Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Faculty of Landscape Architecture, Horticulture and Crop Production Science

Genotyping ethylene production genes *Md-ACS1* and *Md-ACO1* for marker-assisted selection in apple

Genotypbestämmning av etenproduktionsgenerna *Md-ACS1* och *Md-ACO1* för markörbaserat urval i äppelförädling.

Johan Lundmark

Independent Project • 15 credits
Hortonomprogrammet
Alnarp 2019

Genotyping ethylene production genes *Md-ACS1* and *Md-ACO1* for marker-assisted selection in apple

Johan Lundmark

Supervisor: Larisa Gustavsson, SLU, Department of plant breeding

Co-supervisor: Firuz Odilbekov, SLU, Department of plant breeding

Examiner: Helena Persson Hovmalm, SLU, Department of plant breeding

Credits: 15 credits
Project level: G2E
Course title: Independent Project in biology
Course code: EX0855
Programme: Hortonomprogrammet

Place of publication: Alnarp
Year of publication: 2019
Cover picture: Johan Lundmark
Online publication: http://stud.epsilon.slu.se

Keywords: *Malus domestica*, Ripening, Fruit firmness, Shelf-life, Storage, Nordic, Baltic

SLU, Swedish University of Agricultural Sciences
Faculty of Landscape Architecture, Horticulture and Crop Production Science
Department of Biosystems and Technology

Acknowledgements

I would like to thank my supervisor Larisa Gustavsson for the opportunity to be a part of this project, and for all the help and support I received writing this thesis, without her input it would not have been the same.

I would also like to express my gratitude to my co-supervisor Firuz Odilbekov for being patient with me, and for teaching, guiding and assisting me through the lab work.

Big thanks to Helle Turesson for running the capillary electrophoresis and providing the final data.

This work is a part of the international project "NORDFRUIT". Financial support was received from the Nordic Ministries of Food and Agriculture through the Nordic collaboration on Public-Private Partnership for pre-breeding, PPP, administered by NordGen.

Abstract

Texture, firmness and storability are among the most important traits for fruit quality in apple. Consumers are increasingly demanding apples with a "crispy texture", and the market is offering a premium for a longer shelf life, which has as a result generated an increased interest from plant breeders to meet these demands. The traits are internally regulated by the fruit's inherent ethylene production, the suppression of which results in higher firmness retention. Thus far, the ethylene production genes *Md-ACS1* and *Md-ACO1* represent the best known candidates for enhancing firmness retention during storage. In this study, capillary electrophoresis was used to screen 255 cultivars from Nordic and Baltic germplasm collections for alleles at the *Md-ACS1* and *Md-ACO1* loci. This was done in order to enable DNA-marker based selections of these genes in breeding material suitable for cool climates. In addition to compiling genotype data, we performed verifications of previously screened cultivars, and additional evaluations of parentage were made in order to determine any pedigree errors. The analysis of the *Md-ACO1* locus showed false positives for uncertain reasons, and therefore the true allelic compositions could not be determined. Frequencies of the normal ethylene allele *Md-ACS1-1* was much higher in the Estonian (0.94), Finnish (0.95) and Latvian (0.90) collections, whereas the low ethylene allele *ACS1-2* was more common in the Lithuanian (0.22), Swedish (0.33) and Norwegian (0.35) collections. Of the previously screened cultivars, 59 showed the expected genotype, with the cultivar 'Eva-Lotta' constituting the only exception. In addition, this cultivar was the only genotype that showed any inconsistencies when comparing it to the presumed parents.

Sammanfattning

Textur, fasthet och lagringsförmåga är bland de mest avgörande egenskaperna för ett äpples fruktkvalitet. Konsumenter kräver alltmer "krispig konsistens" i sina äpplen, och marknaden erbjuder högre pris för längre hållbarhet. Detta har resulterat i ett ökat intresse från växtförädlare för att möta kraven som ställs. Kvalitetskraven påverkas av äpplets egna etenproduktion, som vid nedreglering ökar äpplets förmåga att bevara fasthet vid lagring. Hittills representerar etenproduktionsgenerna *Md-ACS1* och *Md-ACO1* de bästa kandidaterna för att förbättra lagringsförmågan. I den här studien undersöktes *Md-ACS1* och *Md-ACO1* alleler i 255 sorter från nordiska och baltiska samlingar, med hjälp av kapillärelektrofores. Detta gjordes för att möjliggöra DNA-markörbaserade selekteringar för dessa gener i sorter som är lämpliga för kalla klimat. Förutom att sammanställa genotypdata utförde vi verifieringar av tidigare undersökta sorter. Ytterligare utvärderingar av föräldraskap gjordes för att avgöra eventuellt felbestämda härkomster. Av osäkra skäl visade analysen falskt positiva resultat i *Md-ACO1* locuset och allelsammansättningen kunde därför inte bestämmas korrekt. Frekvenserna för allelen *Md-ACS1-1*, som är associerad med normal etenproduktion, var mycket högre i de estniska (0,94), finska (0,95) och lettiska (0,90) samlingarna, medan *Md-ACS1-2* allelen, associerad med låg etenproduktion, var vanligare i litauiska (0,22), svenska (0,33) och norska (0,35) samlingar. Av de tidigare undersökta sorterna upvisade 59 den förväntade genotypen. Sorten "Eva-Lotta" utgjorde det enda undantaget. Dessutom var "Eva-Lotta" den enda sort vars genotyp inte överensstämde med de kända föräldrarnas genotyper.

Table of Contents

1 Introduction

1.1 Apple

Apple (*Malus* x *domestica* Borkh.) is a pome fruit in the Rosaceae family that belongs to the sub-family of Maloideae (Velasco et al., 2010). It is the most economically important fruit crop in the temperate climate zone and was commercially grown in 97 countries 2017 (FAO, 2017). The origin of cultivated apple can be traced back to *Malus sieversii*, which is typically found in wild populations within central Asia. Although the exact ancestry is uncertain, our modern apple is an interspecific hybrid that has various amounts of DNA from crab apple (*Malus sylvestris*) and other members of the *Malus* genus (Velasco et al., 2010).

Nordic countries are considered as the northernmost area of distribution of commercial apple orchards, mainly due to the short and cold vegetative season, coupled with harsh winters. The cultivars that are best suited for these climates require specific attention to certain enabling traits, which are typically not found within the commercial cultivars of continental Europe. The combination of cold-hardiness, frost-hardiness during fluctuating weather and early fruit ripening has to exceed minimum threshold values before considering other traits of quality (Lindén, 2001). Furthermore, to remain of marketable quality throughout the year, the cultivars need to possess good storability traits. If Nordic and Baltic countries are to stay competitive with the international market, it is necessary to maintain breeding programs that specialize on these conditions (Nybom et al., 2016).

Nordic and Baltic countries mainly rely on imported apples to satisfy the market as shown in Figure 1 (FAO, 2013). In Sweden, the total acreage of apples increased by 12% between 2012 and 2017, accounting for 1660 hectares in 2017. In addition, the total number of trees increased by 38% during the same timeframe, indicating an increasing importance of domestically grown apples on the Swedish market. While the domestic market in Sweden consists of predominantly more traditional cultivars, newer plantings are shifting in favour of cultivars from modern breeding programs (Jordbruksverket, 2017).

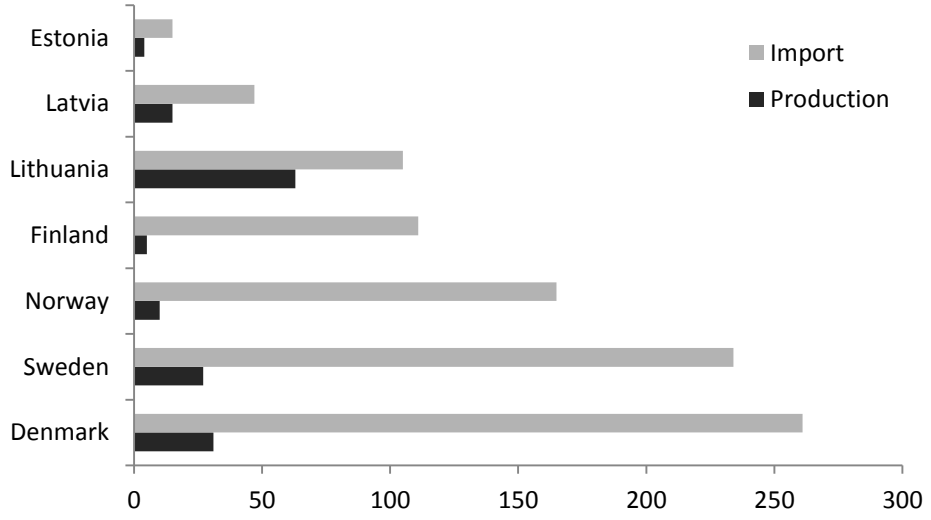

Fig. 1 Amount of produced and imported apples in Nordic and Baltic countries per 1000 tonnes in the year 2013 (FAO, 2013)

1.2 Ethylene and fruit firmness

Texture, firmness and storability are among the most improtant traits for fruit quality in apple. Consumers are increasingly demanding firm and crisp apples, as opposed to soft and mealy ones. The ability to retain firmness is not only important for sensory values, as excessive softening during ripening has a negative impact on storability and shelf life (Nybom et al., 2008; Oraguzie et al., 2009; Costa et al., 2011). Furthermore, softening has been closely associated with susceptibility to post-harvest pathogens, suggesting a strong economic incentive for the enhancement of these traits (Ahmadi-Afzadi et al., 2013).

The production rate of ethylene is a key factor in the process of ripening and softening in climacteric fruit. The ripening process is typically characterized by an increase in respiration rate, triggered by an intensified ethylene production (Bleecker and Kende, 2000). Ethylene production and softening is determined by many factors, notably, horticultural practices and storage regimes. However, great differences in firmness retention between cultivars during storage also suggest a strong genetic basis. While the usage of controlled climate chambers and chemical treatments have proven to be successful at maintaining stable fruit quality over long periods of time, the usage of these methods are also characterized by an increased production cost (Oraguzie et al., 2009). The prospect of partially replacing these methods with a way of genetically suppressing

internal ethylene levels in apple fruits, appear to be the most cost-effective option, especially in organic production where certain chemicals are prohibited.

At the initial stage of ethylene biosynthesis, ACC synthase (ACS) converts S-adenosyl-L-methionine to 1-aminocyclopropane-1-carboxylic acid (ACC), which is an ethylene intermediate. In the presence of oxygen, ACC oxidase (ACO) regulates the last step of converting ACC into ethylene (Bleecker and Kende, 2000). These enzymes are encoded by multigene families, which are expressed in different tissues and at different stages of fruit ripening (Sunako et al., 1999)

1.2.1 Ethylene production genes

The *Md-ACS1* (1-aminocyclopropane-1-carboxylate synthase) and *Md-ACO1* (1-aminocyclopropane-1-carboxylate oxidase) genes are the two most well-studied genes involved in ethylene production (Sunako et al., 1999; Harada et al., 2000; Oraguzie et al., 2004, 2007; Costa et al., 2005). Studies by Sunako et al. (1999) and Harada et al. (2000) have shown that the *Md-ACS1-1* allele (489 bp) results in comparatively normal ethylene production, while the *Md-ACS1-2* (655 bp) allele is associated with a reduced ethylene production. Heterozygous genotypes express a mean ethylene production rate, compared to the homozygous genotypes. Of the two allelic variants at the *Md-ACO1* locus, homozygous cultivars for the *Md-ACO1-1* allele (525 bp) show higher firmness retention, while both heterozygous and *Md-ACO1-2* (587 bp) homozygous cultivars show a higher degree of softening, though their overall effect is less than that of *Md-ACS1* (Costa et al., 2005, 2011; Zhu and Barritt, 2008). Notably, the genotype of *Md-ACO1-1/1* has a synergistic effect in conjunction with *Md-ACS1-2/2*, lowering ethylene production rates to levels that could allow firmness retention in storage for several months (Costa et al., 2005). However, results from previous studies on the allelic distribution of these genes, show that this combination is very rare within the world's apple germplasm (Oraguzie et al., 2004; Zhu and Barritt, 2008; Nybom et al., 2013).

The allelic distinction of *Md-ACS1* and *Md-ACO1* is the result of insertion or deletion mutations (InDel), possibly causing lowered transcription levels (Costa et al., 2005). Functional markers for *Md-ACO1* was developed by Costa et al. (2005) based on its structural polymorphism, and markers for *Md-ACS1* was designed by Harada et al. (2000), both of which have been validated on multiple occasions (Zhu and Barritt, 2008; Longhi et al., 2013). Their availability makes it possible to perform DNA-marker screening of the gene pool, identifying genotypes with the most

valuable allele combinations for low ethylene production and enhanced post-harvest performance (Costa et al., 2005).

1.3 Marker-assisted breeding

Breeding is a long and costly process, especially in crops such as apple, which have a long juvenile phase and are highly heterozygous by their nature (Velasco et al., 2010). However, emerging technologies such as DNA-informed marker-assisted selection (MAS) have been proposed as a valuable tool in conventional breeding programs. Rather than relying on time-consuming and imprecise phenotyping, traits of interest can be selected based on their linkage to certain markers, allowing for optimized selection of parents and subsequent elimination of undesirable genotypes at an early development stage. Because of this, DNA-informed MAS has become routinely incorporated in many conventional breeding programs, improving breeding-efficiency, accuracy and cost-effectiveness (Chagné et al., 2019).

However, MAS can not be efficiently adopted for all traits in all species. Most traits of breeding value are complex and controlled by multiple genes, and are thus very difficult to predict. When multiple genes are involved in regulating a quantitative trait, the phenotypic effect each of them will have and the probability of complex interactions between them escalate to have with increasing numbers of genes involved. Because of this, MAS is best suited for monogenic traits or polygenic traits controlled by a few major genes (Di Guardo et al., 2017).

One of the main bottlenecks for implementation of MAS on a large scale is the inefficient development of functional quantitative trait locus (QTL) markers. These markers are based on the creation of genetic maps, combining phenotypic and molecular data and identifying the loci responsible for the expression of the quantitative traits. Furthermore, confirmation of the position and effect of alleles has to be tested over multiple years on important breeding material in order to determine their predictive power (Peace, 2017).

1.4 Previous studies of Swedish germplasm

It was reported that the relative frequencies of the *Md-ACS1-2* allele has increased in modern apple cultivars, and has been favoured by selection for improved fruit quality in modern apple breeding programs (Nybom et al. 2008). The allelic distribution in cultivars of predominantly Swedish origin reported by Nybom et al. (2008, 2012), revealed a significant skew towards both

of the unfavourable alleles (*Md-ACS1-1* and *Md-ACO1-2*), possibly due to the inclusion of many heirloom cultivars.

2 Aim

The aim of this study was to investigate the allelic configurations of *Md-ACS1* and *Md-ACO1* within apple germplasm suitable for cultivation in Nordic climates. We also performed a verification of the results for cultivars that have been previously investigated for these genes.

The outcome of this study will help breeders in cool climates design MAS programs for low ethylene production and firmness retention during storage, leading to improved accuracy and cost-efficiency when breeding for these traits.

3 Materials and methods

3.1 Plant material

255 apple cultivars of various geographic origins (Table 1) from the germplasm collections of Estonia (31), Finland (41), Latvia (30), Lithuania (23), Sweden (88) and Norway (42) were screened. Material included current and potential parents of the breeding programs as well as cultivars representing a broad genetic diversity. Of these cultivars, 42 reported by Nybom et al. (2008) and 18 additional in Nybom et al. (2012) had already been genotyped. Within the present study, there are 10 cases were both parents and progeny were screened, cultivars ‘Algott’, ‘Eva-Lotta’, ‘Imant’, ‘John-Georg’, ‘Jonagold, Rubinstar’, ‘Konsta’, ‘MA042 10041’, ‘MA962 47003’, ‘MA992 35005’.

Leaves were collected in each country in mid-May-early June of 2018 and immediately shipped to SLU in Alnarp. The leaves were freeze-dried and preserved at -80°C until DNA extraction.

3.2 DNA extraction

About 50mg dry weight of leaf tissue from each apple genotype was used for DNA extraction. The DNA was extracted using the Qiagen Plant Mini Kit following the manufacturer’s protocol. The DNA quality was checked by electrophoresis using a 1% agarose gel stained with Gelred and

the quantity was verified with Nanodrop (Thermo Fisher Scientific). The stock DNA solutions were stored at -80C. Working solutions with 10 ng/uL of DNA were prepared for further use.

Table 1 *Md-ACS1* genotypes of 255 apple cultivars from Nordic and Baltic germplasm collections

Cultivar	Accession Code	Origin	Parentage	*Md-ACS1* Genotype
'Achrenin Syys'	FIN1	Finland		1/1
'Agnes'	SWE80	Sweden	Discovery x Unknown	1/2
'Agra'	LAT5	Latvia		1/1
'Aino'	FIN2	Finland		1/1
'Aldas '	LIT22	Lithuania		1/2
'Alemanda'	LIT20	Lithuania		1/1
'Alesya'	EST39	Belarus		1/2
'Algott '	SWE92	Sweden	Gyllenkrok's Astrakan x Worcester Pearmain	1/2
'Alice'	SWE66	Sweden	Ingrid Marie x Unknown	1/1
'Alkmene'	NOR13	Germany	Dr.Oldenburg x Cox	2/2
'Ananas'	FIN3	Unknown		1/1
'Angold'	SWE18	Czech Republic	A28-39 x Golden Delicious	1/2
'Antei'	LAT9	Belarus		1/1
'Antonovka_EST'	EST22	Russia		1/1
'Antonovka_FIN'	FIN4	Russia		1/1
'Apelsinnoe'	SWE79	Russia	Korichnoe polosatoe x Papirovka	1/1
'Aroma'	SWE69	Sweden	Ingrid Marie x Filippa	1/2
'ARX 412-18'	NOR35	Norway	Discovery x ARX 49-18	1/2
'Auksis '	LIT24	Lithuania		1/1
'Aule'	EST40	Estonia		1/1
'Barchatnoje'	SWE28	Russia		1/2
'Belle de Boskoop'*	SWE83	Germany		1/1/1
'Belorusskoye Malinovoye'	LAT11	Belarus		1/1
'Beržininkų Ananasas'	LIT1	Lithuania		1/1
'Birgit Bonnier'	SWE43	Sweden		1/1
'Björn Lindberg'	FIN5	Finland		1/1
'BM 41497'	LAT12	Sweden		1/1
'BM 55734'	LAT13	Sweden		1/2
'Bogatyr'	LAT14	Russia		1/1
'Bramley's Seedling'	SWE62	United Kingdom		1/2
'Carroll'	NOR30	Canada	Morden_5029-E152 x Melba	1/1
'Cidor'	NOR48	France		1/2

Table 1 (Continued)

Cultivar	Accession Code	Origin	Parentage	*Md-ACS1* Genotype
'Classic Red Delicious'	SWE87	USA		1/2
'Cortland'	SWE59	USA	Ben Davis x McIntosch	1/1
'Criterion'	SWE75	USA	(Golden Delicious x Red Delicious) x Winter Banana	1/2
'Dace'	LAT15	Latvia		1/1
'Dayton'	SWE29	USA		1/1
'Diana'	FIN6	Unknown		1/1
'Discovery'	SWE49	United Kingdom	Worcester Pearmain x Beauty of Bath	2/2
'Dr.Oldenburg'	EST23	Germany		1/1
'Early Geneva'	SWE8	USA	Quinte x Julyred	1/1
'Eir'	SWE71	Norway	Katja x Buckley Giant	1/2
'Eksotika'	LAT16	Latvia		1/1
'Eliakselan Nauris'	FIN7	Finland		1/1
'Elise'	SWE5	The Netherlands	Septer x Cox	2/2
'Ellis Bitter'	NOR49	United Kingdom		1/2
'Els'	EST19	Estonia		1/1
'Elstar'	SWE46	Sweden	Golden Delicious x Ingrid Marie	2/2
'Enterprise'	SWE48	USA	PRI1661-2 x Coop-7	1/2
''Eva-Lotta'	SWE74	Denmark	Cortland x James Grieve	1/2
'Fiesta'	SWE4	United Kingdom	Cox x Idared	1/2
'Florina'	SWE51	France	PRI1661-1 x Jonathan	1/2
'Folke'	SWE77	Sweden		1/2
'Forele'	LAT19	Latvia		1/1
'Fosseple'	NOR4	Norway		1/1
'Franskar'	NOR5	Norway		1/1
'Fredrik'	SWE65	Sweden	Aroma x PRI 1858/102	1/2
'Freedom '	LIT30	USA		1/2
'Frida'	SWE64	Sweden	Aroma x PRI 1858/102	1/1
'Fu Shuai'	NOR14	China	Early_McIntosh x Golden_Delicious	1/2
'Geltonasis Arkadas'	LIT12	Russia		1/1
'Geneva/Race scab'	SWE63	Canada		1/2
'Gita'	LAT20	Latvia		1/1
'Gloster'	SWE58	Germany	Glockenapfel x Delicious	2/2
'Golden Delicious'	SWE57	USA	Grimes Golden x Unknown	1/2
'GoldRush'	SWE60	US		2/2
'Gravensteiner'*	SWE47	Germany		1/1/1

Table 1 (Continued)

Cultivar	Accession Code	Origin	Parentage	*Md-ACS1* Genotype
'Gult Kaneläpple'	SWE10	Russia		1/1
'Gustavs bästa'	FIN9	Finland		1/1
'Gyllenkrok's Astrakan'	SWE68	Sweden		1/1
'Hibernal'	SWE70	Canada		1/1
'Himmelstalund'	SWE85	Sweden		1/1
'Honeycrisp'	SWE50	USA	Keepsake x MN1627	1/2
'Huvitus'	FIN12	Finland		1/1
'Hörnö'	SWE90	Sweden		1/1
'Iedzenu'	LAT21	Latvia		1/1
'Imant'	FIN13	Belarus	Antei x Liberty	1/1
'Imrus'	FIN14	Russia	Antonovka_OB x PRI240-57	1/1
'Inese'	LAT22	Latvia		1/1
'Jalmarin Omena'	FIN15	Finland		1/1
'James Grieve'	SWE53	United Kingdom	Cox x Pott's seedling	1/1
'John-Georg'	SWE89	Sweden	Golden Delicious x James Grieve	1/1
'Jonafree'	SWE26	USA		1/2
'Jonagold, Rubinstar'	SWE56	USA	Jonathan x Golden Delicious	1/2
'Jonathan'	SWE61	USA		1/2
'Julyred'	NOR29	USA	NJ8 x NJ110037	1/1
'Junost'	FIN17	Russia	Gult Kaneläpple x Transparente Blanche	1/1
'Juuso'	SWE9	Finland		1/1
'Kaikuvuori'	FIN19	Finland		1/1
'Kaja'	EST18	Estonia		1/1
'Kallika'	EST36	Estonia		1/1
'Karksi Renett'	EST43	Estonia		1/1
'Karmen'	SWE12	Czech Republic		1/1
'Kasper'	EST6	Estonia		1/1
'Kaunis'	LIT13	Lithuania		1/1
'Kaupanger'	NOR6	Norway		1/1
'Kenttämies'	FIN22	Finland		1/1
'Kersti'	FIN23	Finland		1/1
'Kikitriinu'	EST20	Estonia		1/1
'Kim'	SWE84	Sweden	Cortland x Ingrid Marie	1/2
'Kingston Black'	SWE11	England		1/1
'Kirkniemen Talvi'	FIN24	Finland		1/2
'Konfetnoe'	LAT23	Russia		1/1

Table 1 (Continued)

Cultivar	Accession Code	Origin	Parentage	*Md-ACS1* Genotype
'Konsta'	SWE73	Finland	Lobo x Antonovka	1/1
'Korichnoe Novoe'	LAT24	Russia		1/2
'Kosztela'	LIT2	Poland		1/1
'Kovalenkovskoye'	LAT25	Belarus		1/1
'Krista'	FIN26	Estonia	L25 x Unknown	1/1
'Krügeri Tuviõun'	EST38	Unknown		1/1
'Kuku'	EST8	Estonia		1/1
'Lantun Talvi'	FIN27	Finland		1/1
'Lavia'	FIN28	Finland		1/1
'Lembitu'	EST30	Estonia		1/1
'Lepaan Liereä'	FIN29	Finland		1/1
'Liberty'	SWE16	USA	Macoun x PRI54-12	1/1
'Lietuvos Pepinas '	LIT3	Lithuania		1/2
'Ligol'	LIT33	Poland		1/2
'Liivi Kuldrenett'	EST1	Unknown		1/1
'Liivi Sibulõun'	EST13	Unknown		1/1
'Liivika'	EST2	Estonia		1/1
'Linda'	SWE13	Canada	Wealthy x Unknown	1/2
'Lobo'	FIN31	Canada	McIntosh x Unknown	1/1
'Lovisa'	SWE76	Sweden	Katja x Priscilla	1/2
'Luotsi '	FIN32	Russia		1/1
'MA042 10041'	NOR52	Norway	Martaeple x Rubinstep	1/2
'MA962 02073'	NOR34	Norway	Discovery x ARX 49-18	2/2
'MA962 47003'	NOR51	Norway	Pink Pearl x Pristine	2/2
'MA982 05043'	NOR38	Norway	Discovery x ARX 49-18	2/2
'MA983 04010'	NOR44	Kazakhstan / Norway		1/1
'MA983 05002'	NOR47	Kazakhstan / Norway		1/1
'MA985 03023'	NOR45	Kazakhstan / Norway	PRI14-510 x NJ123249	1/1
'MA992 03006'	NOR46	Kazakhstan / Norway		1/1
'MA992 35005'	NOR39	Norway	Tohoku 2 x Rubinstep	1/2
'MA992 39008'	NOR36	Norway	Aroma x Rubin	1/2
'MA992 45017'	NOR50	Norway	NA_12-68 x Murray	1/1
'MA992_37013'	NOR12	Norway	Freedom x Realka	1/2
'Madli'	EST37	Estonia		1/1
'Maigold'	SWE14	Switzerland	Fraurotacher x Golden Delicious	1/2

Table 1 (Continued)

Cultivar	Accession Code	Origin	Parentage	*Md-ACS1* Genotype
'Maikki'	SWE2	Finland	Melba x Huvitus	1/1
'Maimu'	EST16	Estonia		1/2
'Maj-Britt'	SWE54	Sweden		1/1
'Make'	SWE3	Finland	Atlas x Keltainen Syyskalvilli	1/1
'Mantet'	SWE6	Canada		1/2
'Martaeple'	NOR7	Norway		1/1
'Martsipan'	EST11	Unknown		1/1
'Meelis'	EST27	Estonia		1/1
'Monta'	LAT28	Latvia		1/1
'Mutsu'*	SWE7	Japan	Golden Delicious x Indo	1/2/?
'NA 42-51'	NOR41	Norway	Discovery x Julyred	1/2
'Nanna'	SWE30	Norway	Katja x Buckley Giant	1/1
'NB 6-4'	NOR40	Norway	Prins x Carroll	1/1
'Noris'	LIT28	Lithuania		1/2
'Norland'	FIN35	Canada	Resque x Melba	1/1
'NY 184121'	NOR18	USA.	Macoun x Antonovka	1/1
'Opalescent'	SWE23	USA		2/2
'Oranie'	SWE44	Sweden		1/1
'Orlinka'	FIN36	Russia	Stark's Earliest x Pervyj saljut	1/1
'Orlovim'	LIT16	Russia	Antonovka_OB x SR0523	1/1
'Oye'	NOR42	Norway	Discovery x NY18491	1/2
'Panemunės baltasis'	LIT5	Lithuania		1/1
'Papirovka'	LIT25	Russia		1/1
'Paprastasis antaninis'	LIT4	Russia		1/1
'Paulis'	LAT29	Latvia		1/1
'Pekkalan Puu'	FIN37	Finland		1/1
'Pepin Shafranniy'	SWE41	Russia		1/1
'Pink Pearl'	NOR19	USA	Surprise x Unknown	1/2
'Pirja'	SWE35	Finland	Huvitus x Melba	1/1
'Poema'	LIT21	Lithuania		1/1
'Polli Kaunitar'	EST21	Estonia		1/1
'Prairifire'	SWE42	USA		1/1
'Prima'	SWE45	USA		1/2
'Priscilla_LAT'	LAT30	USA		1/2
'Priscilla_SWE'	SWE22	USA		1/2
'Pristine'	NOR20	USA	Cauzat x Coop-10	1/2
'Punakaneli '	FIN39	Russia		1/1

Table 1 (Continued)

Cultivar	Accession Code	Origin	Parentage	*Md-ACS1* Genotype
'Pure Ametist'	LAT31	Latvia		1/1
'Raud Prins'	NOR2	Norway		1/1
'Reanda'	SWE20	Germany	Clivia x Unknown	1/2
'Rebella'	SWE25	Germany		2/2
'Red Delicous'	LAT33	USA		1/2
'Remo'	LAT34	Germany		1/2
'Rescue'	SWE36	Canada	Blushed Calville x Unknown	1/1
'Rigas Rozabele'	LAT32	Latvia		1/1
'Risäter'	SWE78	Sweden		1/1
'Rouville'*	SWE38	Canada	Melba x PRI69-52 x McIntosh	1/2/?
'Rubin'	NOR21	Czech Republic	Golden_Delicious x Lord_Lambourne	2/2
'Rubin (Kazakhstan cv.)'	LAT35	Kazakhstan		1/1
'Rubinola'	SWE1	Czech Republic	Rubin x Prima	2/2
'Rubinstep'	NOR33	Czech Republic	Clivia x Rubin	2/2
'Rudenis '	LIT26	Lithuania		1/2
'Rudens Dryžuotasis'	LIT6	Baltic States		1/1
'Rödluvan'	SWE 15	Sweden		1/2
'Samo'	SWE33	Finland		1/1
'Šampion'	LIT34	Czech Republic	Golden Delicious x Lord Lambourne	1/2
'Sansa'	NOR22	Japan	Gala x Akane	2/2
'Santana Balsgård'	SWE 19	The Netherlands	Elstar x Priscilla-NL	1/1
'Sariola'	FIN41	Finland		1/1
'Scarlet O'Hara'	SWE 39	USA	PCFW2-134 x PRI669-205	2/2
'Sidrunkollane Taliõun'	EST32	Estonia		1/1
'Sierinka '	LIT7	Baltic States		1/1
'S'igne Tillisch'	SWE86	Denmark		1/2
'Silva'	NOR23	Sweden	Melba x Stenbock	1/1
'Sinap Orlovsky'*	LAT38	Russia		1/1/1
'Sipolins'	LAT39	Latvia/Estonia		1/1
'Skaistis'	LIT27	Lithuania		2/2
'Slava Petersburg'	SWE34	Russia		1/1
'Snövit'	SWE32	Sweden	Stenbock x Peach Summer Apple	1/2
'Sokerimiron '	FIN43	Finland		1/1
'Spenser'	SWE40	Canada	McIntosh x Golden Delicious	1/2
'SR 0523'	LAT42	Sweden		1/1
'Štaris'	LIT23	Lithuania		1/1

Table 1 (Continued)

Cultivar	Accession Code	Origin	Parentage	*Md-ACS1* Genotype
'Stars'	LAT40	Latvia		1/2
'Streifling Herbst'	LAT41	Baltic States		1/1
'Stølen'	NOR8	Norway		1/1
'Suislepp'	EST12	Estonia		1/1
'Sultanat'	SWE17	Kazakhstan		1/1
'Svezhest'	FIN44	Russia	Antonovka Krasnobochka x PRI54-22	1/1
'Sügisdessertõun'	EST31	Estonia		1/2
'Särsö'	SWE55	Sweden		1/1
'Sävstaholm'	SWE81	Sweden		1/1
'Talvenauding'	EST5	Estonia		1/1
'Tellissaare'	EST26	Estonia		1/1
'Tiara'	NOR37	Norway	Pink_Pearl x K_2-24	2/2
'Tiina'	EST3	Estonia		1/1
'Tohoku 2'	NOR24	Japan	McIntosh x Worcester_Pearmain	1/1
'Trailman'	SWE24	Canada		1/1
'Trulsa'	SWE72	Sweden	Eva-Lotta x B4:1547	1/2
'Tsaarin Kilpi'	FIN47	Finland		1/1
'TSR18T13'	SWE91	USA		2/2
'Tuscan'	NOR11	United Kingdom	McIntosh_Wijcik x Greensleeves	1/1
'USA: NY55140-12'	SWE27	USA	Macoun x PRI54-12	1/1
'Vaasan Talvi'	FIN48	Finland		1/1
'Vahur'	EST28	Estonia		1/1
'Valge Klaarõun'	EST10	Unknown		1/1
'Valkealan Syys'	FIN49	Finland		1/1
'Wealthy'	EST24	USA		1/2
'Veniaminovskoye'	FIN50	Russia		1/2
'Vetle'	NOR10	Norway		1/1
''William's Pride'	NOR25	USA	PRI1018-101 x NJ50	1/2
'Virve'	EST34	Estonia		1/1
'Vista Bella'	NOR28	USA	77349 x Julyred	1/1
'Worcester Pearmain'	SWE21	United Kingdom		1/2
'Vuokko'	FIN51	Finland	Melba x Huvitus	1/1
'X 4876'	NOR26	France	Jonathan x Malus_pumila_niedzw	1/1
'Y9330' ('Valtti')	FIN53	Finland		1/2
'Y936'	FIN54	Finland	Pirja x BM8834	1/2
'Y9369' ('Sokeripapu')	FIN55	Finland		1/1

Table 1 (Continued)

Cultivar	Accession Code	Origin	Parentage	*Md-ACS1* Genotype
'Y9397' ('Hertta')	FIN57	Finland		1/1
'Y9510' ('Kymppi')	FIN58	Finland		1/1
'Yläkautun_Omena'	FIN59	Finland		1/1
'Zailiyskoe'	LAT45	Kazakhstan		1/1
'Zarya Alatau'	LAT46	Kazakhstan		1/1
'Žemaičių grietininis'	LIT8	Baltic States		1/2
'Åkerö'	SWE82	Sweden		1/1
'Ölands Kungsäpple'	SWE 37	Sweden		1/1

* - Known triploid cultivars

3.3 PCR amplifications

Amplification of DNA was carried out following a PCR protocol provided by Zhu et al. (2008). Primer sequences for amplification of the target fragments of the *Md-ACS1* gene (Md-ACS1-5'F 5'AGAGAGATGCCATTTTTGTTCGTAC3'; Md-ACS1-5'R 5'CCTACAAACTTGCGTGGGGATTATAAGTGT3') were obtained from Harada et al. (2000), and for the *Md-ACO1* gene (Md-ACO1F: 5'TCCCCCCAATGCACCACTCCA'3; Md-ACO1R: 5'GATTCCTTGGCCTTCATAGCTTC3), from Costa et al. (2005).

3.4 Gel electrophoresis

Three cultivars of different allelic configurations were used as control for successful amplification of target DNA-fragments:

- 'Wealthy', heterozygous for both genes
- 'Elstar', homozygous for favourable alleles (allele 2) at *Md-ACS1*, and homozygous for non-favourable allele (allele 2), at *Md-ACO1*.
- 'Silva', homozygous for non-favourable allele (allele 1) at *Md-ACS1*, heterozygous at *Md-ACO1*

The PCR products from the control cultivars were separated by electrophoresis (93 V for 50 minutes) on a 1.7% agarose gel stained with Gelred, followed by examination under UV-light, using GeneRuler 1000 bp Plus DNA Ladder to control product sizes.

3.5 Capillary electrophoresis

Final analyses determining the allelic configuration was carried out using capillary electrophoresis with Genetic Analyzer 3500 (Thermo Fisher Scientific) with eight capillaries.

Samples were mixed with a size marker, GeneScan™ 1200 LIZ™ dye Size Standard in Hi-Di Formamide, (Applied Biosystems) and denatured prior to electrophoresis.. The results were analyzed using GeneMarker 2.7.0 (Softgenetics).

3.6 Data analysis

Five cultivars that were known to be triploid ('Belle de Boskoop', 'Gravensteiner', 'Mutsu', 'Rouville', 'Sinap Orlovsky') were excluded from the statistical analysis, due to presence of an additional allele, which was unknown in the heterozygous ones. Count and frequency of each allelic configuration was determined for each germplasm collection. For fully genotyped sets of mother, father and progeny, an additional evaluation of heredity was used to determine any pedigree errors. A verification of previously identified genotypes by Nybom et al. (2008, 2012) was performed.

4 Results

4.1 Allele detection

In the present study, the authenticity of the *ACO1* amplifications could not be verified. In the capillary electrophoresis output, every cultivar was falsely identified as heterozygous at the *Md-ACO1* locus. Circumstances leading to this outcome remain uncertain and will have to be further evaluated.

Detection of *Md-ACS1* allele configurations proceeded as expected and allele presence could easily be assessed using the GeneMarker 2.7.0 toolkit (Figure 2). Among cultivars previously studied, 59 of 60 genotypes matched with our findings. The only exception was 'Eva-Lotta', which had an expected allelic configuration of *Md-ACS1-1/1*, but in our study turned out to be *Md-ACS1-1/2*.

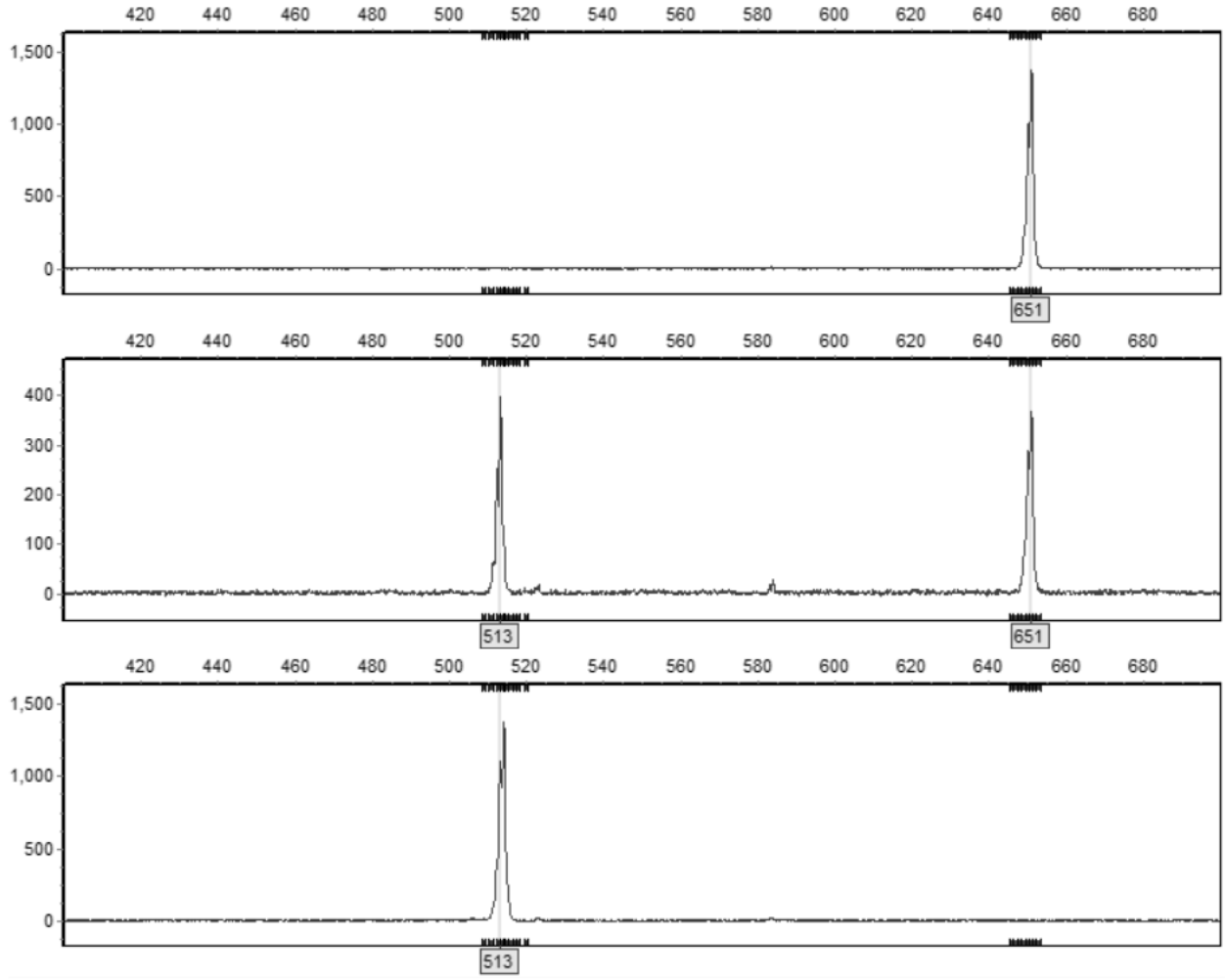

Fig. 2 GeneMarker 2.7.0 display of control cultivars, constituting all three different *Md-ACS1* genotypes: 'Elstar', 'Wealthy' and 'Silva', from top to bottom. Y-axis represents the fluorescence intensity and the X-axis represents fragment the size of an amplified sequence. *Md-ACS1-1* was detected at 513 bp and *Md-ACS1-2* at 651 bp.

4.2 Evaluation of heredity

In the cases were both parents and progeny were genotyped, cultivars 9 out of 10 cultivars displayed expected allelic configurations in regards to heredity. However, once more, 'Eva-Lotta' demonstrated an unexpected variation. Genotyping data on the parents (Table 1) revealed homozygosity of the *Md-ACS1-1* allele in both cases, which is inconsistent with the allele outcome of *Md-ACS1-1/2* in 'Eva-Lotta'.

4.3 Genotype data

The frequency of the *Md-ACS1-1* allele was especially prominent in the Estonian (0.94), Finnish (0.95) and Latvian (0.90) collections (Figure 3). Conversely, the frequencies of the reduced ethylene production allele *Md-ACS1-2* were noticeably higher in the Lithuanian (0.22), Swedish

(0.33) and Norwegian (0.35) collections, although only the Swedish and Norwegian collections comprised any significant amounts of the favourable, *Md-ACS1-2/2* genotype, whereas the Lithuanian *Md-ACS1-2* alleles was mainly present in heterozygous cultivars (Figure 3).

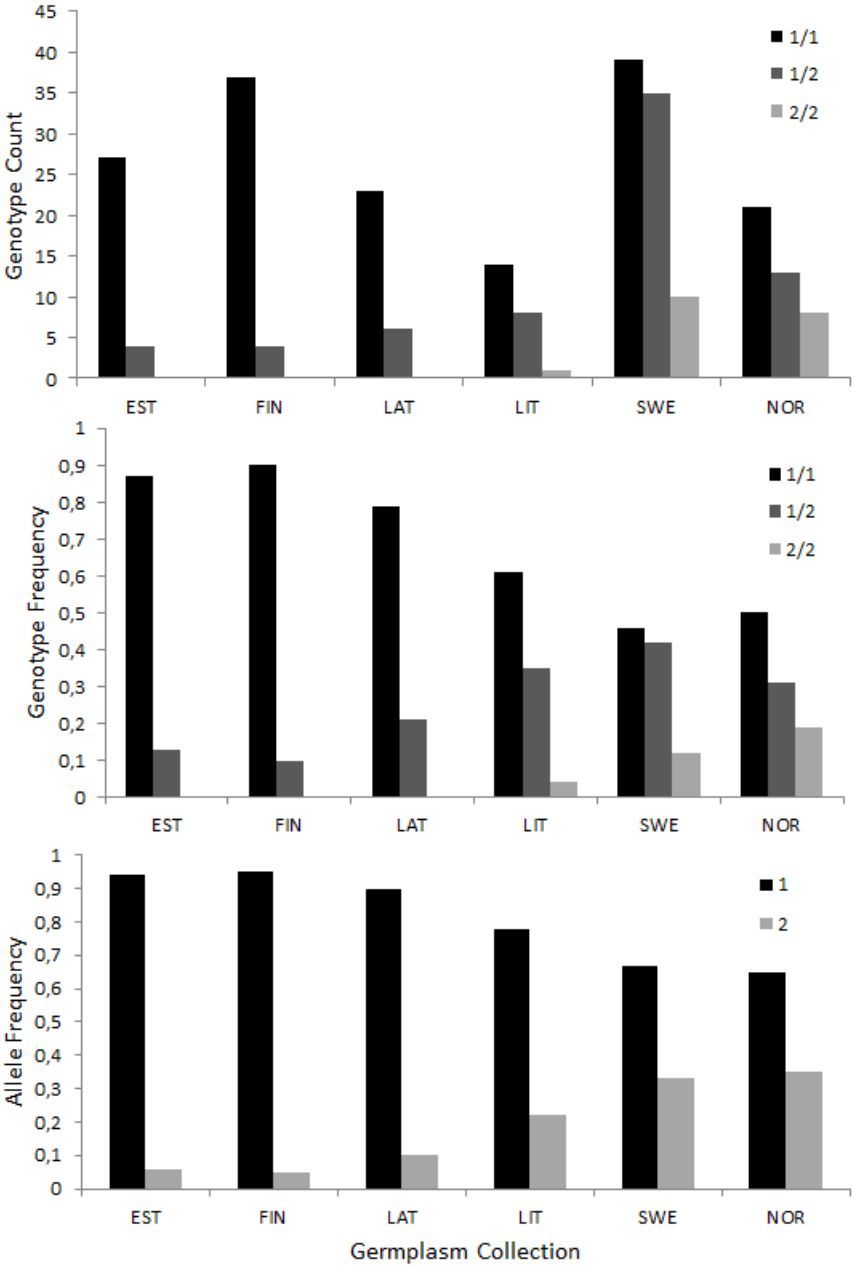

Fig. 3 Histograms of 250 apple cultivars and the low ethylene allele *Md-ACS1-2,* and normal ethylene allele *Md-ACS1-1*. The legend is represented by *ACS1* genotypes 1/1, 1/2 and 2/2 in the first two histograms, and alleles *Md-ACS1-1* (1) and *Md-ACS1-2* (2) at the bottom. Categories are arranged by germplasm collection origin of each sample: EST- Estonia, FIN –Finland, LAT-Latvia, LIT-Lithuania, SWE-Sweden, NOR-Norway. Total count of each genotype is presented at the top, followed by frequency percentages of genotype and allelic distribution thereafter.

5 Discussion

5.1 Genotypes of screened material

255 cultivars from Nordic and Baltic germplasm collections were screened for *Md-ACS1* and *Md-ACO1* allelic configurations, however genotypes could not be determined at the *Md-ACO1* locus, possibly as a result of an unknown contaminant or errors in some part of the protocol. The discrepancy between the reported *Md-ACS1* fragment-sizes (489 bp, 655 bp) and the values observed in Figure 2 (513 bp, 651 bp), could be explained by calibration settings and/or previous studies evaluating allelic configuration solely based on gel electrophoresis (Sunako et al., 1999), as opposed to capillary electrophoresis.

Five triploid cultivars were excluded from the data analysis since the exact allelic composition was unknown in two of them, 'Mutsu' and 'Rouville'. Exclusion of cultivars were limited to known triploids, and because evaluation of ploidy was outside of the scope of this study, it is still possible that undetected triploids may remain in the dataset.

In the 250 remaining cultivars, the normal ethylene allele configuration (*Md-ACS1-1/1)* remained the most numerous throughout every germplasm collection, similarly to previous studies by Nybom (2008, 2012) and Zhu and Barritt (2008). The discrepancy between allele frequencies in Nordic countries and Baltic countries could possibly be explained by differences in historical selection practices. Moreover, several of the *Md-ACS1-2/2* cultivars within the Swedish and Norwegian collections are considered popular commercial cultivars in Europe and important breeding parents.

The overall genotype frequencies observed in the present study are not significantly different from previous reports (Nybom et al., 2008, 2012). However, Estonian, Finnish and Latvian collections feature less of the *Md-ACS1-2* allele compared to the previous studies (Nybom et al., 2008, 2012), while in contrast the Swedish and Norwegian collections are overrepresented regarding the *Md-ACS1-2* allele. Because of this, and the absolute count of cultivars with a favourable genotype (Figure 3), the Swedish and Norwegian collections represent the main bulk of promising breeding material.

Verifications of previously screened cultivars (Nybom et al., 2008, 2012), revealed a single mis-match in 'Eva-Lotta'. Moreover, the deviating cultivar 'Eva-Lotta' also displayed genotype

inconsistencies compared to the presumed parents. Given the fact that both disparities are associated with the same cultivar, the deviating results are most likely caused by a sampling error or user error made in this study. However, the trueness-to-type of the tree in the germplasm collection should be confirmed.

5.2 Marker-assisted selection of *Md-ACS1* and *Md-ACO1*

Ethylene is generally accepted as the main factor influencing fruit ripening and softening in apple (Bleecker and Kende, 2000). Based on current knowledge, the ethylene production gene Md-ACS1 and Md-ACO1 represent the best candidates for enhancing firmness retention during storage, and has been implemented in several breeding programs (Peace, 2017). The breeding material presented in this study (Table 1) contains many cultivars that have a long proven track record of cold climate suitability, and the screening for *Md-ACS1* alleles in this material constitutes a significant progress towards implementation of MAS in Nordic and Baltic countries.

The genotype data presented in Table 1 can be used for optimal selection of parents when designing apple breeding programs, focusing on the fruit firmness potential of the genotypes in the progeny. Given the fact that the low ethylene *Md-ACS1* and *Md-ACO1* alleles most often can be found in a heterozygous configuration (Table 1; Nybom et al., 2008, 2012), the usage of DNA-markers for parent selection and early elimination of undesired genotypes in a segregating population could certainly be beneficial for increasing the breeding efficiency. Savings from the resulting reduction in phenotyping requirements could be used to scale up subsequent field trials, or to intensify evaluations on a limited subset of progenies, many of which may carry additional valuable production traits.

Imported apples comprise a majority of apples sold in the Nordic and Baltic countries (Figure 1), suggesting that there is huge potential to increase national market shares, provided that each country manage to improve their competitiveness at the global market. The opportunity to obtain cultivars with a high degree of fruit firmness and storability through MAS of *Md-ACS1* and *Md-ACO1*, could significantly increase their competitiveness in that regard.

6 References

Ahmadi-Afzadi, M., Tahir, I., Nybom, H., (2013). Impact of harvesting time and fruit firmness on the tolerance to fungal storage diseases in an apple germplasm collection. Postharvest Biol. Tech. 82, 51-58.

Bleecker, A.B. & H. Kende, (2000). Ethylene: A gaseous signal molecule in plants. Annu. Rev. Cell. Dev. Biol., 16, 1–18.

Chagné D. Vanderzande S., Kirk C., Profitt N., Weskett R., Gardiner S.E., Peace C.P., Volz R.K., Bassil N.V., (2019). Validation of SNP markers for fruit quality and disease resistance loci in apple (Malus×domestica Borkh.) using the OpenArray platform. Hortic. Res., 6, 30-30.

Costa, F., Stella, S., Van de Weg, W.E., Guerra, W., Cecchinel, M., Dalla Via, J., Koller,B., Sansavini, S., (2005). Role of the genes Md-ACO1 and Md-ACS1 in ethylene production and shelf life of apple (Malus domestica Borkh.). Euphytica, 141, 181–190.

Costa, F., Cappellin, L., Longhi, S., Guerra, W., Magnago, P., Porro, D., Soukoulis, C.,Salvi, S., Velasco, R., Biasioli, F., Gasperi, F., (2011). Assessment of apple (Malus x domestica Borkh.) fruit texture by a combined acoustic-mechanical profiling strategy. Postharvest Biol. Tech., 61 (1), 21–28.

Di Guardo M., Bink M., Guerra W., Letschka T., Lozano L., Busatto N. et al., (2017). Deciphering the genetic control of fruit texture in apple by multiple-family based analysis and genome-wide association. J. Exp. Bot., 68(7),1451–1466. https://doi.org/10.1093/jxb/erx017.

FAO., (2013). Food and Agriculture Organization of the United Nations, FAOSTAT Statistic Database.

FAO., (2017). Food and Agriculture Organization of the United Nations, FAOSTAT Statistic Database.

Harada, T., Sunako, T., Wakasa, Y., Soejima, J., Satoh, T., Niizeki, M., (2000). An allele of the 1-aminocyclopropane-1-carboxylate synthase gene (Md-ACS1) accounts for the low level of ethylene production in climacteric fruits of some apple cultivars. Theor. Appl. Genet., 101, 742–746.

Jordbruksverket., (2017). Fruktträd 2017, JO 33 SM 1802, 2018-10-23.

Longhi S, Cappellin L, Guerra W, Costa F., (2013). Validation of a functional molecular marker suitable for marker-assisted breeding for fruit texture in apple (Malus × domestica Borkh.). Molecular Breeding, 32, 841–852.

Lindén, L., (2001). Re-analyzing historical records of winter injury in Finnish apple orchards. Can. J. Plant Sci., 81, 479–485.

Nybom H., Sehic J., Garkava-Gustavsson L., (2008). Modern apple breeding is associated with a significant change in allelic ratio of the ethylene production gene Md-ACS1.J. Hortic. Sci. Biotechnol., 83, 673–677.

Nybom, H., Ahmadi-Afzadi, M., Sehic, J., Hertog, M., (2012). DNA marker-assisted evaluation of fruit firmness at harvest and post-harvest fruit softening in a diverse apple germplasm. Tree Genet. Genom., 9, 279–290. doi:10.1007/s11295-012-0554-z.

Nybom, H., Ahmadi-Afzadi, M., Sehic, J., Hertog, M., (2013). DNA marker assisted evaluation of fruit firmness at harvest and postharvest fruit softening in a diverse apple germplasm, Tree Genet. Genom., 2013, 9, 279–290.

Nybom, H., Røen, D., Karhu, S., Garkava-Gustavsson, L., Tahir, I., Haikonen, T., Røen, K., Ahmadi-Afzadi, M., Ghasemkhani, M., Sehic, J., Hjeltnes, S.-H., (2016). Pre-breeding for future challenges in Nordic apples: susceptibility to fruit tree canker and storage diseases. Acta Hortic., 1127, 117-124. DOI: 10.17660/ActaHortic.2016.1127.20.

Oraguzie, N.C., Iwanami, H., Soejima, J., et al., (2004). Inheritance of Md-ACS1 gene and its relationship to fruit softening in apple (Malus domestica Borkh.), Theor. Appl. Genet., 108, 1526–1533.

Oraguzie N.C., Volz R.K., Whitworth C.J., Bassett H.C.M., Hall A.J., Gardiner S.E., (2007). Influence of Md-ACS1 allelotype on fruit and harvest season within an apple germplasm collection on fruit softening during cold air storage. Postharvest Biol. Tech., 44, 212–219.

Oraguzie, N., Alspach, P., Volz, R., Whitworth, C., Ranatunga, C., Weskett, R., Harker,R., (2009). Postharvest assessment of fruit quality parameters in apple using both instruments and an expert panel. Postharvest Biol. Tech., 52 (3), 279–287.

Peace CP, (2017). DNA-informed breeding of rosaceous crops: promises, progress and prospects. Hortic. Res., 4, 17006.

Sunako, T., Sakuraba, W., Senda, M., Akada, S., Ishikawa, R., Niizeki, M., Harada,T., (1999). An allele of the ripening-specific 1-amino-cyclopropane-carboxylic acid synthase (ACS1) in apple fruit with a long storage life. Plant Physiol., 119, 1297–1303.

Velasco R., Zharkikh A., Affourtit J., Dhingra A., Cestaro A., Kalyanaraman A., Fontana P., Bhatnagar S.K., Troggio M., Pruss D., et al., (2010). The genome of the domesticated apple (Malus domestica Borkh). Nat. Genet., 42,833–839.

Zhu, Y., & Barritt, B., (2008). Md-ACS1 and Md-ACO1 genotyping of apple (Malus x domestica Borkh.) breeding parents and suitability for marker-assisted selection. Tree Genet. Genom., 4, 555–562.