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# "Detection of Drought Tolerant Genes in some Apple Rootstocks Seedlings in Syria"

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## ABSTRACT

This investigation was conducted to detect the presence of three genes associated with drought tolerance within seedling apple rootstocks derived from five apple genotypes, including Syrian apple cultivars. Polymerase chain reaction (PCR) results showed that the amplicon for *MdPepPro* (a cyclophilin) was found in all studied genotypes and their progenies except for one plant in genotype B. This was followed by the amplicon for an apple heat shock protein gene (*MdHSP18*), while amplicons of *MdDhn1* (Dehydrin1) were detected in fewer of the progenies of the studied genotypes. On the other hand, plants derived from a local apple cultivar Sukari 2 (S2) and genotype A were distinguished by a high number of plants amplifying all three genes, while the genotype B plants had the lowest number of individuals amplifying these genes, in particular *MdDhn1* and *MdHSP18*, compared to the other genotypes. Phenotypic data showed a significant decrease in leaf number in response to deficit irrigation treatment in genotype B. Consequently, this study provides a rapid method for screening apple genotypes for potential drought tolerance and provides initial results for selecting desired genotypes for a further study. Future studies documenting expression of these genes under deficit water will provide additional information towards identifying apple rootstocks with better drought tolerance.

**Keywords**: drought tolerant genes, MdPepPro, MdHSP18, MdDhn1, Apple rootstocks.

## INTRODUCTION

Agriculture is a major user of water resources in many regions of the world, therefore a better understanding of the effects of drought on plants is vital for improving management practices and breeding efforts in an agriculture undergoing rapid climate change (Chaves *et al*., 2003). In addition, success in breeding varieties better adapted to abiotic stresses depends upon the concerted efforts of workers in various research fields, including plant and cell physiology, molecular biology, genetics, and breeding. Of equal importance is the use of modern molecular biology tools for elucidating the control mechanisms of abiotic stress tolerance and for engineering stress tolerant crops based on the expression of specific stress-related genes (Amudha and Balasubramani 2011).

Mild drought in plants stimulates regulation of water loss and uptake, allowing maintenance of leaf relative water content within the limits where photosynthetic capacity shows no or little changes. But severe drought induces unfavorable changes in plants leading to inhibition of photosynthesis and growth, and the most severe drought stress results in desiccation from which a plant may or may not recover (Yordanov *et al*., 2003). Therefore, strategic improvement of water use efficiency

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(WUE) and drought tolerance in perennial crops, like fruit trees, could reduce water use without compromising yield or quality (Bassett *et al*., 2011). However, predicted climatic changes resulting in more frequent and severe drought episodes with prolonged periods of water deficit will lead to many demanding challenges for temperate zone fruit growers in the future. Selection of superior stress-resistant rootstocks for sustainable fruit growing is an important goal for improving the productivity of fruit trees under increasing abiotic stress conditions (Hrotkó, 2007). In apple plantations, which are currently multiplying on a worldwide scale, water supply becomes the limiting factor at unirrigated sites experiencing short term or long term deficits (Nemeskeri *et al*., 2010). This is not surprising as the fact that water relations are critical to the function of the apple tree, as water is the greatest component of the tree by mass, and even essential processes can be limited by inappropriate water status (Lakso, 2003). Hence, drought induced changes in morphological, physiological and biochemical traits are important for identifying mechanisms of drought resistance in susceptible and resistant varieties (Wisniewski *et al*., 2008; Jaleel *et al*., 2009; Nemeskeri *et al*., 2010).

Expressed sequence tag (EST) analysis of the response of apple (*Malus x domestica* 'Royal Gala') to water deficit showed that the genes that were upregulated in the water deficit libraries fell mainly into the functional categories of stress (heat shock protein, dehydrins) and photosynthesis (Wisniewski *et al*., 2008). Dehydrins are proteins induced by environmental stresses such as drought or low temperature, and many plants have several dehydrin genes (Artlip and Wisniewski 1997). A new peach (*Prunus persica* (L.) Batsch) dehydrin gene (*PpDhn2*) was identified which was strongly induced by water deficit (Wisniewski *et al.,* 2006).

Recently, some apple breeding programs have concentrated on the response of rootstocks to deficit water and on water use efficiency. However, most dwarfing rootstocks have shallow root systems and are usually irrigated in commercial orchards. Nevertheless, rootstock behavior under water deficiency is different from well-watered controls (Sakalauskaite *et al*., 2006). The drought tolerance of the commercial apple (*Malus domestica* Borkh.) rootstocks and some new selections from the rootstock breeding program at HRI-EastMalling, have been previously studied. As a result, two of the extremely dwarfing and one of dwarfing rootstocks, i.e. AR628-3, AR295-6 and AR486-1, produced considerably more root mass than evident with more vigorous rootstocks, particularly M26 under deficit water condition (Atkinson *et al*., 1999).

Currently, most apple-producing countries, such as Germany, Poland and America, are in the process of establishing their own rootstock breeding programs, depending heavily on local genetic resources and old cultivars well adapted to their local environments (Feuerhahn and Jesch 2000 ; Johnson *et al*., 2001; Czynczyk and Jakubowski 2007). Likewise, valuable gene sources are to be found among the old cultivars in genetic resource collections (Kiraly *et al*., 2012).

Seedling-raised rootstocks of several *Malus* species have proven to be of value in enhancing the tolerance of apple trees to drought conditions (Wertheim and Webster 2003). Such rootstocks are critically important in regions with substantial dependence on rainfed agriculture. For example, in Syria 66.6% of the total apple growing area is not irrigated (Annual Agricultural Statistical Abstract, 2011). Since most of its apple trees are grown on seedling rootstocks, the present study is aimed at detecting drought tolerant genes within a group of seedling genotypes, including some local apple cultivars, to screen genotypes in the apple rootstock breeding program.

**Materials and methods:**

*Plant Material:* The study was carried out by using one year old apple seedlings derived from five apple genotypes, including Syrian apple cultivars as shown in Table (1), in addition to the Royal Gala cultivar as a control (Wisniewski *et al*., 2008). All seeds of the apple genotypes were obtained from the germplasm collection maintained by the General Commission for Scientific Agriculture Researches in Sweida, Syria.

*Genomic DNA Extraction and PCR amplification:*

Twelve plants derived from each genotype (except for genotype B containing 11 plants), in addition to the mother plants and 'Royal Gala' were used. Total DNA was extracted from mature leaves of one year-old seedlings using a CTAB protocol according to Porebski *et al*. (1997). To remove RNA contamination, RNase A (10 mg/ml, Sigma,USA) was added to the DNA buffer and incubated at 37° C for 30 min. The extracted DNA was deproteinized by adding proteinase K (10 mg Sigma, USA) and incubating at 37°C for 2 h. Estimation of the DNA concentration in different samples was done by using biophotometer plus (Eppendorf, Germany).

A set of 3 specific primer pairs (Table 2) related to drought tolerance genes as described by Wisniewski et al. (2008) was used in the detection of tolerant genes among the five apple genotypes and their progenies. The amplification reaction was carried out in 10 µl reaction volume 0.2 ml containing: 5X PCR buffer, 0.2 mM dNTPs, 10 pmole primer, 1 unit Go-Taq DNA polymerase and 250 ng template DNA. PCR amplification was performed in PCR gradient (Eppendorf , Germany), programmed using touch down PCR to fulfill 35 cycles after an initial denaturation cycle for 4 min at 94° C. Each cycle consisted of a denaturation step at 94° C for 45 sec., an annealing temperatures according to each primer sets as shown in Table (2), for 45 sec. (-1° C for annealing temperature over the first 10 cycles), and an extension step at 72°C for 1 min, followed by extension cycle for 5 min at 72° C in the final cycle.

The PCR products were detected by electrophoresis on agarose gels (1% w/v in 1X TBE buffer: 75 mM Tris-base, 90 mM Boric acid and 0.2 mM Na2-EDTA ), then stained with ethidium bromide (0.3 ug/ml), and the gel was photographed using Gel documentation (VILBER LOURMOT Germany)

*Phenotypic Characters of Genotypes:*

The ability of one year old apple seedling rootstocks derived from the indicated genotypes to respond to deficit water was studied to estimate drought tolerance. Two levels of water treatment were attained by applying two irrigation regimes: 100% of field capacity (control), and 75% of field capacity (deficit irrigation treatment). After irrigation was stopped, shoot length and leaf number of central and lateral shoots were calculated for each genotype and its progenies in the two treatments **.**

*Statistical analysis:*

Chi square test was used for gene detection which calculated by the formula:

$$X^2 = \sum \frac{(\text{Observed value} - \text{Expected value})^2}{\text{Expected value}}$$

Degree of freedom (df) = n-1 where n the number of classes.

Relative Shoot Length (RSL) was calculated by dividing the average shoot length of deficit water-treated trees by the shoot length of control trees. Relative Leaf Number Differences (RLND) was calculated by dividing the average number of leaves from trees under deficit water by the average number of leaves from control trees.

**Results and Discussion:**

The results showed the ability of the three primer sets to detect the genes associated with drought tolerance within studied genotypes (figure 1). The MdPepPro gene

encodes a peptidyl-prolyl-*cis/trans* isomerase (also known as cyclophilin). Peptidyl-prolyl-*cis/trans* isomerases are ubiquitous proteins associated with stabilization of the *cis-trans* transition state during protein folding at XaaPro bonds (Brandts *et al*., 1975). Increased mRNA levels and protein activity have been observed in response to drought and other stresses in several plants (Marivet *et al*., 1992; Meza-Zepeda *et al*., 1998; Wisniewski *et al*., 2008). Significant increases in peptidyl-prolyl-*cis/trans* isomerases in response to water stress have also been associated with a drought tolerant sorghum cultivar (Sharma and Singh 2003). MdPepPro was Significantly presented in all genotypes and their progenies, including the control ('Royal Gala'), except for a single individual plant of genotypes B and H (Table 3). This observation suggests that the presence or absence scoring for MdPepPro is not associated with drought resistance or susceptibility in any of the genotypes tested.

The low molecular weight heat shock gene, 18.1, (*HSP18.1*) encodes a heat shock protein which plays an important role in protecting cellular biochemical processes during abiotic stress by preventing protein aggregation and promoting protein refolding to reconstruct functional enzymes (Ritenour *et al*., 2001). Induction of both high and low molecular weight heat shock responsive genes has been observed to occur in response to other abiotic stresses, including drought (Gorantla *et al*., 2005; Bogeat-Triboulot *et al*., 2006). Although we have not seen any other reports of *HSP18.1* induction in response to drought, it has been reported that the *HSP18.1* promoter is a target of the heat stress-activated transcription factor, HsfA3, which itself can be activated by the drought-responsive DREB2 transcription factors (Schramm *et al*., 2008).

All studied genotypes and their progenies significantly revealed the gene *MdHSP18.1,* since all individuals (12/12) derived from the A and S2 genotypes amplified *MdHSP18.1*, while the amplicon was found in only 7 out of 11 plants of genotype B and 10 out of 12 plants of genotypes C and H (table 3). The size of the band detected was about 400 bp in agreement with the predicted size.

Several LEA-like (Late Embryogenesis Abundant) protein genes are induced in response to abiotic stresses. In Arabidopsis the family of LEA protein genes known as dehydrins consists of ten members with different regulatory responses. The synthesis of dehydrins is tightly associated with the onset of abiotic stresses, and may serve a protective function, especially during dehydrative events (Artlip and Wisniewski 1997). For example, in Arabidopsis *Xero2* (*Dhn1*) is predominantly up-regulated by low temperature (Chung and Parish 2008), whereas *Xero1* (*Dhn2*) is more responsive to drought (Kawaguchi and Bailey-Serres 2005; see also GDS1382 / 252137_at / XERO1 of the Gene Expression Ominibus Profiles at NCBI).

*MdDhn1* encoding a *Xero2*-like dehydrin, was amplified in all of the apple genotypes surveyed, as well as the 'Royal Gala' control. Most of the plants derived from the A and S2 genotypes (11 out of 12) amplified *MdDhn1*, while it was amplified in only half (6/11) of the plants of genotype B (table 3). Three quarters of the individual plants from genotypes C and H amplified the gene. The molecular weight of the detected band was about 400 bp and was the size predicted by sequence information and illustrated in Figure 2 of Wisniewski *et al*. (2008).

The previous three genes play an important role in defense of the cell during drought stress (Wisniewski *et al*., 2008). For example, LEA proteins can protect specific cellular structures or ameliorate the effects of drought stress because they are highly hydrophilic (Ingram and Bartels 1996).In addition, HSP's have been

reported to serve as molecular chaperones that participate in ATP-dependent protein unfolding or assembly/disassembly reactions and prevent protein denaturation during stress (Santacruz, 2006).

The S2 and A genotypes revealed the highest number of plants amplified three genes studied (Eleven plants for each genotype), and just one plant with band for two of the genes studied (Table 4). Plants from genotype B showed the lowest number of amplicons in comparison with the other genotypes (six plants), while the other plants amplified between 0-2 genes (Table 4). The plants of genotype C were divided into three groups: two plants amplifying one gene, one plant amplifying two genes, and nine plants amplifying three genes. Finally, genotype H showed nine plants amplifying three of the genes. Moreover, the number of genes significantly presented in the genotypes A, C , S2 and H (Table 4).

One of the most challenging aspects of identifying drought resistant plants is finding reliable phenotypic measurements of drought responses. Furthermore, linking molecular genetic data with phenotypic measurements to identify drought resistance has been more difficult than originally thought due in part to the enormous complexity of this trait. The genotypes in this study were examined for shoot and leaf traits known to be associated with drought response (Table 5). Fewer leaves and shorter branches are a common adaptation to water limitation, most likely reflecting decreasing growth and reduced transpiration through leaf shedding. A recent study (Rivero *et al*., 2007) confirmed the link between drought tolerance and delayed leaf senescence, suggesting that trees maintaining growth and canopy size during drought periods would be more likely to be drought resistant.

A decrease in leaf number in response to deficit irrigation treatment was significant in genotype B, particularly with regard to lateral shoots; this result is in agreement with Atkinson *et al*. (1999). Leaves on these shoots were likely expanding during the drought period and may have been more directly impacted by the treatment. No differences in leaf number were observed for any of the other genotypes in response to deficit water treatment. Likewise, a greater reduction in shoot length in the 75% irrigation treatment was seen with

genotype B (Table 5), although the response was not as dramatic as that of leaf number. Comparing the molecular genetic data with this phenotype suggests that MdDhn1 and MdHSP18 might work together synergistically to enhance the development of drought tolerance in apple. In depth study of these genes will be necessary to confirm this hypothesis.

**Conclusion**:

The present investigation reflected the variance in the ability of studied genotypes for drought tolerance, since the stress tolerance is controlled by a large number and variety of genes acting additively and/or synergistically. The genetic background required for functional expression of tolerance is also present in non-tolerant plants, a fact which argues for the existence of highly specific and specialized genes in some tolerant plants (Santacruz, 2006). Therefore, the genotypes and their progenies investigated in our study provide an important genetic base for apple breeding programs since they apparently possess genotypic differences among three genes generally shown to be responsible for drought tolerance in apple and other plants. These results revealed the need for further research related to expression of these and other drought-responsive genes under deficit water conditions to determine if there are additional links to drought tolerance which can be exploited to improve water use and lower production costs.

**Table 1. Studied genotypes, description and sources**

| Genotype | Description | Source |
|---|---|---|
| A | Open pollinated seedling genotype (unknown parents ) | Apple rootstock mother plants field in Sweida- Syria |
| B | Open pollinated seedling genotype (unknown parents) | Apple rootstock mother plants field in Sweida- Syria |
| C | Open pollinated seedling genotype (unknown parents) | Apple rootstock mother plants field in Sweida- Syria |
| 'Sukari 2' (S2)[a] | Local apple cultivar, vigorous tree, regular bearing with small fruits, it has good tolerance for abiotic and biotic stresses. Maturity date :July. grown in the south of Syria | Germplasm collection in the Agricultural Scientific Research Center in Sweida-Syria |
| H | Derived from the crosses between MM 106 X Skarji (SK)<br>MM 106[b] : semi-vigorous apple rootstock, derived from (Northern spy X M1). High yield efficiency, good anchorage; few suckers, some drought tolerance, resistant to woolly apple aphid.<br>'Skarji': Local apple cultivar, vigorous tree, regular bearing, it has good tolerance for spring frost. Maturity date: August .grown in the south of Syria | Crosses were made at the Agricultural Scientific research Center in Sweida, for apple rootstock breeding program. |

[a] Muzher *et al*.,2007; El-Halabi *et al*., 2009
[b] Webster and Wertheim, 2003

**Table 2. List of primers, their sequences and the product size[a].**

| Gene [b] | Primer | Primer sequence (´5 →3) | Product size (bp) | PCR condition |
|---|---|---|---|---|
| MdPepPro (peptidyl-prolyl-*cis/trans* isomerase) | Peptidylfor(219)<br>Peptidylrev(534) | ctccggcaagcccctccactacaa<br>ccccaccttctcgatgttcttcac | ~315 | TD 62-52° C |
| MdDhn1 (Dehydrin1) | Xero2for(2361)<br>Xero2rev(2740) | ctggtggttggacgtagggatgac<br>cactcgcgcacgtaagaaagaaat | ~400 | TD 62-52° C |
| MdHSP18 | 18.1kDaHSP(136)<br>18.1kDaHSP(560) | cccggcaaaaacccagcatcta<br>cagttcagcaccccgttctccatc | ~434 | TD 55-45°C |

[a] Product size was predicted from sequence information (Wisniewski et al. 2008).
[b] Assembled sequences for the genes used in this study can be found at NCBI under BioProject PRJNA210935.

**Table 3. Number of individuals with bands for each primer.**

| Primer ID[a] | Genotype A | Genotype B | Genotype C | Local Cultivar (S2) | M.M106 X SK (H) | $\chi^2$ | Probability 5% |
|---|---|---|---|---|---|---|---|
| MdPepPro | 12/12 | 10/11 | 12/12 | 12/12 | 11/12 | 52.26 | 3.84 |
| MdHSP18.1 | 12/12 | 7/11 | 10/12 | 12/12 | 10/12 | 32.26 | 3.84 |
| MdDhn1 | 11/12 | 6/11 | 9/12 | 11/12 | 9/12 | 19.26 | 3.84 |

[a] Primers were designed against sequences obtained from 'Royal Gala' (Wisniewski *et al*. 2008). MdPepPro: apple peptidylprolyl*cis-trans* isomerase; MdERC: apple ethylene-responsive trans-criptional co-activator; MdDhn1: apple dehydrin probable homolog of Arabidopsis Xero2; MdHSP18: apple 18.1 kDa heat shock protein gene.

**Table 4. Number of individuals with studied genes for each genotype.**

| No. of genes studied | Genotype A | Genotype B | Genotype C | Local Cultivar (S2) | M.M106 X SK (H) |
|---|---|---|---|---|---|
| | Individual Plants oferNumb | | | | |
| **3** | 11 | 6 | 9 | 11 | 9 |
| **2** | 1 | 1 | 1 | 1 | 1 |
| **1** | - | 3 | 2 | - | 1 |
| **0** | - | 1 | - | - | 1 |
| $\chi^2$ | 28.67 | 6.09 | 16.67 | 28.67 | 16 |
| **Probability 0.05** | 7.82 | 7.82 | 7.82 | 7.82 | 7.82 |

**Table 5. Effect of deficit water treatment on shoot length and leaf number.**

| Genotype | Relative Shoot Length (cm)[a] | | Relative Leaf Number Difference[b] | |
|---|---|---|---|---|
| | Central Shoot | Lateral Shoot | Central Shoot | Lateral Shoot |
| A | 0.92 | 0.82 | 0.99 | 0.91 |
| B | 0.81 | 0.64 | 0.89 | 0.71 |
| C | 0.97 | 0.82 | 0.95 | 0.90 |
| S2 | 1.00 | 0.77 | 1.05 | 0.97 |
| H | 1.04 | 0.77 | 0.96 | 0.94 |

[a] RSL was calculated by dividing the average shoot length of deficit water-treated trees by the shoot length of control trees.

[b] RLND was calculated by dividing the average number of leaves from trees under deficit water by the average number of leaves from control trees.

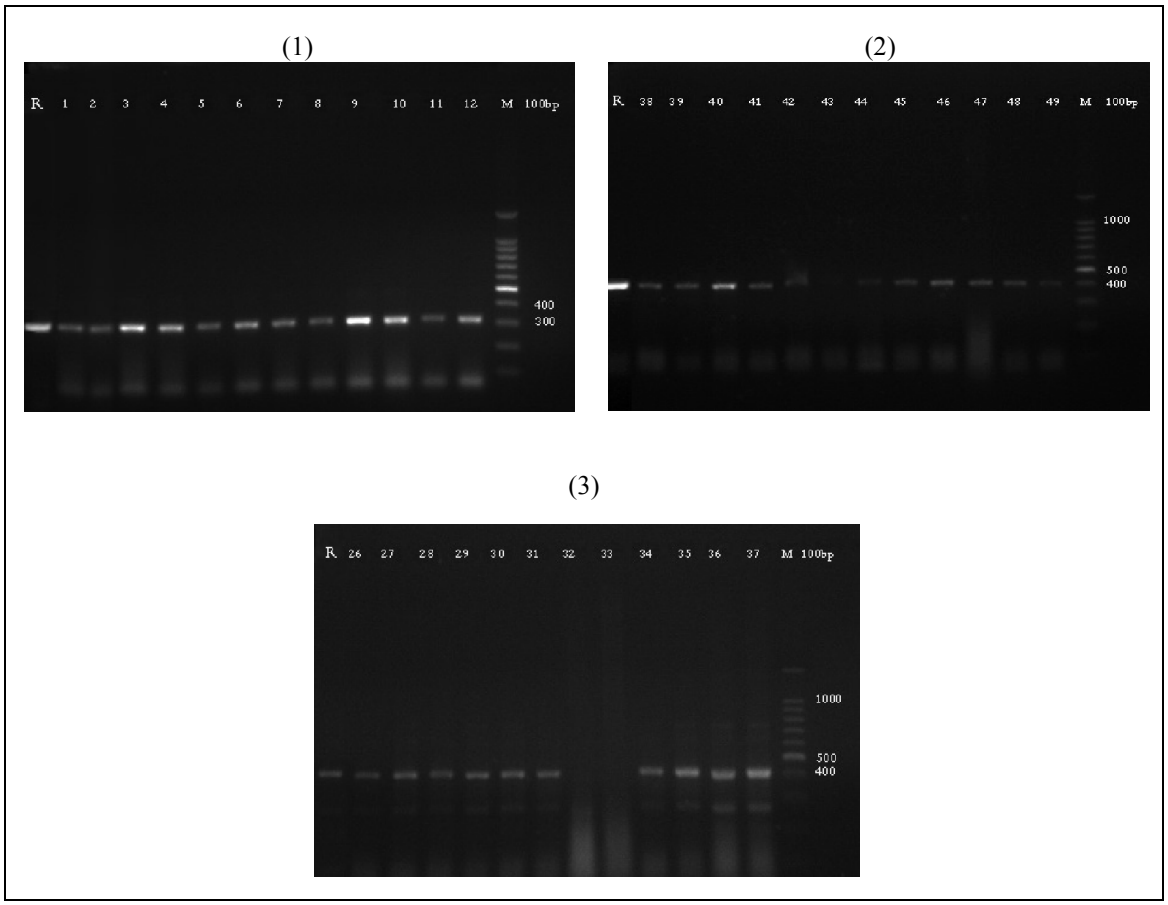


**Figure 1. PCR analysis of genomic DNA from different apple genotypes.**
(1) profile of genotype A with primer Peptidyl, (2) genotype S2 with primer Xero, (3) genotype C with primer 18.1kDaHSP, R: Royal Gala (control), lane numbers represent the plants in each genotype, M: Marker 100 bp ladder.

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# الكشف عن مورثات التحمل للجفاف في بعض أصول التفاح البذرية في سورية

***علا توفيق الحلبي [1] وبيان محمد مزهر [1] و كارول باسيت [2]***

## ملخــص

أجري هذا البحث للكشف عن وجود ثلاث مورثات مسؤولة عن التحمل للجفاف في أصول التفاح البذرية الناتجة عن خمسة طرز من ضمنها أصناف تفاح محلية. وقد أظهرت نتائج تفاعل البلمرة المتسلسل (PCR) أن المورثة *MdPepPro* (a cyclophilin) وجدت في كافة الطرز المدروسة والنباتات الناتجة عنها، عدا نبات واحد في الطراز B، تلتها المورثة(*MdHSP18*) heat shock protein، في حين وجدت المورثة(Dehydrin1) *MdDhn1* بشكل أقل في النباتات الناتجة عن الطرز المدروسة. ومن جهة أخرى فقد تميز صنف التفاح المحلي سكري2(S2) والطراز A بإعطاء أعلى عدد من النباتات التي تحمل المورثات الثلاثة المدروسة، في حين أعطى الطراز B أقل عدد من النباتات التي تحمل هذه المورثات، وبخاصة المورثتين *MdDhn1* و *MdHSP18* بالمقارنة مع باقي الطرز. وكان هناك نقص معنوي في عدد أوراق الطراز B كاستجابة لمعاملة إنقاص ماء الري. وبالنتيجة تزودنا هذه الدراسة بطريقة سريعة لغربلة طرز التفاح بناءً على قدرتها الكامنة لتحمل الجفاف، وكذلك بالمعلومات الأساسية لانتخاب الطرز المرغوبة لمتابعة الدراسة عليها، مما يتطلب دراسات مستقبلية تتناول التعبير المورثي لهذه المورثات تحت ظروف نقص الماء لتوثيق أصول التفاح الأكثر تحملاً للجفاف.

**الكلمات الدالة:** مورثات التحمل للجفاف، MdPepPro، MdHSP18، MdDhn1، أصول التفاح.

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