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# Transcriptome analysis uncovered fruit wax biosynthesis regulatory genes in highbush blueberry (*Vaccinium corymbosum* L.)

## INTRODUCTION

The highbush blueberry (*Vaccinium corymbosum* L.) is common soft fruit species cultivated in many countries of world temperate regions.

This small fruit crop belongs to the Ericaceae family and includes blueberry, cranberry (*V. macrocarpon*), lingonberry (*V. vitis-idaea*), rhododendron and over 400 other species (Rowland et al. 2012). The most commercially produced are: lowbush (*V. angustifolium*), highbush (*V. corymbosum*) and rabbiteye (*V. ashei / V. virgatum*). Most of them originated from North America but they grow also in Asia, Europe, South America, Australia, Africa, New Zealand and China. According to official data from the Central Statistical Office (GUS, 2024), the annual production of highbush blueberries in Poland is estimated at over 63,000 metric tons (eighth rang place in the World).

In general, the main goals of the breeding program of *V. corymbosum* L. are to obtain new / innovative cultivars, well adapted to local climate changes and soil conditions, high yielding, with different periods of fruit ripening, resistant or less susceptible to basic fungal diseases and producing good quality, firm fruits with long shelf life. In addition the wild blueberry - bilberry (*V. myrtillus* L.) represent diploid genome (2n=2x=24 chromosomes) while the cultivated highbush blueberry (*V. corymbosum* L.) is mainly tetraploid (2n=4x=48) as well as hexaploid (2n=6x=72) (Li et al. 2016) which makes the genetic and molecular studies more complex.

Taking into consideration the fruit quality and durability, and for the purpose of blueberry transcriptome analysis, and in the first scope of our study we have selected different genotypes producing fruits with a low and high intensity wax coating (cuticle) on the surface.

Within the framework of the highbush blueberry genetic and breeding research conducted at the Department of Horticultural Crop Breeding of the National Institute of Horticultural Research (InHort) in Skierniewice, Poland, and based on the *V. corymbosum* transcriptome reads analysis a specific genes were uncovered, that may be considered as candidates for early seedling selection and marker assisted selection (MAS).

## MATERIAL and METHODS

❖ **GENERAL BREEDING PROGRAM CONDUCTED AT InHort, SKIERNIEWICE**

**Conventional Breeding**

**Classical, hybridization breeding methods, ~~no GMO~~**

1. Crossing of selected parental forms (according to DNA polyphormism, phenotypic evaluation in the collection and genetic studies
2. Evaluation of $F_1$ seedling progenies
3. Selection of breeding material (best individual are selected) and propagated
4. Further evaluation and selecting of best clones

**Crossing programs are mainly done under cover (high-plastic tunnel)**

**CROSSING PROGRAM**

**Traditional breeding –** crossing of parental forms, production of $F_1$ seedlings and selection of valuable individual and clones

Maternal form x

Paternal form

- 25-50 flower buds are emasculated and pollinated with pollen, labeling and bag isolation,
- Ripening of fruits, collecting and seed extraction,
- Sawing of seeds immediately or stratification of seeds, germination of seeds in the glasshouse conditions,
- Production of $F_1$ seedlings in glasshouse (tunnel) for 8-12 months,
- Planting of seedlings in breeding/selection fields at the Experimental Orchard at Dabrowice for further evaluation and selection the best materials.

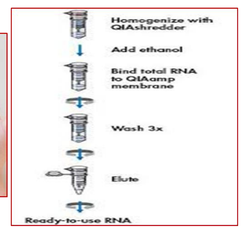
❖ **TRANSCRIPTOME SEQUENCING - WORKFLOW**

▪ **Plant material selection based on the breeding program**

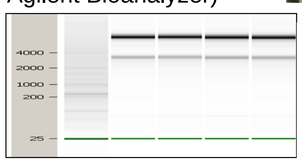
Peel samples from ripe fruits of 'Aurora' (low wax intensity fruits) and 'Bluegold' (intensively waxy fruits)


MinSeq – Illumina Genetic Ananlyzer

▪ **RNA isolation** (Qiagen column – kit applied protocol)



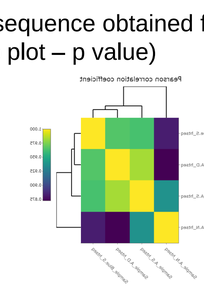

RNA concentration and integration (2100 Agilent Bioanalyzer)

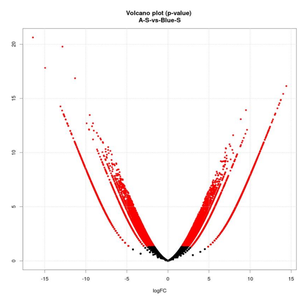




❖ **BIOINFORMATIC ANALYSIS**

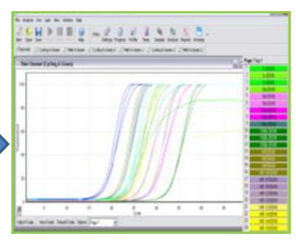
- Comparative bioinformatics analysis of libraries - FASTQ sequence obtained for 'Aurora' (A-S) and ‚Bluegold' (Blue-S) (heat map, volcano plot – p value)
- Cutadapt adapter removal
- Mapping of obtained Hisat2 reads.
- Mapping to reference genome of 'Draper', (https://www.vaccinium.org/analysis/49)
- Searching for significant sequences in Uniprot protein databases - BLASTX and BLASTP.
- Final processing of results – edgeR package






❖ **EXPRESSION PROFILING OF GENES INVOLVED IN PLANT WAX BIOSYNTHESIS**

- Plant material – fruit peel of 'Aurora', 'Bluegold', 'Bonifacy', ‘Jorma’, 'Liberty', 'Rubel', 'Toro' – different fruit wax coat intensity.
- Reverse transcription of 1µg of total RNA into stable cDNA - performed using the Affinity Script QPCR cDNA Synthesis Kit (Agilent).
- Amplification of RT-qPCR products, number of gene transcript calculation (Rotor-Gene 6000 Series Software v. 1.7 (Corbett)).



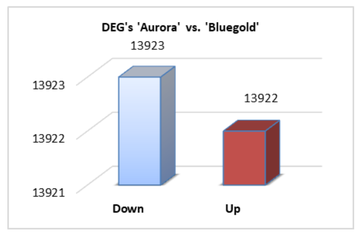
**Thermal profile:**
95 $^{o}$C for 5 min. – polymerase activation, 15 s for 95 $^{o}$C – sample denaturation, oligonucleotide annealing at 60 $^{o}$C for 20 s – 40 cycles.
**Number of transcript calculation**:
performed by measuring the concentration of the amplicons appearing in each RT-qPCR cycle, and using the Rotor-Gene 6000 Series Software v. 1.7 (Corbett).

- The relative expression: $^{-2\Delta\Delta}$Ct method.
- Data visualization: GraphPadPRISM 8.1, calculated as an average relative gene in comparison to the ‘Aurora’ cultivar (fruits with low-intensity wax layer) and normalized to the *GADPH* (glyceraldehyde-3-phosphate dehydrogenase) reference gene, assigned as the mean standard error ±SEM.

## RESULTS

❖ **GENERAL DATA**

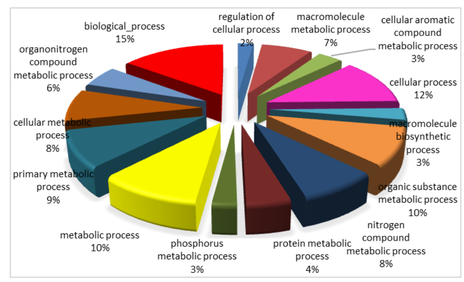
As a result of comparative analysis of ‚Aurora' vs. ‚Bluegold' fruit samples, in total 73 428 raw sequence reads were mapped on the reference genome.
13,923 showed overexpression and 13,922 down regulation in the low waxy fruit samples of the ‘Aurora’



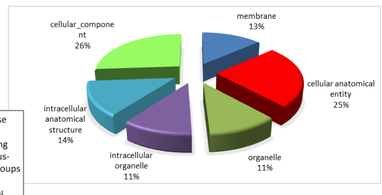

❖ **DEG'S ANNOTATION**

▪ **GO enrichment analysis**

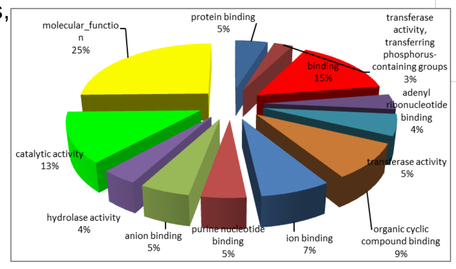
Three main functional groups.
**BP - biological processes**
(totaly 1,199 genes), -
35% - genes regulating general biological processes,
15% metabolic processes
(10%) involved in the production of organic substances.
**CC- cellular components**
(totaly 245 genes) –
51% proteins of basic cell components,
26% - cellular organelles
13% - cellular membrane proteins.
**MF - molecular factors**
(totally 626 genes) –
22% general molecular factors,
13% binding factors
11% as factors participating in catalytic reactions.




**BP**




**CC**

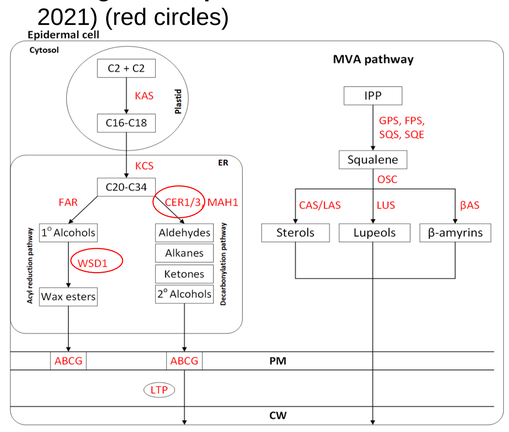


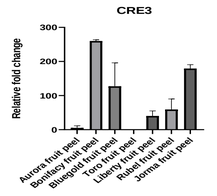

**MF**

Genes with recognized identifiers in KEGG (Kyoto Encyclopedia of Genes and Genomes) metabolic pathways www.genome.jp/kegg/pathway.html, were chosen for their expression profiling.

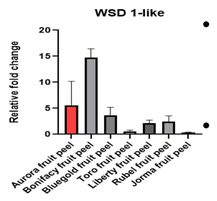
❖ **GENE EXPRESSION PROFILING – validation of blueberry genotypes selected in InHort breeding program**

- **Changes in expression of known two structural genes –** involved in wax biosynthesis pathway (Trivedi et al. 2021) (red circles)



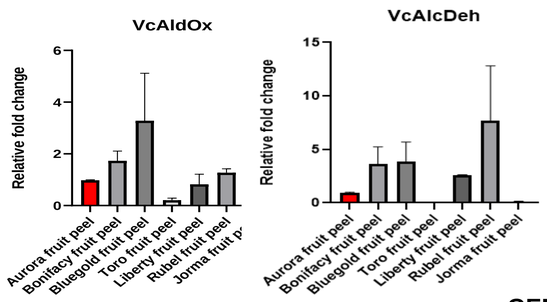

- Significant overexpression of ***CRE3*** (fatty acyl-CoA reductase) gene noted in fruit skin of – ‘Bonifacy’ and ‘Bluegold’ (fruits with higher wax intensity) as well as ‘Rubel‘ and ‘Jorma’ (medium wax intensity)




- Down regulation of ***WSD1-like*** gene (wax synthase) – in ‘Bluegold’, ‘Toro’,‘Liberty’,‘Rubel’ and ‘Jorma’ (fruits with waxy coating).
- ***WSD1-like*** activity in ‘Aurora’ and ‘Boniface’ was respectively 2-15 times higher, compared to varieties with more waxy fruits.

**REGULATION OF NEWLY SELECTED GENES**

Genes from BP group, annotated as: Alcohol dehydrogenase, **gene ID: VaccDscaff14-augustus-gene-165.25**) and Aldehyde oxidase; **gene ID: VaccDscaff19-augustus-gene-144.16**) and downregulated in low waxy fruits of *‚Aurora'.*




- Both selected genes, showed significantly higher number of transcripts in the fruit peel samples of 'Bonifacy', 'Bluegold' and 'Rubel' (thus in cultivars producing fruit with intensive wax coat).
- Selected genes are involved in metabolism of the molecules from the alcohols to wax esters as welll as aldehydes into long chain lipids.

**GENERAL SUMMARY**

In general, our results of expression profiling of the structural genes from wax biosynthesis pathway as well as newly selected ones from the *V. corymbosum* transcriptome analysis, confirm the their activity directly depends on the genotype studied and the level of waxiness intensity of highbush blueberry fruit.

## CONCLUSIONS

1. The highbush blueberry breeding program at the InHort in Skierniewice, Poland is already bringing the first effects in the form of selected valuable individuals and breeding clones.
2. The analysis of specific genes activity allows initial recognition of the mechanism of wax coat formation on the fruit surface of the blueberry cultivars from the breeding collection.
3. Selected genes seems to be potential functional molecular markers, and could be applied for marker assisted selection (MAS breeding proces) in monitoring waxy fruit trait of highbush blueberry.

## LITERATURE

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Rowland, L.J., Alkharouf, N., Darwish, O., Ogden, E.L., Polashock, J.J., Bassil, N.V., Main, D. (2012). Generation and analysis of blueberry transcriptome sequences from leaves, developing fruit, and flower buds from cold acclimation through declamation. BMC Plant Biol. 12(1): 46–64. DOI: 10.1186/1471-2229-12-46.
Trivedi, P., Nguyen, N., Klavins, L., Kviesis, J., Heinonen, E., Remes, J., Jokipii-Lukkari, S., Klavins, M., Karppinen, K., Jaakola, L., Häggman, H. 2021.Analysis of composition, morphology, and biosynthesis of cuticular wax in wild type bilberry (*Vaccinium myrtillus* L.) and its glossy mutant,Food Chemistry, Vol. 354, https://doi.org/10.1016/j.foodchem.2021.129517.

## ACKNOWLEDGEMENT

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