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Original research paper

Ocena zmian profilu ekspresji genów kandydujących w podkładkach jabłoni o odmiennym stopniu tolerancji mrozowej

Evaluation of changes in the expression profile of candidate genes in apple rootstockss with a different degree of frost tolerance

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Celem przeprowadzonych badań była identyfikacja genów sprzężonych z cechą mrozoodporności podkładek jabłoni. Ocenę zmian w poziomie ekspresji wyizolowanych genów przeprowadzono metodami RNAseq i qRT-PCR, dla podkładek zróżnicowanych po względem stopnia tolerancji mrozowej: P 66 (tolerancyjna) i M.9 (wrażliwa).
W wyniku przeprowadzonych odczytów sekwencji RNA (sekwencjonowanie *de novo* w systemie Illumina Solid) dla w/w podkładek zidentyfikowano około 167 milionów odczytów unikatowych sekwencji, z których do wstępnych badań weryfikacyjnych wytypowano 15 o zróżnicowanym profilu ekspresji. Sekwencje poddano adnotacji funkcjonalnej. Wytypowane geny kodują: białka strukturalne i integralne błon komórkowych i wakuoli komórkowych, czynników transkrypcyjnych, białek regulujących transport międzykomórkowy i wewnątrzkomórkowy, białek hydrolizujących wiązania C-O i C-N oraz białek wiążących makro- i mikroelementy. Celem weryfikacji typu regulacji sekwencji transkryptomu uzyskanych z sekwencjonowania nowej generacji (NGS), dla tych samych prób przeprowadzono ilościową analizę transkryptu genów (qRT-PCR). Spośród badanych genów, trzy reprezentowały identyczny typ regulacji w badanych układach eksperymentalnych RNA-seq i qRT-PCR. Wytypowane geny stanowią potencjalne sekwencje kandydujące do sporządzenia markerów funkcjonalnych, umożliwiających wczesną selekcję podkładek jabłoni tolerancyjnych na mróz.

Słowa kluczowe: adnotacja funkcjonalna genów, *Malus domestica* Borkh., sekwencjonowanie, profil ekspresji, ilościowa reakcja amplifikacji

The aim of presented study was to identify putative candidate genes associated with apple rootstock winter hardiness. The assessment of changes in expression profile of isolated differentially expressed genes, was performed using two subsequent experiments: RNAseq (based on New Generation Sequencing, NGS) and qRT-PCR (Real Time transcript amplification). In terms of traits of interests two apple rootstocks P 66 (frost tolerant) and M.9 (frost sensitive) were evaluated. As a result of the RNA sequence readings (*de novo* sequencing, Illumina Solid system), approximately 167 million reads of unique sequences were identified. Finally, fifteen functionally annotated expressed tags, representing different expression profile, were chosen. Selected putative genes coding: structural and integral proteins of cell membranes and cellular vacuoles, transcription factors, proteins regulating intercellular and intracellular transport, C-O and C-N bonds hydrolyzes, and proteins binding macro- and microelements.

In order to verify the type of regulation of the transcriptome sequences obtained in NGS technology, qRT-PCR tests were carried out for the same samples layout. Three of studied sequences, represented identical type of regulation in both RNA-seq and qRT-PCR experiments. The selected genes seems to represent potential candidate sequences (functional molecular markers), enabling the early selection of frost-tolerant apple rootstocks.

Key words: expression profile, gene annotation, *Malus domestica* Borkh., new generation sequencing (NGS), quantitative transcript level (qRT-PCR)

Introduction

Winter damage to perennial plants grown in the temperate climate zone is an important problem in horticulture. Prolonged winter frosts, often without snow cover, rapid warming and subsequent spring temperature drop cause significant damage both to the above-ground plant organs and to the root systems of trees. Moreover, frost damage during flowering of plants leads to a significant reduction in yields and is more dangerous than the long-term effect of sub-zero temperatures during winter (Aygu, 2005).

One mechanism of plant adaptation to frost relies on the regulation of biochemical changes associated with transcriptomic reprogramming of cellular functions that make plants tolerant to the formation of ice crystals in tissues (Wisniewski et al. 2003, Kalberer et al. 2006).

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The second mechanism involves the regulation of freezing avoidance during deep supercooling (-38°C/-42°C), changing the cell water into an ice emulsion, thus increasing the concentration of cryoprotective molecules such as sugars, salts, etc. In addition, the regulated migration of water molecules between cells and in extracellular space can also gradually inhibit the sudden formation of ice crystals in cellular structures (Wisniewski et al. 2003).

This complex and polygenic mechanism of cold hardiness in plants is not fully understood, and genetic studies has been so far conducted mainly in the USA, New Zealand, Norway and Japan. Consequently, several groups of genes were identified e.g. in plant genomes of peach and apple, the expression of which is modified by the influence of low temperatures. These include, for example, genes coding dehydration response elements binding protein (DREB) activated in vascular bundles (Feng et al. 2012, Takata et al. 2007, Zhao et al. 2012), C-repeat binding factor genes such as: CBF - the transcription factors (Wisniewski et al. 2007, 2011), MADS-box genes activated in the floral buds of perennial plants (Du et al. 2008), and cold regulated genes (COR) (Wisniewski et al. 2007).

Because the molecular mechanism determining traits associated with plant cold hardiness is insufficiently explained, our study employed techniques that enable an in-depth analysis of the sequence transcripts of apple rootstocks. New generation sequencing (NGS) and the analysis of changes in the level of gene transcripts using Real-Time quantitative PCR in plants representing different phenotypic traits allowed for the rapid identification of exons sequences linked to various functional traits of the genus *Malus* (Bai et al. 2014).

The identification of unique genes expressed at different levels, allowed us to determine the type of their regulation and select transcriptome fragments of the genome of apple rootstock, involved in the regulation of their cold hardiness. In addition, the precise description of 'Golden Delicious' genome sequence (Velasco et al. 2010), and partially also its transcriptome (Bai et al. 2014) allowed for the functional annotation of these unique sequences.

The aim of the study carried out at the National Research Institute of Horticulture was to assess apple rootstocks kept at the local collection for the expression profiling of putative candidate genes associated with cold hardiness. The regulation type was also preliminarily verified by comparing the results obtained in the RNAseq experiment and estimation of transcript level of the selected candidate genes by qRT- PCR array.

Material and Methods

The plant material used in the research comprised RNA templates isolated from two apple rootstocks with different tolerance to frost: frost-sensitive M.9 and frost-tolerant P 66. Selected rootstocks were frozen under controlled conditions in a special freezing chamber (BINDER GmbH). Samples were consecutively treated with three temperatures (-10°C, -12°C and -14°C) for 3 h, with a temperature drop of 2°C/h. Rootstocks not exposed to frosts stress were used as controls. After the treatment, the plants were transferred for acclimation in a cold storage room for samplings (0°C), and then were moved into field condition. RNA samples were isolated from treated and untreated (control) plants following a procedure described by Zeng and Yang (2000). The produced precipitate was dissolved in RNase-free water (DEPC). The quality, integrity and concentration of RNA were assessed by microchip electrophoresis (2100 Bioanalyzer system), and analysed with 2100 Expert software, (Agilent).

The RNA template isolated for the control rootstock M.9 and samples frozen at -10, -12, and -14°C, as well as for the control rootstocks of P 66, and samples frozen at -10, -12, and -14°C (a total of 8 experimental samples sequenced) were used for the transcriptome sequencing process, using the Solid Genome Analyzer, Illumina (Genomed, S.A). Next, the obtained differentially expressed genes (DEG) were mapped using BBmap software, and the number of reads for individual genes was counted using HTseq software. Final results were processed in the R environment [2] and standardized using the DEseq2 package. The correlation between the tested samples was estimated using Pearson's coefficient (from -1 to 1).

qRT-PCR assays were performed using RNA (1 µg) isolated from the same set of genotypes of apple rootstocks, which was then reverse transcribed into stable cDNA using the Affinity Script QPCR cDNA Synthesis Kit (Agilent). The reaction was carried out with the universal oligo-dT primer (0.1µg/µl) and reverse transcriptase (RT) at optimized temperatures: 25°C/5 min, 42°C/5 min – oligo-dT primer annealing, 55°C/15 min – RT, 95°C/5 min – enzyme inactivation (Biometra Basic thermocyclers). Stable cDNA was used as the template for the quantitative amplification reaction (qRT-PCR).

qRT-PCR assays for selected transcript sequences were carried out on a cDNA template in a 6000 RotorGene thermocycler (Corbett) with

Sybr Green I fluorescent dye and a universal Kapa SybrqPCR kit (Kapa Biosystems). In each experimental setup two specific primer pairs, complementary to the revealed DEG sequences were used for the multiplex assay together for the reference gene *PAL* and the gene of interests. The cDNA template was prepared in aliquots of known concentrations, allowing for the generation of a standard amplification curve of qPCR reactions. The analysed experimental setup - reference *PAL vs.* target gene consisted of: cDNA and oligonucleotides designed for the sequences of differentially expressed genes (DEG) revealed by RNA-seq experiment.

The thermal profile of the quantitative amplification reaction was as follows: 95°C/5 min (polymerase activation), 95°C for 15 s, 60°C for 20 s (oligonucleotide annealing), 72°C for 20 s (reading the fluorescence level).

The expression level for each of the tested samples was evaluated by analyzing the amplification curves (standard curve comparison ΔΔCt, RotorGene 6000 Series Software 1.7.), plotted based on the measured fluorescence of the SybrGreen dye in each reaction cycle. Statistical analysis was performed for biological replicates (at least two samples of plant material) and technical replicates (at least 2 replicates of reaction mixtures of the same composition). For each of the samples, standard deviation (SD) was calculated, while for the mean readouts the relative change (fold change) in the amount of amplicon for the analysed reaction layout was established using RotorGene 6000 Series Software 1.7..

The expression profiles for the selected genes obtained in the RNAseq and qRT-PCR experiments were then compared to verify the type of gene regulation (up or down).

Results

Database of annotated transcripts selected from NGS layout

Preliminary analyses of the correlation between the RNA samples from the frost-tolerant P 66 and frost-sensitive M.9 apple rootstocks revealed a total of 166,987,057 sequences (i.e. more than 500,000 different transcripts). The total number of sequence readings for each single sample is presented in Table 1. The Table 2 shows the type of regulation (comparison of the transcript level between samples), sequences and their functional annotation. The size of identified sequences was estimated in the range of 20–380 bp (base pairs).

Functional annotations were created for 4,000 upregulated genes and 4,000 downregulated genes in the analysed comparative setups of the RNAseq experiment. Gene ontology (GO) identifiers were assigned to individual sequences based on a .gff file with a description of the genes selected for the *Malus domestica* genome.

The comparison was made for object files containing: gene ID for *Malus domestica*, raw data on the number of reads mapped for the gene, the *fold change* estimated with the DEseq2 software for the first sample relative to the second sample (logarithmic scale), the (p) value of the statistical test performed using DEseq2 software, *contig* (coverage) to the reference genome of *Malus domestica,* which includes the gene of interest, coordinates of the start and end of the gene read in *contig,* and gene ontology terms (GO terms) associated with data for their putative function.

Tabela 1
Table 1

Liczba odczytanych sekwencji w badanych próbach.
Number of sequences read out in the tested samples.

	Genotyp/traktowanie podkładki Genotype/treatment of apple rootstock	liczba odczytanych sekwencji Number of sequence reads
Wrażliwa	M.9 Kontrola zbiorcza	16 768 301
	M.9 (-10°C)	32 732 590
	M.9 (-12°C)	19 494 823
	M.9 (-14°C)	16 260 592
Tolerancyjna	P 66 Kontrola zbiorcza	19 587 737
	P 66 (-10°C)	25 391 667
	P 66 (-12°C)	16 512 288
	P 66 (-14°C)	20 238 759
Razem		**166 987 057**

Tabela 2
Table 2

Typ regulacji w eksperymencie RNAseq, odczyty sekwencji oraz adnotacja funkcjonalna fragmentów DEG.

Type of regulation in the RNAseq experiment, sequence and functional annotation of DEG fragments.

Identyfikator genu/regulacja w badanym układzie porównawczym Gene ID/ type of gene regulation observedin comparable layout	Sekwencja Sequence	Adnotacja funkcjonalna Functional gene annotation
MDP0000883315 *Up-M.9K vs. M.9 -10*	ATTCACATTCCCATTCCCATTCCCACTCCCCATTCCCCGACGTTTCTCCTCCACAACGGC TTCAGCGACGAGGTCGATGTCTCTGACATCGAGATGATCACGATCCAGACCGTCACCGGA AAGGTTGTCATTGGGATGGATGTTGCTGCCT	Białko transmembramowe, transportujące jony potasu, komponent błony komorkowej. Transmembrane protein transporting potassium ions, a component of the cell membrane
MDP0000254705 *Down-M.9K vs. M.9 -10*	CATTCCCATTCCCATTCCCACTCCCATTCCCCGACTCGTTTCTCCTCCACAACGGCTTCA GCGACGAGGTCGATGTCTCTGACATCGAGATGATCACGATCCAGACCGTCACCTACACCA GCCTCAGGGACCTCCTCCCGCCGTCGCCGCAGCCATCGATCTCGC	Białko proliferacji komórek o właściwościach czynnika wzrostowego. Cell proliferation protein with the properties of growth factor.
MDP0000208730 *Up-M.9K vs. M.9-12*	CATTCCCATTCCCATTCCCACTCCCATTCCCCGACGTTTCTCCTCCACAACGGCTTCAGC GACGAGGTCGATGTCTCTGACATCGAGATGATCACGATCCAGACCGTCACCTACACCACG	Białko wiążące jony wapnia Ca^{2+} (kalmodulina), regulujące procesy sekrecji. Calcium ion binding protein Ca^{2+} (calmodulin), regulating secretion processes.
MDP0000310262 *DownM.9K vs. M.9-12*	CCACAACGGCTTCAGCGACGAGGTCGATGTCTCTGACATCGAGATGATCACGATCCAGAC CGTCACCTACACCAGCCTCACGGGACTCCTCCGGCGTCGCCGCAGCCATCGATCATGTCA CCGATTCACAATTCCAGCTGGCACACGACGAGACTTTCCGATAAAGAAACCC	Esteraza biorąca udział w hydrolizie estrów zawierających domenę SGNH. Esterase involved in the hydrolysis of esters containing SGNH domain.
MDP0000195260 *Down-M.9 K vs M.9 -14*	CCACAACGGCTTCAGCGACGAGGTCGATGTCTCTGACATCGAGATGATCACGATCCAGAC CGTCACCTACACCAGCCTCAGGGACCTCCTCCCGCGTCGCCGCAGCCATCGATCATGTCA CCGATTCACAATTCCAGC	Białko wiążące jony cynku, biorące udział w regulacji odporności na choroby. Zinc ion binding protein involved in the regulation of disease resistance.
MDP0000920400 *Up- M.9 K vs M.9 -14*	CGTTTCTCCTCCACAACGGCTTCAGCGACGAGGTCGATGTCTCTGACATCGAGATGATCA CGATCCAGACCGTCACCTACACCAGCCTCAGGGACCTCCTCC	Białko sekrecyjne, aktywowane w tkankach zranionych. Secreted protein activated in injured tissues.
MDP00007527209 *Down-P66K vs P 66 -10*	AGGAATGGAACTGGCTGTGGTGCATGCTACCAGGTGAGGTGCAAGATCCCTCAGCATTGC AGCAGTGATGGGGTAACTACGGTGGTGACAGACCACGGCGAAGGCGACAAGAACTGAACT TCATCTTCAGCCCAAGATCTTATGCAAAGTGGACAAACAC	Białko wiążące jony cynku. Zinc-ion binding protein.
MDP0000301184 *Up-P 66K vs P 66 -10*	ACATGGAGTTTTTCCTTAAGCACATAACTTGGTTTCTTTGTATCACGGTACTCTTGCCTG CTCTATGTACCTCTCAATACACATATGTATCCTCTAGAGCAACATATTATGGTAGCCCAG ATTGCTACGGGAACCCAACTGGAGCTTGTGGGTTTGGAGAATATGG	Białko integrujące błonę komórkową. Cellular membrane integrating protein

Tabela 2 cd.

Table 2 cont.

Typ regulacji w eksperymencie RNAseq, odczyty sekwencji oraz adnotacja funkcjonalna fragmentów DEG.

Type of regulation in the RNAseq experiment, sequence and functional annotation of DEG fragments.

Identyfikator genu/regulacja w badanym układzie porównawczym Gene ID/ type of gene regulation observedin comparable layout	Sekwencja Sequence	Adnotacja funkcjonalna Functional gene annotation
MDP0000165364 *Down-P66 vsP66 -12*	TTAAGCACATAACTTGGTTTCTTTGTATCACGGTACTCTTGCCTGCTCTATGTACCTCTC AATACACATATGTATCCTCTAGAGCAACATATTATGGTAGCCCAGATTGCTACGGGAACC CAACTGGAGCTTGTGGGTTTGGAGAATATGGCTC	Białko strukturalne błony komórkowej. Cell structural membrane protein.
MDP0000392485 *Up-P66 vsP66 -12*	ACAGCGATCGTTGGAGGAAGATTTCAAGATCATGGTTGCAATCAGCCTTTAATGTGGTTT GCGGTAGTCATTTTTTATGCTCGTCGATGTGTATGGTTGGCATGTATATTTCTGGCTATC CTATGTTCCACTCTTGGCCGTTCTGGTTCTAGGAGCCAAACT	Białko o aktywności liazy, katalizuje cięcie wiązań węglowych (C-C, C-O, C-N). Protein with lyaze activity, catalysis the cleavage of carbone bonds (C-C, C-O, C-N).
MDP0000948602 *Down-P 66 K cs P66 -14*	AGACTCATATTCTGACACAGGAACAACGGGAAATCAGTGGCCAGTTCTTGGAAACAACCC TATGGAATCACATTCCCGGGAAAAACCCAGATGGCCGGTTTCTTCCGATGGCCGGGTCCT CACCGATTTCCTTGCTAGGTTTATAGGAGTGAAGTCTCC	Czynnik transkrypcyjny, regulujący procesy zależne od DNA. Tramscription factor, regulating DNA dependent processes.
MDP0000575908 *Down-P 66 K cs P66 -14*	AGACCATCCTTCACTCAAAAACGACGCTGTCGGGGTGTCGTTAGGGTCGCCCGAGATTGA GTCAGCACCGCCGCTGCTTAAAGCCCTACTCGACTGCGCCAGGCTCACCGAGTCGGACAC CGACCGCGCCGTCAAATCACTGATTCGACTCAGGGATCGATCTCAGACCACGGAGATC	Białko strukturalne błony komórkowej. Cell structural membrane protein.
MDP0000127750 *Up-M.9 K vs M.9 -14*	TCACTCAAAAAACGACGCTGTCGGGGTGTCGTTAGGGTCGCCCGAGATTGAGTCAGCACC GCCGCTGCTTAAAGCCCTACTCGACTGCGCCAGGCTCACCGAGTCGGACACCGACGCGCC GTCAAATCACTGATTCGACTCAGGGAATCGATCTCAGACCACGGAGATCCAACCG	Czynnik transkrypcyjny. Transcription factor.
MDP0000228546 *Up-M.9 vs M. -14*	TATGCTGCTTTCGACACTACACCATGTTGTATGGCCCAAGAGTGGATGGTATGCAGTTCC CTTGACAAGCCTAATAGAAGGTTCACAGGTGAAGAAAGCGTATCAGAAAGCAAGGCTGTC GCCTCCAACCCA	Białko strukturalne błony komórkowej. Cell structural membrane protein.
MDP0000695032 *Down P 66 K vs. P 66 -14*	AATCCAACATTCGTATGCTGCTTTCGACACTACACCATGTTGTATGGCCCAAGAGTGGAT GGTATGCAGTTCCCTTGACAAGCCTAATAGAAGGTTCACAGGTGAAGAAAGCGTATCAGA AAGCAAGGCTGTGCCTCCACCCA	Biłko o aktywności kinazy serynowo-treoninowej, fosforylujące reszty aminokwasowe w odpowiedzi na czynniki zewnętrzne. Protein with serine-threonine kinase activity, phosphorilating aminoacids residues in responce to external factors.

Assessment of gene expression profiles revealed by NGS analysis

dscDNA fragments were amplified using a total of 15 pairs of oligonucleotides individually designed for this study (Tab. 3) according to the selected annotated sequences presented in Table 2.

Differences in expression profiles were found for 9 analysed genes. In case of M.9 rootstock, four identified sequences with the defined annotation: MD883315 (Figure 1b), MD7527209 (Figure 1e), MD228546 (-12°C) (Figure 1g) and MD392485 (-14°C) (Figure 1d) expressed the high level of transcripts, while for another three sequences MD127750 (-14°C) (Figure 1a), MD575908 (-10°C) (Figure 1i) and MD301184 (-10°C) (Figure 1h) the level of transcripts decreased.

For the P 66 rootstock, an increased transcript level was recorded for four DEG fragments: MD883315, MD392485, MD165364 (for samples treated at -10°C) (Figures 1b, d-f) and MD920400 (for samples treated at -12°C) (Figure 1c), while four analysed genes: MD127750, MD7527209, MD301184 (samples treated at -10°C) and MD165364 (samples treated at -12°C) were downregulated (Figures 1a, e, h, f).

Moreover, three of the identified gene sequences: MD920400, MD163564 and MD7527209 (Figures 1c, f, e.) were upregulated and one was downregulated (MD575908, Figure 1i) in the genomes of both analysed rootstocks. No changes in expression profiles were found for other identified genes.

Verification of the level of gene transcripts selected based on the RNA-seq experiment through the comparative analysis of the results obtained with qRT-PCR

Data from analyses of expression profiles for the selected sequences were used for the verification of putative markers, potentially regulating the frost tolerance of apple rootstocks. Among the transcriptome sequences for which the expression profile was assessed by the qRT-PCR test, three represented the same type of regulation as in the analysed setups of the RNA-seq experiment.

Decreased transcript levels (downregulation) in both conducted tests induced by exposure to low temperatures were observed for the following genes: MD7527209 (Figure 3) (rootstock P 66), MD575908 (Figure 5) (rootstock P 66), MD127750 (Figure 4) (rootstock M.9).

Tabela 3
Table 3

Sekwencje zaprojektowanych oligonukleotydów (qRT-PCR).

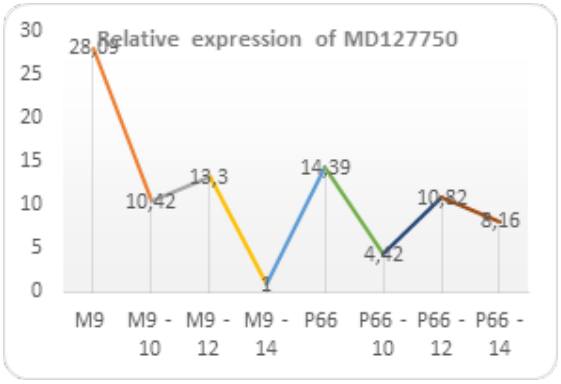
Sequences of designed oligonucleotides (qRT-PCR).

Identyfikator genu/ EST ID of the gene/ EST	SEKWENCJA 5' Sequence of Oligos 5'	SEKWENCJA 3' Sequence of Oligos 3'
PAL (gen ref.)	*tatcgacccaatttctgggttg*	*atgcagcatgtaaaccgtga*
MD883315	*tcacattcccattcccattcc*	*catccatcccaatgacaacct*
MD254705	*cattcccattcccattccca*	*aggaggtccctgaggct*
MD208730	*cccattcccactcccattc*	*tgtaggtgacggtctggat*
MD310262	*cacaacggcttcagcga*	*ctttatcggaaagtctcgtcgt*
MD195260	*cacaacggcttcagcga*	*gctggaattgtgaatcggtga*
MD920400	*ctcctccacaacggcttc*	*aggaggtccctgaggct*
MD7527209	*aggaatggaactggctgtg*	*gtgtttgtccactttgcat*
MD301184	*ttccttaagcacataacttggtttc*	*aaacccacaagctccagtt*
MD165364	*tggtttctttgtatcacggtact*	*catattctccaaacccacaagc*
MD392485	*gcgatcgttggaggaagatt*	*tttggctcctagaaccagaac*
MD948602	*agactcatattctgacacaggaac*	*gagacttcactcctataaacctagc*
MD575908	*ttagggtcgcccgagatt*	*tctccgtggtctgagatcg*
MD127750	*ttagggtcgcccgagatt*	*ttggatctccgtggtctga*
MD228546	*atgctgctttcgacactaca*	*tgggttggaggcgacag*
MD695032	*ccaacattcgtatgctgctttc*	*gtggaggcacagccttg*

Wykresy 1 a-i. Zmiany w poziomie ekspresji genów wytypowanych ma podstawie eksperymentu RNA-seq.

Figures 1 a-i. Changes in the level of expression of the selected genes are based on the RNA-seq experiment.

a



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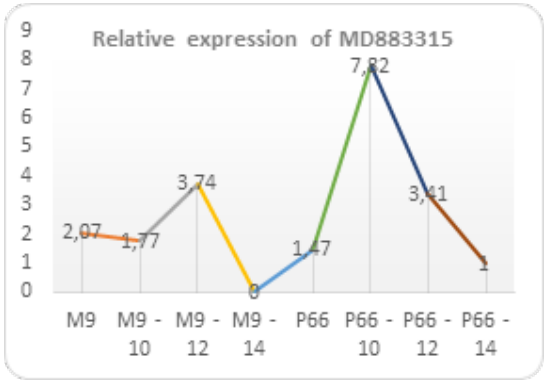

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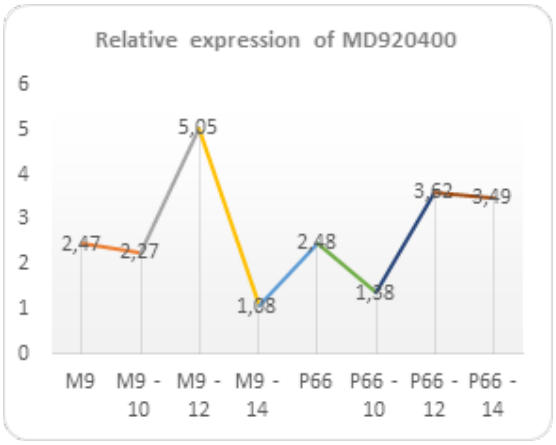

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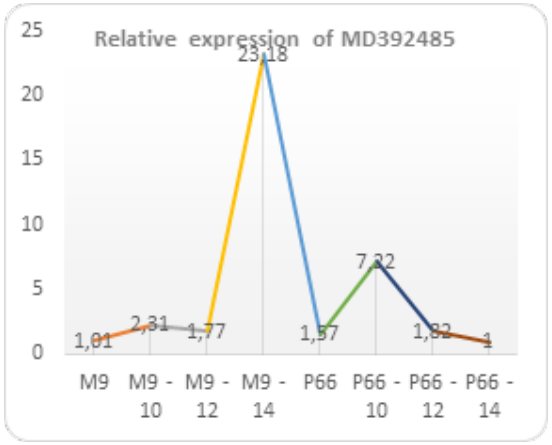

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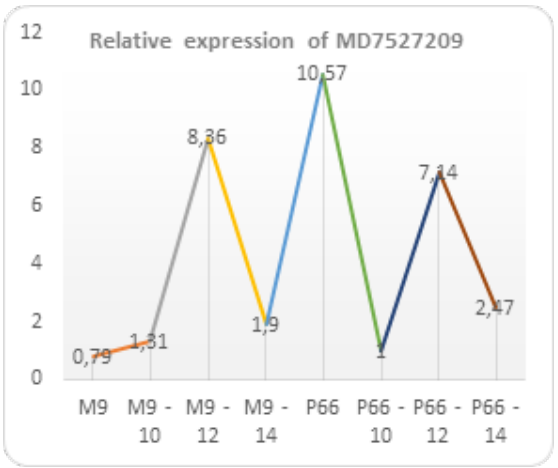

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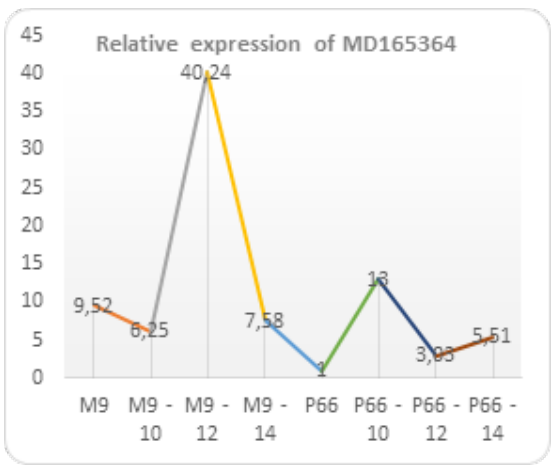
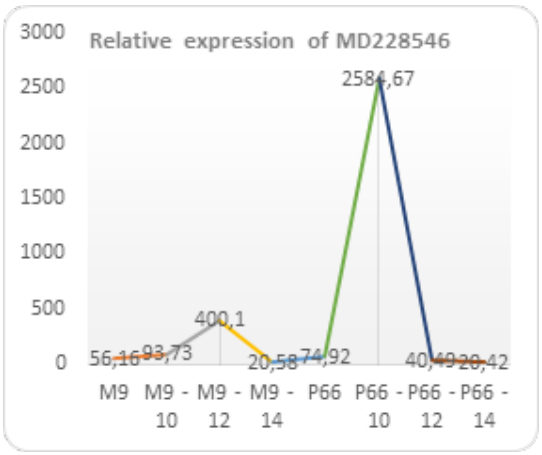
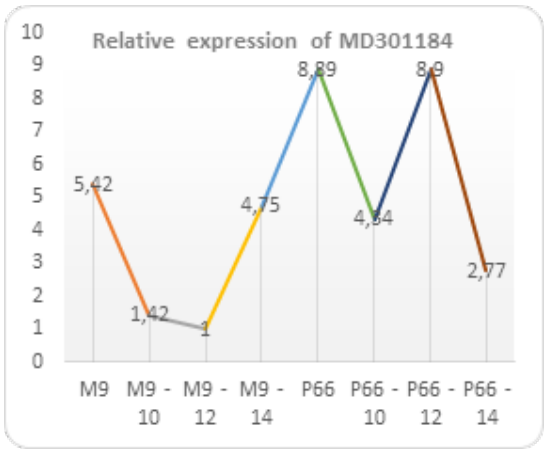


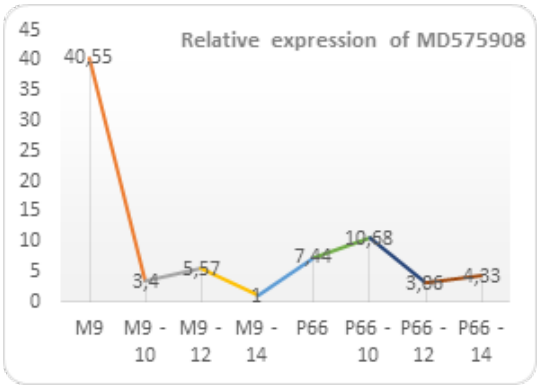
g

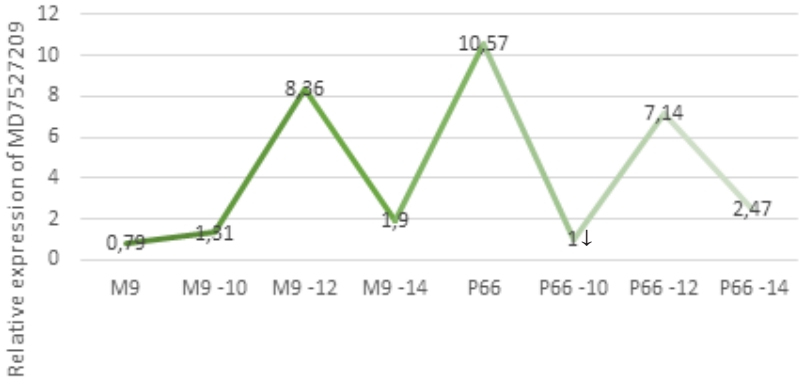


h



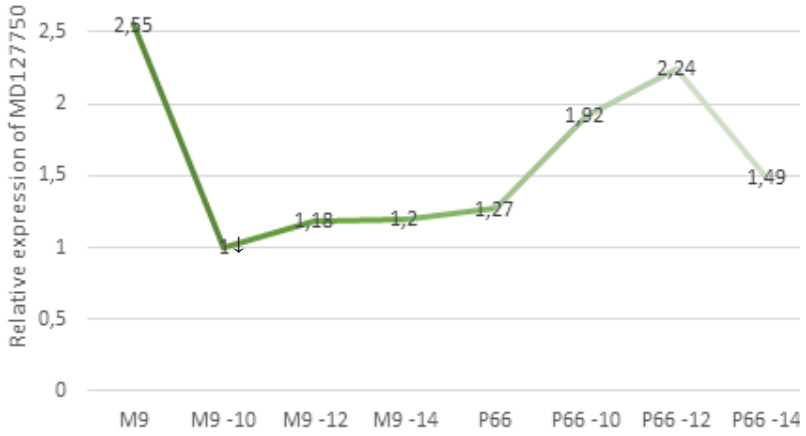
i





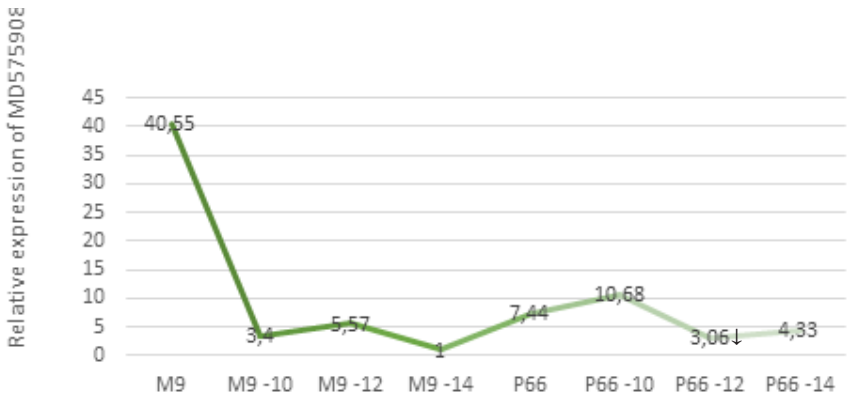
Wykres 2. Profil ekspresji genu MD7527209 uzyskany metodą qRT-PCR. Strzałkami wskazano typ regulacji *(down regulation)* genu w układzie eksperymentalnym prób porównanych po analizie transkryptomu podkładek jabłoni (P66 vs. P66 -10°C, tabela 2).

Figure 2. Expression profile of MD7527209 gene obtained by qRT-PCR method. The arrows indicate the type of down regulation of the gene in the experimental setup of samples compared after transcriptome analysis of apple rootstocks (P66 vs. P66 -10°C, Table 2).



Wykres 3. Profil ekspresji genu MD254705 uzyskany metodą qRT-PCR. Strzałkami wskazano typ regulacji (*down regulation*) genu w układzie eksperymentalnym prób porównanych po analizie transkryptomu podkładek jabłoni (M.9 vs. M.9 -10°C. tabela 2).

Figure 3. Expression profile of MD254705 gene obtained by qRT-PCR method. The arrows indicate the type of down regulation of the gene in an experimental set of samples compared after transcriptome analysis of apple rootstocks (M.9 vs. M.9 -10°C. Table 2).



Wykres 4. Profil ekspresji genu MD575908 uzyskany metodą qRT-PCR. Strzałkami wskazano typ regulacji (*down regulation*) genu w układzie eksperymentalnym prób porównanych po analizie transkryptomu podkładek jabłoni (P66 vs. P66 -12°C).

Figure 4. Expression profile of MD575908 gene obtained by qRT-PCR method. The arrows indicate the type of down regulation of the gene in an experimental set of samples compared after transcriptome analysis of apple rootstocks (P66 vs. P66 -12°C).

Discussion

The mechanism of cold hardiness in fruit crops is regulated by many genes and has not been fully explained, while the molecular assessment of changes in the plant genome caused by exposure to stressors is difficult (Luby, 1991). Researchers have pointed out that the level of gene expression depends on factors like for example: plant genotype, cultivation method and extrinsic/climatic conditions (Girardi et al., 2013). Moreover, Kalberer et al. (2006) observed that temperature can regulate the frost-tolerance processes in various ways in acclimated and non-acclimated plants.

Recently, only a few groups of genes regulating these processes have been identified in model plants, i.e. *Arabidipsis thaliana*, tomato (*Lycopersicon* Mill.), and in some fruit crops such as peach (*Prunus persica*), strawberry (*Fargaria x ananasa* Duch) and apple (*Malus domestica* Borkh.) (Medina et al. 1999, 2011; Thomashow 1998; Chinnusamy et al. 2006). These include genes coding dehydrins (Takata et al. 2007, Artlip et al. 1997), C-repeat binding factor genes (CBF genes) (Wisniewski et al. 2007, 2011, Gilmour et al. 1998), *MADS-box* and *DREB* genes (Du et al. 2008), and cold regulated genes (COR) (Wisniewski et al. 2007, Zhao et al. 2012, Feng et al. 2012). Recent studies have also confirmed the influence of ERF genes (ethylene

response factors: AP2/ERF involved in the ethylene synthesis pathway), in the regulation of frost tolerance in plants (Girardi et al. 2013).

Due to the very complex and poorly investigated mechanism of cold hardiness of fruit plants, the conducted study relied on very sensitive techniques for the analysis of the functional transcriptome sequences of apple rootstocks extremely responsive to stress induced by low temperatures.

In our study we analysed genetic material from two apple rootstocks: frost-sensitive M.9, widely used in Polish orchards, and P 66, characterised as highly tolerance to frost. Conducted field experiments also confirmed that the M.9 rootstock responds much more strongly to low-temperature stress than, for example, P 2, P 16, P 66 rootstocks or Antonówka (Quamm. 1990, Callesen, 1996). Such a diversity of material allowed for the correct verification of the obtained sequence reads for their transcripts level regulation.

An important aspect of this type of research is the availability of the reference genome sequence of the species under investigation. Recent studies precisely describes the size of the reference apple genome (diploid, 2n = 2x(17) = 34 at size of 742.3 Mb) sequenced for the ' Golden Delicious' variety (Velasco et al. 2010). This information was fundamental for the research comparing transcript sequences to the Malus reference genome, carried out by Girardi et al. 2013 and Bai 2014, which identified nearly 60,000 functional genes activated or inhibited in 'Royal Gala' and 'Golden Delicious' (Wisniewski et al. 2008, Girardi et al. 2013, Bai et al. 2014).

In addition, more than 800 million base pairs of the *Malus* genome (Velasco et al. 2010) have been identified and described by using a variety of available platforms, such as Illumina Solexa, 454 Roche and ABI-Solid P (Kumar and Blaxter, 2010). Nevertheless, *de novo* sequencing (without previous knowledge of the reference gene) also made it possible to read more than 71,000 new functional genes/transcripts with differential expression in the *Malus* genome (Imelfort and Edwards, 2009, Xu 2010, Edwards and Batley, 2010, Bai et al. 2014).

As reported by researchers, before sequencing the whole genomes, fragments with high molecular weight DNA (a DNA molecule of several Mb) are digested into small pieces (500 bp to 135 Kbp) and used to construct genomic libraries. Xu (2010) used the Illumina system for the *Malus* genome and obtained 39.2 million sequence reads for such libraries, which accounted for approximately 13 billion newly generated and sequenced nucleotides. The bioinformatics assemble analysis of 39.2 million reads led to recover 122,146 contigs with a cumulative size of 604 Mb sequence reads suitable for annotation (Xu, 2010). The conducted research revealed more than 500,000 diverse transcripts with an identified biological function, and allowed considerably extended the database of transcriptome sequences for the two analysed apple rootstocks. The NGS technique used in the presented study allowed for the selection of genes coding, among others, structural membrane proteins, integral membrane proteins and transcription factors. Previous studies have demonstrated that these groups of genes are strongly involved in the plant response to low temperatures and are the first to be activated when the plants are exposed to stressors (Orvar et al. 2000).

Despite the high sensitivity of the RNAseq method, offering the identification of a specific set of genes, individual measurements of expression levels carried out by means of NGS and qPCR are sometimes inconsistent (Everaert et al. 2017). A weak correlation (only a few %) between the expression level of genes identified with qRT-PCR and RNAseq was also observed in our study, however no detailed reports exist in this field. Verification of the selected sequences with diverse expression in apple rootstocks frost sensitive and tolerant to low temperatures, led to the selection of three (out of nine analysed) genes with known annotations: MD7527209, MD127750 and MD575908, for which a stable type of regulation was verified.

Regarding the fact that apple breeding programs should be directed on the production of frost-tolerant rootstocks, results of our study provide new information on the regulation of the plant cold hardiness mechanism at the molecular level, and thus can significantly contribute to genetic and breeding research in this area.

In addition, the created database of sequences will be useful for the development of functional molecular markers, enabling the early selection of frost-tolerant genotypes of apple rootstocks.

Conclusions

1. Differences in the expression profiles of the analysed genes were observed for the investigated apple rootstocks.
2. Three of selected sequences (identified in the RNA-seq experiment) seems to be a putative candidates for the development of functional markers, applied for the monitoring of apple cold hardiness.

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