Identification of dwarfism loci Dw1 and Dw2 in clonal apple rootstocks using molecular markers

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Abstract. The paper presents the results of identification of quantitative trait loci (QTL) of Dw1 and Dw2 involved in the control of dwarf growth in clonal apple rootstocks using the molecular markers. In total, 14 forms of rootstocks were analyzed. The microsatellite markers Hi01c04, Hi04a08, CH03a09 (for the Dw1 locus), MDP0000365711, and MDP0000243703 (for the Dw2 locus) were used in the study. Analysis of the results did not reveal a relationship between the presence of a marker and the manifestation of a sign. Of the three markers of the Dw1 locus, only Hi01c04 amplifies a fragment of the expected size of 120 bp. It was found in 9 forms of rootstocks (PB-4, 57-491, 83-1-15, M9, G16, 2-12-10, 2-9-102, 4-6-5 and 70-20-20). The Hi01c04 marker was identified in both dwarf and medium-sized forms. Of the two markers of the Dw2 locus, the desired fragment is amplified in MDP0000365711. It is typical for almost all genotypes. The exception is 83-1-15, 2-12-10 and 70-20-20, which have a null allele. To assess the genetic diversity of the Dw1 and Dw2 loci, 6 microsatellite sequences Hi01c04, Hi04a08, CH03a09, CH02d08, MDP0000365711, and MDP0000243703 were used. In 14 studied samples, 29 allelic variants ranging in size from 102 bp. up to 170 bp were identified. The number of alleles per locus varied from 1 (for the MDP0000365711 locus) to 7 (for the Hi04a08 locus). No rare alleles were identified. All alleles were observed more than three times. Based on the analysis of SSR spectra, a dendrogram reflecting the similarity of the genotypes under study was built.

1. Introduction
Although dwarf clonal rootstocks are widely used in modern horticulture, the basic mechanisms by which traits such as vigor and early flowering are controlled have not been identified. The influence of the dwarf rootstock on the apple graft is unclear. After inoculation, changes occur at the cellular level and at the level of expression of hundreds of genes [1-4]. Dwarfism manifests itself as a faster physiological aging associated with a reduction in the length of the first annual shoot of the lower branches and a high level of flowering of this shoot [5-7].

Hormones have the greatest influence on the plant architecture. It was established that the more auxin is produced, the more vigorous the growth [8-10]. The dwarf growth of the most common M9 rootstock is associated with a low level of expression of the MdPIN genes, which encode auxin carrier proteins, which limits the basipetal transport of nutrients to the roots. The low content of
zeatin in the rootstock roots caused by the low expression of the MdIPT gene encoding isopentenyltransferase weakens the vegetative growth of the scion [11, 12].

Studies were carried out to map the dwarf character for accelerated selection of new genotypes. In the hybrid M9 × Robusta 5 population, the quantitative trait locus (QTL) Dw1 was identified in the fifth linkage group. This locus includes 547 genes and has a size of 4.74-7.62 Mb. Another QTL Dw2 was identified in linkage group 11 and includes 1141 genes. The total size of QTL Dw2 is 1.88-8.98 Mb [13].

The phenotypic analysis shows that the combination of Dw1 and Dw2 has the strongest effect on the dwarf character in rootstocks. Moreover, Dw1 has a stronger effect than Dw2. Genetic markers were generated for both QTLs. They were tested on 41 rootstock samples. Most of the dwarf and semi-dwarf rootstock accessions carried marker alleles associated with Dw1 and Dw2 [13].

QTLs with a little effect have been found on other linkage groups (chromosomes 6, 9, 10, 12, and 13) [14].

A number of genes linked to QTL Dw1 and Dw2 were found in 64 genotypes of Malus plants. In the Dw1 locus, a domain controlling the boundaries of plant branching (MdLBD3) and a response factor to auxin (MdARF6) were identified. In the Dw2 locus, the MdG3OX3 gene was identified. It controls the production of gibberellin 3-beta dioxygenase. For these genes, functional microsatellite markers were created. They were tested on hybrid plants and recommended for the marker selection. However, in a number of rootstock forms and hybrids, no markers were identified, but phenotypically they had all the signs of dwarfism. This indicates the complex nature of the mechanism of dwarf growth in the apple tree and requires more in-depth studies [10].

Weak clonal stocks obtained by Michurin State Agrarian University are widely used in the industrial horticulture both in Russia and abroad. However, there are no works on the dwarfism at the molecular level. The aim of this study is to conduct a molecular genetic analysis of weak clonal apple tree rootstocks to identify the QTLs Dw1 and Dw2.

2. Materials and methods

The biological objects were forms of clonal rootstocks of an apple tree from the Michurin State Agrarian University’s collection. In total, 14 forms of rootstocks were analyzed.

The DNA was extracted using the a Quick-DNA Plant / Seed Miniprep Kit (Zymo Research, USA) according to the manufacturer's protocol.

To identify the loci of quantitative traits Dw1 and Dw2, microsatellite markers were used (Table 1) [13].

| Name     | Sequence                        | Size   |
|----------|---------------------------------|--------|
| Hi01c04 F | 6FAM-GCTGCCGTTGACGTTAGAG        | 114-124 |
| Hi01c04 R | GTTTGAGAAGTGCGTTTGGAGG          |        |
| Hi04a08 F | R6G-TTGAAGAGGTTCGTTGTTTG        | 228-249 |
| Hi04a08 R | GTTTCACCTCTGTGCTGGATAATTCG      |        |
| CH03a09 F | TAMRA-GCCAGGTTGACTCCTTCTC       | 142-162 |
| CH03a09 R | CTGCAGCTGCTGAAAACCTGG          |        |
| MDP0000365711F | 6FAM-TCTCCTCTCCTCGTTCTCA | 137-152 |
| MDP0000365711R | TTTTGCAGATCTCGTGATCGTA  |        |
| MDP0000243703F | TAMRA-AAACCCCATGCTCCATCCTCA  | 160-181 |
| MDP0000243703R | CAAATGGGATCCGCTGCTAT       |        |
Amplification was performed in a SimpliAmp device manufactured by Applied Biosystems (USA) using the Type-it Microsatellite PCR Kit (Qiagen) and primers manufactured by Syntol LLC, including those containing a fluorescent label (FAM, R6G, TAMRA). Fragment analysis was performed on the ABI Prism 3130xl automatic genetic analyzer (Thermo Fisher Scientific). The results were analyzed in Peak Scanner v1.0 (Thermo Fisher Scientific). The genetic differentiation was analyzed in PAST 3.10.

All the methods are reproducible and ensure the high reliability of the results.

3. Results and Discussion

The Dw1 and Dw2 loci were identified using the microsatellite markers. This study is preliminary and aimed at identifying the effectiveness of markers for the marker-mediated selection of low-growing clonal apple rootstocks.

To identify the QTL Dw1, the presence of three markers Hi01c04, Hi04a08, and CH03a09, and for Dw2, two markers MDP0000365711 and MDP0000243703 was analyzed. These markers were selected from 13 microsatellite sequences. They are associated with the signs of dwarfism and early flowering of apple trees. The association percentage is 68.6% [13].

Most researchers assume that the Dw1 locus exerts the greatest influence on the dwarf signs, while the Dw2 locus enhances its effect [13, 15]. In this case, an important factor is the allelic state of the marker. The homozygous form is dominant in the identification of the growth trait [9].

The expected fragment sizes in the presence of a dwarf growth locus are shown in Table 2.

| Locus | Marker        | Expected size |
|-------|---------------|---------------|
| Dw1   | Hi01c04      | 120           |
|       | Hi04a08      | 230           |
|       | CH03a09      | 158           |
| Dw2   | MDP0000365711| 153           |
|       | MDP0000243703| 167           |

14 genotypes of clonal apple rootstocks with a phenotypic manifestation of the restrained growth trait were selected. The results of the molecular analysis are presented in Table 3.

An analysis of the results showed a discrepancy between the data obtained and the expected result. Of the three markers of the Dw1 locus, only Hi01c04 amplifies a fragment of the expected size of 120 bp. It was found in 9 forms of rootstocks (PB-4, 57-491, 83-1-15, M9, G16, 2-12-10, 2-9-102, 4-6-5 and 70-20-20). At the same time, the Hi01c04 marker was identified in both dwarf and medium-sized forms. In form 2-12-10, the microsatellite is in a homozygous state. The sizes of the fragments obtained for other two markers do not coincide with the expected ones.

Of the two markers of the Dw2 locus, the desired fragment is amplified at MDP0000365711. It is typical for almost all genotypes. The exception is 83-1-15, 2-12-10 and 70-20-20, which have a null allele. All samples in which the marker was identified are homozygous. The fragment sizes were different from the expected one.

The absence of a clear relationship between the presence of a marker and the presence of a trait when using data from microsatellite sequences was observed in other studies. Thus, the presence of QTL Dw2 influenced the strength of apple plant growth in the studies conducted by Fazio [15]. In [13], a number of genotypes with a phenotypic manifestation of the dwarfism trait lacked loci markers.

The microsatellite genome sequences are most often used to analyze the genetic diversity and to assess the degree of similarity of samples [16-19]. An analysis of a small amount of SSR markers is sufficient to separate the samples [20].

To assess the genetic diversity for Dw1 and Dw2, 6 microsatellite sequences Hi01c04, Hi04a08, CH03a09, CH02d08, MDP0000365711, and MDP0000243703 were used. In the 14 studied
samples, 29 allelic variants were identified. Their size ranged from 102 bp. up to 170 bp. The number of alleles per locus varied from 1 (for MDP0000365711) to 7 (for Hi04a08). In two samples (83-1-15 and 2-12-10) out of 14, the null allele of the microsatellite MDP0000365711 was found. No rare alleles were identified. All alleles were observed more than three times.

The results of the analysis of SSR spectra were used to build a dendrogram that reflects the similarity of the genotypes under study (Figure 1).

**Table 3.** Results of the fragment analysis of apple tree clonal rootstocks

| Genotype name | Plant Growth Strength | QTL Dw1 | QTL Dw2 |
|---------------|-----------------------|---------|---------|
|               | Hi01c04   | Hi04a08 | CH03a09 | MDP0000365711 | MDP0000243703 |
| PB-4          | super dwarf       | 120     | 234     | 155         | 153          | 182          |
|               | 122         | 237     |         |             |              |
| PB            | dwarf        | 122     | 234     | 155         | 153          | 164          |
|               | 123         | 239     | 163     |             | 177          |
| 57-491        | dwarf        | 120     | 239     | 153         | 153          | 164          |
|               | 105         | 263     | 163     |             | 177          |
| MB            | dwarf        | 122     | 222     | 141         | 153          | 164          |
|               | 124         | 234     | 155     |              | 172          |
| 70-20-21      | dwarf        | 123     | 233     | 155         | 153          | 177          |
|               | 129         | 239     | 163     |              |              |
| 83-1-15       | dwarf        | 120     | 234     | 155         | 0            | 172          |
|               | 122         | 272     |         |              | 177          |
| M9            | dwarf        | 120     | 234     | 151         | 153          | 172          |
|               | 122         | 272     | 155     |              | 177          |
| G16           | dwarf        | 120     | 233     | 149         | 153          | 182          |
|               | 122         | 234     | 155     |              | 188          |
| 62-396        | dwarf / semi-dwarf | 122   | 222     | 141         | 153          | 164          |
|               | 124         | 234     | 155     |              | 172          |
| 54-118        | Semi-dwarf   | 124     | 222     | 141         | 153          | 164          |
|               | 239         | 163     |         |              | 172          |
| 2-12-10       | Semi-dwarf   | 120     | 233     | 145         | 0            | 170          |
|               | 123         | 263     | 155     |              | 172          |
| 2-9-102       | Semi-dwarf   | 120     | 222     | 141         | 153          | 170          |
|               | 124         | 263     | 155     |              | 174          |
| 4-6-5         | Semi-dwarf / medium-sized | 120 | 237     | 149         | 153          | 172          |
|               | 123         | 239     | 163     |              | 177          |
| 70-20-20      | medium-sized | 120     | 234     | 149         | 0            | 164          |
|               | 123         | 237     | 155     |              | 177          |
Figure 1. The dendrogram of genetic similarity of apple clonal rootstocks based on the analysis of six microsatellite loci

The dendrogram shows that there is no clear division of collection samples into clusters, which is confirmed by the low bootstrap index value. The genotypes have great genetic similarity or common origin, as evidenced by the lack of unique alleles. To obtain more complete results, it is necessary to expand the number of analyzed samples with more contrasting features. All studied rootstock forms were dwarf and semi-dwarf forms. It is necessary to use vigorous genotypes, as well as apple varieties such as spur. In addition, the system of markers used is specific for determining the loci of quantitative traits of the dwarf type of apple growth.

4. Conclusion
Only some of the markers of dwarf growth loci were identified in the studied samples. However, the phenotypic manifestation of the trait was present in plants. The analysis of genetic relationships based on the assessment of microsatellite genome sequences showed the lack of a clear division into clusters. Such results may be due to a small sample and its uniformity for the assessment of markers, or with a small percentage of association between the QTL and the trait. Since the studies are preliminary, further works should be aimed at expanding the sample, as well as finding more effective markers for the selection of dwarf genotypes of apple tree rootstocks.

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