii) a big gap would be generated when they merged with the selected linkage groups, and hence, the map length could be contributed negatively. Thus, the final genetic map (Fig. 1) consisted of 361 markers (260 DArTs, 74 AFLPs, 4 RFLPs, 1 SNP, and 22 SSRs) that fell into 13 linkage groups, giving a total length of the linkage map of 2042.7 cM. Based on the distribution of anchor markers, 11 linkage groups (LG1, LG1a, LG2, LG3, LG4, LG6, LG7, LG8, LG9, LG10 and LG11) were assigned to 10 tomato chromosomes (except chromosomes 5 and 12) where chromosome 1 was separated into two independent linkage groups. Because LGa and LGb contained no anchor marker, these linkage groups were placed independently.

There was variation in the number of markers, map length, and marker density on the linkage groups. The number of mapped loci ranged from 9 on LG1a to 53 on LG11. The largest chromosome mapped was for LG11 (298 cM); the shortest was for LGb (48.6 cM). The density of markers on the map ranged from 2.7 cM/marker on LG2 to 11 cM/marker on LG1a. Map distances between 2 consecutive markers varied from 0 to 43.8 cM, and 208 of the 237 intervals were less than 20 cM. Among the 29 interval with gaps larger than 20 cM, the largest gaps between markers were observed on LG7 (40.6 cM) and LGb (43.8 cM).

### Segregation distortion

On the whole, the RIL population was not skewed, with 53% for the alleles coming from H7996 and 47% of the alleles from WVa700. However,  $\chi^2$  segregation tests for each locus showed significant ( $P < 0.01$ ) segregation distortion

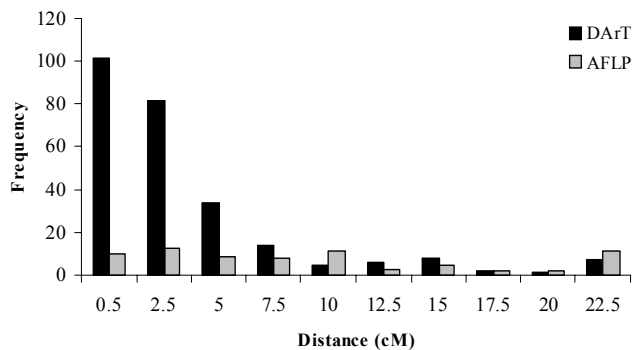
for 165 markers (39.2%). Of these, 154 distorted markers (111 DArTs, 2 RFLPs, 5 SSRs, and 36 AFLPs) were mapped. Markers exhibiting segregation distortion in favor of H7996 alleles were more frequent (54.3%) than those in favor of WVa700 alleles (45.7%). Distorted markers were distributed on all linkage groups except LGb (Fig. 1). The entire region of LG11 showed distorted segregation. The other four linkage groups showing major regions (about 50-80% of the entire length) with distorted segregation were LG2, LG4, LG 9, and LGa.

### Marker distribution

The distribution of markers between linkage groups was unequal (Table 1). The DArT markers were most frequent on chromosome 7 (LG7) and followed by chromosome 11 (LG11), 2 (LG2), 9 (LG9), 3 (LG3), 4 (LG4), and 1 (LG1), whereas AFLP markers were mainly distributed on chromosome 11 (LG11), 4 (LG4), and 6 (LG6). Exception of anchor markers, only DArT markers distributed on chromosomes 1 (LG1), 2 (LG2), and LGb. Clear clustering of DArT markers was observed in the genetic linkage map (Fig. 2). A total of 260 DArT markers were mapped and distributed into 13 LGs. Of these, 38.8% and 31.5% of the markers segregated into clusters within an interval between adjacent markers smaller than or equal 0.5 cM and 2.5cM, respectively. Clustering of DArT markers was observed in all linkage groups, while AFLP markers were clustered in 3 linkage groups (LG4, LG6 and LG11). The frequencies of clustering in DArTs markers at intervals less than or equal 0.5 cM and 2.5 cM were about 3 and 2 times higher than AFLPs, respectively.

**Table 1.** Distribution of genetic markers in the tomato linkage map.

Chromosome/ linkage group	cM	AFLP	DArT	RFLP	SNP	SSR	Number of markers	Marker density (cM/interval)
LG1	109.5		21			1	22	5.0
LG1a	99.4	2	6		1		9	11.0
LG2	91.1		32			2	34	2.7
LG3	278.1	10	22			1	33	8.4
LG4	209.8	15	22			7	44	4.8
LG6	263.3	15	9	4		1	29	9.1
LG7	172.4	3	46			1	50	3.4
LG8	174.4	3	15			3	21	8.3
LG9	131.6	1	24			2	27	4.9
LG10	88.4	5	11			3	19	4.7
LG11	298.0	19	33			1	53	5.6
LGa	78.1	1	9				10	7.8
LGb	48.6		10				10	4.9
Total	2042.7	74	260	4	1	22	361	5.7



**Fig. 2.** Distribution of Kosambi map distance between 2 consecutive DArT and AFLP loci over all linkage groups.

## Discussion

### Map comparison

In tomato, several linkage maps have been constructed using different mapping populations and marker types (Foolad 2007); however, many interesting traits are not segregating in those populations (e.g. bacterial wilt resistance) or many of the markers in those maps are not polymorphic in other populations. Therefore, a major goal of this paper was to apply a set of DArT markers and to study the distribution of their sequences in the tomato genome.

Although tomato has 12 chromosomes, the 362 markers split into 13 linkage groups in this study. We expect that the small linkage groups will be merged into larger linkage groups when more markers are assigned. Complete delineation of the linkage groups with tomato chromosome, however, would be hard to achieve with an interspecific *S. lycopersicum* × *S. pimpinellifolium* mapping populations because the degree of marker polymorphism is lower than in other interspecific mapping populations (i.e. *S. lycopersicum* × *S. pennellii*). In an attempt to coordinate our map with other tomato maps, we screened 84 anchor SSR markers; however, only 22 were placed in the framework map. Even if all SSR markers revealed straight-forward homologies both in marker order and distribution between 10 linkages groups (LGs 1, 2, 3, 4, 6, 7, 8, 9, 10 and 11) of the present map and 10 chromosomes of the reference map (Fulton et al., 2002), a few differences of the marker positions would still be observed. This difference seems acceptable considering that genetic maps provide only an indication of the relative marker positions and genetic distance. Moreover, inconsistency in map position could be explained by the presence of additional loci in the tomato genome. More comprehensive delineation of the linkage map would be helpful for quantitative trait locus analysis and the use of molecular markers in tomato breeding.

The total genome length of tomato was estimated to be 1276 cM (Tanksley et al., 1992). Thoquet et al. (1996) reported

a linkage map covering 600 cM using  $F_2$  population, whereas Balatero (2000) reported a map covering 378.1 cM using  $F_6$  RIL population derived from the same cross as in the present study. Thus, clearly, the level of marker saturation of the two mapping populations is very low to allow marker-assisted selection. There is a need to saturate the map and to identify markers that could be tightly linked to interesting traits. In this study a molecular linkage map was constructed with a total of 2042.7 cM in map length. The resultant map coverage is about 1.5 times of the latest tomato linkage map (Frary et al., 2005). This large coverage could be due to the large gaps within linkage groups. This is the first linkage map of tomato that utilizes DArT marker technology.

### Segregation distortion

Deviation from expected Mendelian segregation ratios has been reported previously in mapping populations (Lee et al., 2006; Lu et al., 2002; Pradhan et al., 2003; Törjék et al., 2006). Segregation distortion has been found in most plant pedigrees when large numbers of markers were mapped (Bradshaw and Stettler, 1994). The cause of skewed segregation could be physiological and genetic factors (Lu et al., 2002). In tomato, factors associated with the distorted segregation ratio are gametophytic selection, viability selection of segregating plants (Foolad, 1996) and spore function (Tanksley and Loaiza-Figueroa, 1985). Distorted segregation in tomato has been reported in many interspecific crosses and proposed to be greater in wilder crosses compared with crosses between closely related species, and generally higher in filial than in backcross population. Several studies confirmed this proposition; e.g. 8.3% distortions were observed in *S. lycopersicum* × *S. pimpinellifolium*  $BC_1$  population (Grandillo and Tanksley, 1996), 20% in a *S. lycopersicum* × *S. habrochaites*  $BC_1$  population (Bernacchi and Tanksley, 1997), and 75% in *S. lycopersicum* × *S. cheesmanii* RIL population (Paran et al., 1995). Thus, the observed segregation distortions in previous studies were higher than those in the present study (39.2%). This could be due to the population used derived from a cross between closely related species.

The level of distortion (about 48%) of AFLP markers in the present study is quite high compared with other crops using the same marker technique, silver staining (Becker et al., 1995; Maheswaran et al., 1997), but in accordance with results of Carlos (1998), who found 50% segregation distortion for AFLP markers in a  $F_7$  RIL population of tomato; whereas the observed segregation distortion (42%) of the DArT markers in the present study is much higher than those found in previous mapping studies (Mantovani et al., 2008; Semagn et al., 2006). Markers deviating from the expected segregation ratio are generally believed to be linked to genes that are

subject to direct selection; for example: a lethal allele in *Populus* spp. affecting embryo development was the cause of segregation distortion of markers (Bradshaw and Stettler, 1994); markers cosegregating with the Melampsora resistance gene also showed a significant deviation (Cervera et al., 2001). Therefore, distorted markers in this study were used in the mapping process to avoid missing parts of the linkage groups.

#### Distribution of DArT markers in the tomato genome

The genetic linkage map consisting of DArTs, AFLPs, SSRs, RFLPs and SNP demonstrates that the new tomato DArT markers behave in a Mendelian manner. The total number of DArT markers mapped was higher than other markers, therefore, a large number of DArT loci showed a tendency to cluster. The number of clustered DArT markers at intervals  $\leq 0.5$  cM was about 50% of markers mapped and 3-fold higher than AFLP markers (Fig. 2). Thus, the frequency of clustering of DArT markers in the tomato genome was similar to those in the wheat genome. The high proportion of clustering of DArT markers may be indicative of gene-rich regions or representative of redundant clones in the whole genome (Semagn et al., 2006).

The total length of the linkage map was 2042.7 cM, with an average DArT marker density of 1 per every 7.9 cM. Although the total map length is longer than previous genetic linkage maps (Foolad, 2007), DArT markers are distributed across the genome in a similar way to other markers. The density of DArT markers appeared to be highest in chromosomes 2 (LG2), which corresponds to density of markers on chromosome 2 in the reference map (Fulton et al., 2002). Beside SSR markers, only DArT markers contributed to chromosomes 1 (LG1) and 2 (LG2). This indicates the co-linearity between DArT markers in the present map and other markers in the reference map (Fulton et al., 2002) is conserved. The present map provides insights regarding the distribution of DArT markers in comparison with the published SSR markers for linkage mapping.

#### Usefulness of DArT as a marker system

One of the criteria for genetic markers that are to be used for fingerprinting and marker-assisted selection is a high level of polymorphism. Clearly, DArT meets this requirement, with a single DArT assay simultaneously typing hundreds of thousands of SNPs and InDels polymorphism spread across the genome. DArT is a new technique and most commonly used in wheat and barley for construction of linkage maps (Semagn et al., 2005; Wenzl et al., 2006).

The efficiency of molecular markers for genetic mapping depends on their ability to detect polymorphism. A low number

of polymorphism was detected using SSR and AFLP markers in this study. This could be due to the fact that DNA polymorphism between *S. lycopersicum* and *S. pimpinellifolium* is usually lower than between *S. lycopersicum* and either *S. pennelli* or *S. habrochaites* (Miller and Tanksley, 1990), as already demonstrated by Thoquet et al. (1996), given that 25 SSRs and 76 AFLPs might be enough to frame the map but not enough to saturate the linkage map. The study resulted in discovery of 313 DArT markers that were polymorphic among the parents. The high number of DArT markers generated not only provides a precise estimate of genetic relationships among genotypes, but also their distribution over the genome offers real advantages for a range of molecular breeding and genomic applications. Using the H7996  $\times$  WVa700 recombinant inbred line population, 260 new DArT markers were mapped, improving coverage relative to the previous maps. The DNA sequences of DArT clones could be used to convert DArT markers to single-marker assay formats for applications in breeding programs. The number of loci targeted by marker-assisted selection will increase in tomato breeding programs.

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## 토마토 유전자연관지도 상의 DarT 마커 분포

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**초 록.** 토마토꽃마름병에 저항성인 *Solanum lycopersicum* H7996와 극도감수성인 *S. pimpinellifolium* WVa700 간의 교배를 통해 획득한 재조합순계통 F<sub>9</sub> 세대의 188개체를 이용하여 유전자연관지도를 작성하였다. 유전자지도는 DarT 260종, AFLP 74종, RFLP 4종, SNP 1종 및 SSR 22종 등 총 361종의 마커로 구성되었다. 작성된 유전자지도는 총 13개의 연관군(LG)에 2042.7cM을 포함하였으며 마커간의 평균지도거리는 5.7cM이고 이중 DarT마커는 평균 7.9cM당 1개가 분포하였다. SSR 마커의 분포를 기초로 작성된 11개 연관군들은 토마토 염색체의5번과 12번을 제외한 10개 염색체에 해당하였다. DarT 마커는 다른 마커들처럼 토마토 유전체 상에 고르게 분포하였으며, 인접 마커와의 상호분석(≤ 0.5cM) 결과 클러스터링 빈도가 13.5%인 AFLP 마커보다 3배 정도 높은 38.8%의 빈도로 최고치를 나타내었다. 본 연구를 통해 토마토에서 최초로 DarT 마커를 이용한 유전자연관지도를 작성하였다.

**추가 주요어 :** diversity arrays technology(DArT), Hawaii 7996, 마커분포, 재조합순계통