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PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF ETHYLENE BIOSYNTHESIS IN APPLE

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1.1 The fruit

The fruit is, by anatomical definition, a mature ovary and therefore typically includes carpel tissues in part or in whole (Giovannoni, 2004). A more accurate and inclusive definition encompasses extracarpellary tissues that are included at the mature fruiting stage. Fruits can be additionally separated into dehiscent or dry fruits and non-dehiscent or fleshy fruits (Giovannoni, 2001). In the fleshy fruit, the pericarp is differentiated into three distinct layers: the epicarp, the mesocarp and the endocarp (the outer, median and inner layers, respectively). Common to all fruits is the developmental process that involves three basic phases. In the first phase (fruit set) there is the development of the ovary and the initiation of cell division. In the second phase, cell division is the predominant feature. During the third phase, fruit increases in size mainly by cell expansion. Once the fruit cells have fully expanded and the fruit matured, the ripening process ensues. Each phase of fruit development and ripening involves specific gene activity as revealed by transcriptomic analyses (Handa et al., 2012). Ripening has received most attention from geneticists and breeders, as this important process activates a whole set of biochemical pathways that make the fruit attractive, desirable, and edible for consumers (Bouzayen et al., 2010).

1.2 Fruit ripening

Fruit ripening is a developmental complex process which occurs in higher plants and involves a number of stages displayed from immature to mature fruits that depend on the plant species and the environmental conditions. During fruit ripening deep metabolic changes occur in the biochemistry, physiology and gene expression of the fruit such as chlorophyll degradation and pigment biosynthesis, conversion of starch to simple sugars, accumulation of flavours and cell wall softening, simple sugar and organic acid accumulation, volatile production and flesh softening (Palma et al., 2011). These changes are under the control of both external (light and temperature) and internal (developmental gene regulation and hormonal control) factors.

According to the regulatory mechanisms underlying the ripening process, fleshy fruits can be divided into two groups: climacteric and non-climacteric. The climacteric fruits exhibit an upsurge in the rate of respiration at the onset of ripening that is associated with

increased biosynthesis of ethylene (Lelievre et al., 1997). The role of respiratory surge in climacteric fruits at the onset of ripening is still a mystery, but it has been proposed to be related to coordination and synchronization of the ripening process in these fruits. Both climacteric and non-climacteric fruits show similar changes in various metabolic processes leading to pigment alterations, sugar accumulation, textural change, fruit softening, volatiles production, and enhanced susceptibility to pathogens, but although ethylene plays a significant role in the development of ripening attributes in climacteric fruit, it is not required for the metabolic shifts seen in non-climacteric fruits. The molecular distinctions underlying climacteric versus non-climacteric ripening are poorly understood (Giovannoni, 2004).

Examples of common climacteric fruits that require ethylene for ripening include tomato, apple, banana, and most stone fruits, whereas non-climacteric fruits, including grape, citrus, and strawberry, are capable of ripening in the absence of increased ethylene synthesis.

1.3 Ethylene and ripening

The role of ethylene as the “ripening hormone” in climateric fruits has been firmly established (Giovannoni, 2001). Affecting biosynthesis of ethylene during ripening in fleshy fruit has been the foremost attempt for arresting post harvest deterioration. One of the most striking characteristics of climateric as compared with nonclimateric fruits is their capacity to produce autocatalytic ethylene. It has been speculated that two regulatory systems of ethylene production exist. System I, operating in both climateric and non climateric fruits as well as in vegetative tissues, would be responsible for basal and wound-induced ethylene production, while system II would be responsible for the upsurge of ethylene production during ripening of climateric fruit (Pech et al., 2003)

Ethylene (C_2H_4) is a simple gaseous plant hormone that is biologically active in trace amounts, as little as 10 nL L-1 air (Pech et al., 2003). It is important both in normal development and for plant response to stress. During normal development ethylene is thought to coordinate events such as senescence, abscission, and fruit ripening. It also affects growth of vegetative tissues including stems, roots and petioles. Ethylene biosynthesis is increased in response to a large number of stimuli including wounding, pathogen attack, mechanical stimulation and drought and is known to exert its effects, at least in part, by altering gene expression. Effects on both transcriptional and post-transcriptional processes have been identified (Deikman, 1997).

The ethylene biosynthesis pathway has been intensively studied for its role in fruit ripening. It’s catalysed by two enzymes, *ACS* (1-aminocyclopropane-1-carboxylate

synthase) and *ACO* (1 aminocyclopropane-1-carboxylic acid oxidase) both encoded by multi-gene families (Figure1). The first step is the formation of 1-aminocyclopropane-1carboxylic acid (ACC), the immediate precursor of ethylene, from S-adenosyl-L-methionine (SAM) by *ACS*. The second step converts ACC to ethylene through the action of *ACO*. The former is considered the rate-limiting step of ethylene production (Kende, 1993).

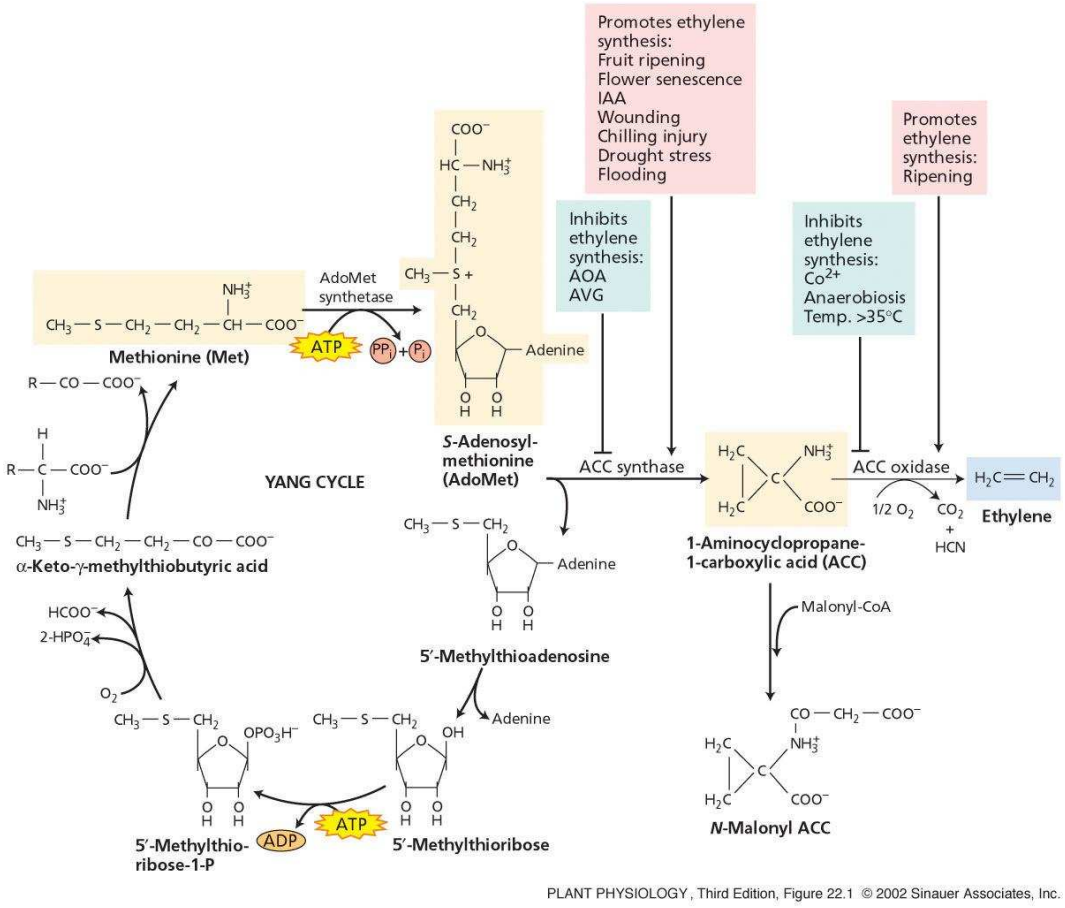


Figure 1. Ethylene biosynthetic pathway.

The competence of climateric fruits to synthesize autocatalytic ethylene is developmentally regulated and requires as primary step the stimulation of *ACS* and *ACO* gene expression by non ethylene regulatory factors. Then autocatalytic ethylene production proceeds via the up regulation by ethylene of its biosynthetic genes. Since both *ACO* and *ACS* are encoded by multigene families it can be speculated that this transition to autocatalytic ethylene production may be related to a cascade of expression of different members of the gene families (Pech et al., 2003).

1.4 Apple ripening

Apple (*Malus* x *domestica* Borkh) is a member of the Rosaceae family, sub family pomoideae, which includes many crop species such as pear, strawberry, cherry, peach, apricot, almond, forest and ornamental crab apple species. Members of the pomoideae have a fruit that consists of two distinct parts: ovary-derived tissues restricted to the center of the mature fruit (core) and the cortex or edible portion of the fruit which is derived from the fused base of stamens, petals and sepals, which expands to surround the ovary.

Apple fruit develop over a period of 150 days from pollination to full tree ripeness with a simple sigmoid growth curve (Janssen et al., 2008). During apple fruit development different overlapping physiological events, such as cell division and expansion, starch accumulation, starch decline and ripening, occur and lead to the fully ripe fruit.

Apple is a typical climateric fruit, whose ripening is associated with an upsurge in the rate of respiration and ethylene production (Figure 2).

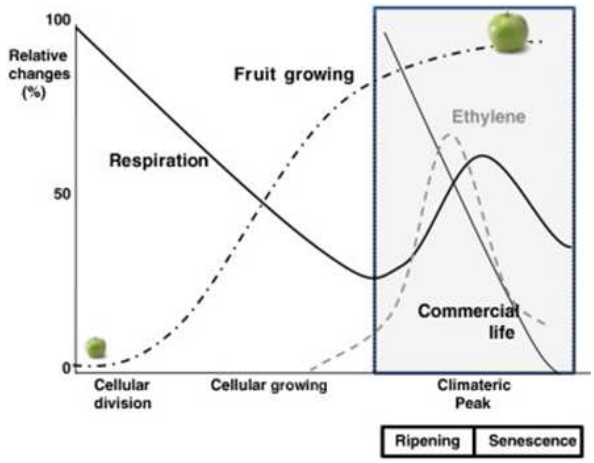


Figure 2. Qualitative evolution of respiration rate, fruit growing, ethylene levels and commercial life of Granny Smith apple (from M.Castro-Giraldez et al., 2010).

Due to its long history and widespread cultivation and more importantly its out-crossing nature, apple exibits a high level of heterozygousity and great variation in ripening behavior and quality attributes (Zhu et al., 2012). The ripening season of apple can differ up to 3 months among the elite apple cultivars under the same weather condition (Zhu et al. 2012). The rates of ethylene biosynthesis in apple fruits differ considerably among cultivars leading to varied storage properties ranging from rapid post-harvest deterioration to cultivars that can be stored for up to a year under optimal conditions.

A positive correlation exist between ethylene production during storage and softening (Barry and Giovannoni, 2007). Though apples can be stored under the controlled conditions for considerably long periods, its storage capability depends on cultivars. In apple breeding programs, genotypes with inherent long-term storage capability have therefore been used to develop new commercially acceptable varieties.

Considering the prevalent role of genotype in the determination of apple ripening behaviour, the knowledge of the mechanisms underlying ripening in each cultivar has to be considered essential to improve growing and commercial handling practices. Due to the preponderant role of ethylene in ripening process the two enzymes involved in its synthesis during fruit ripening, *MdACS1* and *MdACO1* have been deeply studied. Both genes are reported to affect the loss of firmness after harvest and thereby fruit shelf life. *ACS* is the major gene, *ACO* still having a less strong but still clear effect and acting independently of *ACS* during apple ripening (Costa et al., 2005).

1.5 *ACS* multigene family

1-Aminocyclopropane-1-carboxylate synthase-gene (*ACS*) is the key enzyme in the synthesis of ethylene and has become the hot spot in the study of ethylene. It is a cytosolic enzyme with a very short half-life, its intracellular concentration is low and the active form is labile (Kende,1993). It belongs to a family of proteins that require pyridoxal-5'-phosphate (PLP) as cofactors, known as PLP-dependent enzymes. In particular *ACS* is evolutionary related to a member of this family, the aminotransferase family. The crystal structure of apple *ACS* revealed that the overall folds and catalytic site of this enzyme are very similar to aspartate aminotransferases (Capitani et al., 1999) and that the enzyme forms a homodimer. A lot of *ACS* genes have been isolated and identified from a variety of plant species including tobacco, arabidopsis, rice, apple, potato, tomato, pear, banana, etc. where *ACS* is encoded by a highly divergent multigene family.

A typical *ACS* gene consist of four exon and three introns and most of *ACS* genes has scattered genomic organization. The percent of the shared homology of all *ACS* genes may be very different and even as low as 49% aminoacid identity as reported for *Arabidopsis thaliana.* Some of deduced aminoacid sequences of *ACS* isoenzymes are more similar to those from the other species than to the members of *ACS* family in the same species *(*Jakubowicz and Sadowski, 2002).

The different *ACS* genes are involved in the ethylene synthesis in different tissues and at different plant developmental stages. They have distinct regulation and the main control

point of enzyme activity is at transcriptional level. The differential transcription of *ACS* genes during the course of development and in response to various external cues such as wounding, chilling, drought, auxin, ripening and senescence, is one of the mechanisms that regulates the production of ethylene. However, mechanisms of post translational regulation, such as proteolitic processing and protein phosphorilation/dephosphorilation, have also been proposed to explain the sometimes observed tight correlation between ethylene evolution and transcript accumulation of *ACS* genes (Dal Cin et al., 2005).

1.6 *ACS* genes in apple

In apple at least five *ACS* genes have been reported which include *MdACS1* (accession no. U89156), *MdACS2* (accession no. UO3294), *MdACS3a* (accession no. U73816), *MdACS4* (Kim et al., 1992) and *MdACS5* (MdACS-5A accession no AB034992 and 5B accession no AB034993). However, the isolation of nine and eleven *ACS* genes in tomato and arabidopsis respectively induce to presume that other *ACS* genes are also present in apple genome. Tan et al. (2012) reported to have identified three new *MdACS* genes differentially expressed during apple fruit ripening but these genes are not yet characterized.

The sequence similarity of the overlapping segments of *MdACS1*, *MdACS2*, *MdACS3* and *MdACS5* was found to ranges from 47% to 72,4% indicating that these gene are quite different (Costa et al., 2005). *MdACS2* do not appear to be expressed in the normal development of ripening fruit tissue (Wiersma et al., 2007). *MdACS4* was isolated by Kim et al. (1992) but it has not been publisched in a database. Kondo et al. (2009) reported that *MdACS4* is not produced during fruit ripening but rather is directly induced by auxin. *MdACS5B* was reported to play an important role in apple fruitlet abscission (Dal Cin et al., 2005) as well as in wound response (Sunako et al., 2000). *MdACS1* and *MdACS3* are reported to be expressed in apple fruits (Wakasa et al., 2006; Wiersma et al., 2007), also if with different expression profiles (Tan et al., 2012). They are the better studied members of the *MdACS* multigenes family.

MdACS1 was first found to be expressed in apple fruits (Harada et al., 2000; Wakasa et al., 2006) so most studies to unravel the role of ethylene in apple fruit ripening have focused on this gene. The *MdACS1* expression remains below detection levels throughout all of the earliest fruit development stages and only at the ripening its expression strongly increase, moreover its expression is enhanced by ethylene so it's considered to be involved in the system 2 ethylene biosynthesis (Tan et al., 2012). Two different alleles (*MdACS1-1* and *MdACS1-2*) of this gene have been identified (Sunako et al., 1999). Most of the cultivars

that are homozygous for *MdACS1-2* allele have a very pronounced reduction in ethylene production and this is well correlated with the increase of firmness and with a longer shelf-life in respect to the cultivars, both homozygous or heterozygous, carrying the *MdACS1-1* allele (Harada et al., 2000). However some apple cultivars homozygous for *MdACS1-2* allele can also show different storage capability (Wang et al., 2009). Sunako et al. (1999) also reported the absence of transcription from *MdACS1-2* in a heterozygous cultivars but they supposed that the promoter of this allele still function, since a very low level of transcription was detected in fruit of cultivar Fuji that is homozygous for *MdACS1-2* allele. *MdACS1* was mapped on linkage group 15 but the region was not previously identified as QTL for fruit firmness and it's at a quite large distance from a previously identified minor QTL (Costa et al., 2005). An insertion of a retrotransposon-like sequence (SINE) in the promoter region of the *MdACS1-2* allele has been found and proposed (Sunako et al., 1999; Harada et al., 2000) as the responsible for the reduction of transcription and for the consequent low ethylene evolution. Oraguzie et al. (2004) suggested also that both *MdACS1* genotype and maturity season affect apple fruit softening and that neither alone can determine softening behaviour. *MdACS1* allelic composition is also reported to be responsible for pre-harvest drop rate: apple cultivars of *ACS1-2/2* trees have less fruit drop than the *MdACS1-1-1* or *1-2* trees (Sato et al., 2004).

Only recently *MdACS3* gene has been proposed to have a role in this fine ethylene production regulation. The expression of *MdACS3* is detectable in immature fruit several weeks before harvest, and decreases after the robust expression of *MdACS1* and *MdACO1* and the burst of ethylene production. *MdACS3* is regulated by negative feedback mechanism in apple fruit. So *MdACS3* is supposed to be involved in regulating system-1 ethylene biosynthesis and the transition to system 2 (Tan et al., 2012). Three sub-family *MdACS3* genes, *a, b* and *c* have been isolated but two of them (*b* and *c*) possess a transposon-like insertion in their 5' flanking region which causes failure of their transcription (Wang et al., 2009). *MdACS3a* is the only functional *ACS3* gene in apple and three different alleles were identified in different apple cultivars. Only one of them show activity while the other two are inactive, for the loss of enzyme activity resulting from an aminoacid substitution at the active site and for a still uncharacterized lack of transcription (Bai et al., 2012).

2. TRANSCRIPTION

Transcription is that process by which the information residing in a double stranded DNA molecule (dsDNA) is transferred to a nascent RNA molecule. It's the first step of gene expression and the synthesis of most eukaryotic proteins is regulated at this level. In plant cells genes are transcribed by enzymes known as DNA-dependent RNA polymerase. In eukaryotes there are three different RNA polymerases: RNA polymerases I and III transcribe the genes that encode transfer RNA, ribosomal RNA and various small RNAs. RNA polymerase II transcribes all the other genes, including all those that encode proteins, first producing pre-mRNA which can mature into mRNA molecules. RNA Polymerase II binds to a regulatory portion of plant genes located upstream of the transcriptional start site (TSS) and that is referred as promoter region.

2.1 Promoter region

The promoter region consists of specific DNA sequences that act in the recruitment of protein factors that facilitate transcription of the protein-coding region of the gene. The RNA Polymerase II promoter is composed of three regions: core promoter, proximal and distal promoter regions. Core promoter is a DNA region that contain common core DNA elements, where the initiation complex, RNA polymerase together with general transcription factors, is assembled. It is located -50 to about +40 bp relative to TSS. The DNA elements in the core promoter region are necessary and sufficient for accurate initiation of transcription by RNA Polymerase II but only in an inefficient way. The region located immediately upstream of the core promoter, proximal region, contains the most important regulatory elements. In this region, gene specific regulatory elements assure the binding of regulatory transcription factors which can interact with the general factor and RNA polymerase II to promote transcription. Those DNA sequences located on the same strand as the coding region of the gene are known as *cis*-acting regulatory elements (CARE) while all the transcription factors that are encoded at other genetic loci are referred to as *trans*-acting factors. Other *cis*-acting sequences, distal element, are located further upstream of the proximal promoter sequences and can exert control over eukaryotic promoters in positive or negative way. In plant they are usually located within 1000 bp of the TSS (Komarnytsky and Borisjuk, 2003). In many cases, transcriptional regulation involves the binding of transcription factors at sites on the DNA that are not immediately adjacent to the promoter of interest. These *cis*-acting module, called enhancers or silencers, can be located hundreds

or thousands of base pairs upstream or downstream of the gene they control, within coding region or intronic sequences, and even in regions of the chromosome structurally adjacent to the promoter. They can work in either orientation (Farrell, 2007). It is thought that enhancers act through the binding of activating transcription factors, subsequent recruitment of additional co-activators and the formation of DNA loops that bring these factors in proximity of the relevant promoter. Many enhancer elements have been studied and it was found that they are required for maximal transcriptional activation of a gene. By contrast, very few silencers, that have the ability to repress gene expression, have been characterized. Gene transcription repressors can function by competing for DNA binding with activators, by masking the activation interface or by direct interaction with general transcription factors (Gaston and Jayaraman, 2003).

2.2 Promoter control of transcription

The complexity of specific gene regulation at the level of transcription is therefore dependent on the type, number, position and combination of enhancers and silencers present in and around the coding sequence of a particular gene and on the activation of the enhancer sequences, which is dependent on the presence, absence, or activity within the cell of their associated binding proteins (Potenza, 2004). Moreover, it's difficult to believe that these sequence motifs alone are wholly responsible for RNA Pol–promoter interaction. Experimental evidences suggest that sequence-dependent secondary properties of promoters are also important in their function. Three of such properties that are often involved are stability, curvature and bendability of DNA in promoter regions (Farrell, 2007). A supplementary layer of complexity is added by bringing the transcription factors together on a promoter, by adopting a three dimensional configuration, enabling the interaction with other parts to activate the basal transcription machinary (Rombauts et al., 2003).

2.2.1 Promoter regulatory elements involved in ripening

Only few plant promoter are known to be constitutively expressed in most plant cells, while the majority of them has a temporal and spatial specific expression. This fine regulation is the results of highly coordinated expression networks based on the interaction between transcription factor and *cis*-acting elements. So the promoter of each gene has a specific composition of regulatory elements and genes that are co-expressed or co-regulated are expected to have similar conserved regulatory motif. Different *cis*-acting-elements that

respond specifically to different internal or external stimuli have been reported in plants (Komarnytsky and Borisjuk, 2003).

Different genes involved in the ripening process have been identified but molecular characterization of their promoter region have only recently begun to unravel the mechanism by which genes are regulated. Ripening in climacteric fruit is mainly the results of a combination of ethylene regulation and development control, moreover fruit specific regulation of some ethylene regulated genes and regulatory mechanisms that operate separately from and in addition to ethylene also exist (Giovannoni, 2001). The *cis*-acting elements that are involved in fruit specificity, and that mediate development and ethylene control could be separated. Indeed, in the promoter region of the *cucumisin* gene from melon two distinct regions, one with a fruit specific enhancer and an other that contain I-box-like sequence, that act as negative regulatory element, have been found (Yamagata et. al., 2002). Although fruit-specific, ripening-related and ethylene-induced genes have been isolated and analyzed for a number of species, not many essential *cis*-elements have been identified. A functional ethylene responsive element was identified in the tomato fruit ripening genes *E4* an *E8* (Montgomery et al., 1993; Deikman, 1997) that is different from the GCC box involved in the ethylene activation of plant defense gene. Also some fruit-specific elements have been isolated from different promoter region. Yin et al. (2009) identified a TCCAAAA motif in the promoter region of *ADP-glucose pyrophoshorylase* gene of watermelon, that function as a fruit-specific element by inhibiting gene expression in leaves and the TGTCACA motif, an enhancer element necessary for fruit-specific expression, already reported in the *cucumisin* gene in melon (Yamagata et. al., 2002). In addition, elements that confer expression under specific condition could be present as seen for the *GalUR* gene of strawberry whose expression is restricted to the fruit in a light-dependent manner and whose promoter region is characterized by few known light-responsive elements (Agius et al., 2005).

2.3 Promoter identification and analysis

Identification and functional dissection of the region that harbor regulatory control elements of a gene is an essential step in its full characterization. The promoter analysis should bring to the definition of a minimal promoter region able to drive basal transcriptional activity and to the identification of putative binding site for transcription factors as well as enhancer or repressor regulatory modules. Local chromatin states, and availability of scaffold attachment regions and DNA methylation sites, may further

contribute to the activity of the 5'-flanking region. However, few promoters have been well characterized compared with the number of genes studied, partly due to complicated interactions between a large number of *cis*-elements in promoters and their associated nuclear transcription factors.

Two different strategies are usually used at the same time to study promoter regions: *in silico* analysis using computational methods and *in vivo* laboratory experiments. *In silico* analysis are based on the scan over the entire sequence by a consensus approach. Different web-based databases, such a Plant CARE and PLACE, collect short DNA motif sequences present in plant *cis*-acting regulatory regions, obtained from earlier published research works or from article reviews on the regulatory regions of various plant genes, and allow to search for these *cis*-acting regulatory elements (CARE) in the sequence of interest. Also if the results of this preliminary *in silico* analysis should be carefully interpreted and validated they allow more focused laboratory investigation.

3. *In vivo* ANALYSIS OF PLANT PROMOTER

In vivo analysis is based on the generation of promoter/reporter gene fusion constructs and their expression analysis in plant tissues. Screening the expression level of reporter gene, strength and spatial/temporal pattern of promoter activity could be determined. The promoter region is in general "chopped" in more smaller fragments to study the contribute of different regions to the expression level. These analysis allow also to identify, by progressive deletion analysis, binding sites for transcriptional activator or repressor protein. These results should be validated by gain of function experiments, in which the sequence under study is fused to a minimal promoter-reporter construct, and with gel electrophoresis mobility shift assay (EMSA) that brings to the identification and purification of the correspondent transcriptor factor. The fulfilment of *in vivo* analysis require the cloning of the fragment to be studied in an appropriate expression vector and a protocol for its transfer and analysis in plant tissues.

3.1 Expression vectors

A vector is a DNA molecule that is capable of replication in a host organism, and can act as a carrier molecule for the transfer of genes into the host. Different kind of vectors have been designed to perform specific function, expression vectors have been designed to construct gene fusions that replace native promoter of a gene with another promoter.

An expression vector should have some essential features: a simple ways to construct reporter gene fusion; a reporter gene that allows the detection of promoter in a quantitative and qualitative way; resistance genes for selection of transformed bacteria and plant tissues, and all the sequences necessary for its replication in bacterial cells. If the vector should be transferred in *Agrobacterium tumefaciens* a binary vector is needed (Lee and Gelvin, 2008) that has the same features described above plus left and right borders flanking the region to be transferred and replication function for *Agrobacterium* host.

Different vectors or binary vectors have been generated, to allow the cloning of a promoter region upstream a reporter gene, with a restriction/ligase strategy that require the presence of unique restriction site in vector sequence (http://www.cambia.org/; Hellens et al., 2005). This process however could be labourios and time consuming so to overcame this problem vectors that exploit bacteriophage lambda site-specific recombination system has been generated. In this case target sequences are first captured in a commercially available

'entry vector' and are then recombined into various 'destination vectors' for expression in different experimental organisms (Hartley et al., 2000)

Different reporter genes are available and has been used in promoter analysis: *β*-glucuronidase (*GUS*) from *E.coli* or *Staphylococcus sp.* that are usually used in plant system, luciferase gene (*LUC*) from firefly and green fluorescent protein (GFP) from jellyfish that allow a real time expression analysis. Often two different reporter genes are used simultaneously in transient transformation assay one for the promoter analysis and the other one to check the transformation procedure (Spolaore et al., 2003).

Resistance genes should be chosen in function of availability of reliable selection protocol for the tissue and species used in the transformation assay to avoid the risk of escapes or excessive loss of plant material. The *hptII* gene encoding resistance to hygromycin, or the *nptII* gene encoding resistance to kanamycin are two of the most used resistence genes employed in plant transformation. Plant resistence genes are usually driven by very strong and constitutive promoters such the Cauliflower Mosaic Virus promoter (CaMV35S) to ensure successful selection, so it can occur that the activity of the promoter of interest, often much weaker or more specific, is influenced by that of the plant selection gene. In this case it would be better to remove the selection gene at all, if it's possible, or adopt a co-transformation strategy in which two vectors bringing one the resistance gene and the other the promoter of interest, are separately transformed or co-transformed into plant tissues.

Different expression vector, already prepared for promoter cloning, could be purchased or obtained for free, in case of academic research, or they can be generated by themselves introducing in an existing vector the desired features.

3.2 Transformation strategies

Chimeric promoter/reporter construct for functional analysis can be introduced in plant permanently, by stable transformation methods, or could be expressed only temporary in the new system with a transient transformation strategy. Stable genetic transformation involves two distinct stages: the delivery of DNA into the nucleus of a competent cell and the regeneration of plant from the transformed cell (Jones et al., 2009). Therefore it could be applied only in species not recalcitrant to regeneration and, also if a good protocol for regeneration is available, it could be a lengthy manipulative process, especially in perennial plants as apple. Moreover, the analysis of promoter region require the assay of high number of chimeric constructs bringing to the generation of a huge amount of transformed plants

and also several transgenic lines should be analyzed for each construct studied because copy number and site of integration can affect transgene expression. As consequence cost and time of research considerably increase. Prior to stable transformation, screening of promoter regions using a transient expression assay would be advantageous. Thus the number of constructs to be studied in stable transformed plants could be reduced to a minimum, by disregarding constructs which perform poorly in the transient assay.

Transient expression is based on expression of heterologus DNA that is not integrated into chromosomes. In this case the analysis of gene expression is not confused by position effects, it's free from the interference of chromatin structure and reflect the situation *in planta.* Due to the temporary nature of this kind of transformation it's not required to regenerate transformed cells. These features make transient transformation the better choice for functional genomics and promoter testing especially in woody trees like apple in which stable genetic transformation is a lengthy and tedious process with a low success rate.

The recombinant DNA could be transferred into competent cells by physical (direct) or mediated DNA delivery methods. The first comprise particle bombardment, electroporation, polyethylene glycol (PEG), or microinjection, the second group of methods are based on the natural gene transfer abilities of bacterial and viral pathogens (Jones et al., 2009). Protoplast have frequently been used for transient expression analysis of regulatory sequences in promoters activated by external stimuli (Abel and Theologis, 1994), however to have more reliable information on *in vivo* situation the analysis in intact plant tissues and organs has to be preferred. Two of the most widely used transient transformation system for *in vivo* plant promoter studies has been particle bombardment and *Agrobacterium*-mediated transformation.

By biolistic method biologically inert particles (usually tungsten or gold) coated with DNA of interest are accelerated in a partial vacuum and placing the target tissue within the acceleration path, DNA is effectively introduced (Gan, 1989). Uncoated metal particles could also be shot through a solution containing DNA surrounding the cell thus picking up the genetic material and proceeding into the living cell. A perforated plate stops the shell cartridge but allows the slivers of metal to pass through and into the living cells on the other side. The particle gun methods require specific devices that allow to bombard DNA directly into plant tissues and same parameter such as the size of the gold particle, the distance between the sample and the macro-carrier, the amount of DNA used in each bombardment and the number of shots per samples should be optimized for each plant tissue.

Agrobacterium transformation exploit the naturally occurring gene transfer system present in the common gall-inducing bacterium *Agrobacterium tumefaciens.* This bacteria is able to induce tumors by the conjugative transfer of a DNA segment (T-DNA) from a bacterial tumour-inducing (Ti) plasmid to plant cell. The plasmid T-DNA can be integrated into the genome of the host cell and the tumor morphology genes on the T-DNA are expressed. To allow its use for biotechnological purpose the plasmid has been 'disarmed' by deletion of the tumor inducing genes. The only essential parts of the T-DNA are its two small (25 base pair) border repeats, at least one of which is needed for plant transformation. The genes to be introduced into the plant are cloned into a plant transformation vector that contains the T-DNA region of the disarmed plasmid, together with a selectable marker (such as antibiotic resistance) to enable selection for plants that have been successfully transformed. Plants are grown on media containing antibiotic following transformation, and those that do not have the T-DNA integrated into their genome will die.

Agrobacterium-mediated transient gene expression (agroinfiltration) is based on expression of non-integrated DNA. During early stages of co-cultivation single stranded T-DNA is transferred from the bacteria to plant cells. Once moved into the plant cell this T-DNA becomes double stranded and migrates to the nucleus. Only a tiny part is integrated into the host chromosomes while not-integrated T-DNA copies persist in the nuclei of transfected cells where remain transcriptionally competent for several days (Hellens et al. 2005). Transgene expression and protein accumulation is localized to the site of infiltration and can be detected only 3h after DNA delivery, peaks after 48h and persist for 10 days (Jones et al. 2009). This transformation method has been applied with success in different plant species, however because two living organisms participate in the process the efficiency of transformation is greatly influenced by the compatibility between plant and bacterium and can vary from host to host.

3.3 Transient transformation of fleshly fruits

The increasing number of expression studies in fruits has generated in the last years a big amount of fruit-specific or ripen-related gene sequences. Understand the regulatory properties of their regulatory regions can contribute to unravel complex expression patterns and to planning with more awareness biotechnological studies for fruit quality improvement. Regulation of gene transcription however depends also on interaction between DNA motif and tissue, developmental and species specific transcriptor factors. Also if in some case a conserved heterologous regulation between different species has been demonstrated

(Tittarelli et al., 2009) that is not true for all the gene or plant species (Augias et al., 2005). Gene studies in fruit as been performed especially in the model plant tomato for which transformation and regeneration protocols are common and easy to be applied. However, the generation of transformed ripe fruit can take different years for perennial fruit tree species. So the set up of good transient transformation protocols for the analysis of promoter in fruit of different species has became a big challenge for biotechnological researchers. The specific physiological and anatomical features of fruits required an adaptation of the existing protocol for transformation with *Agrobacterium* or particle gun, in particular when ripe fleshy fruit are adopted. These fruits are indeed characterized by large vacuolated cell whose walls undergo marked changes in their structure.

Augias et al. 2005 were able introducing a step of osmotic treatment in the protocol for particle gun to transform with success ripe strawberry. This methodology was used successfully also for the transformation of immature melon (Yamagata et al., 2000), and watermelon (Yin et al., 2009) and juice sacs from green mature fruits of acid lemon and acidless lime (Sorkina et al., 2011).

Specific adjustment to the *Agrobacterium*-transient transformation method has also been done to allow its application in fleshy fruit. Spolaore et al. in 2001 published an agroinjection transformation method of intact fruits. It's based on the injection with a syringe with a needle of transformed agrobacteria in a specific infiltration media directly in detached intact fruits. The transformation rate was evaluated with two reporter gene GUS and LUC in a qualitative way on apple, pear and orange, and with a quantitative assay in peach, and strawberry. This method was applied with success for the analysis of promoter in peach in which it allowed also to study regulation by temperature of cold-inducible promoter (Spolaore et al., 2003; Tittarelli et al., 2009). It's applicability was also tested in fruit of rough lemon (Ahamad and Mirza, 2005), and banana (Matsumoto et al. 2009). A modified injection methods with infection of strawberry fruits still attached to the plant demonstrated that this method is also suitable for analyzing gene functions during the development of strawberry fruits (Hoffmann et al. 2006).

Despite agroinjection has been applied with success in some species, some problems has still to be resolved. A high variability between fruit at different stage of development and between different tissues has been observed in agroinjection of mature and immature fruits of rough lemon (Ahamad and Mirza, 2005). The spatial expression patterns observed with agroinjection seem at least partially governed by constraints imposed by fruit architecture and the ability of the bacteria to reach the different tissues in the fruit (Spolaore et al., 2001;

Orzaez et al., 2006). Consequently, interpretation of the spatial expression patterns obtained by agroinjection should take these considerations into account. Particular attention should also be paid to the choice of reporter gene in function of the species in which the transformation assay is performed. Spolaore et al. 2001 reported that in strawberry the reporter activity related to protein amount was higher for GUS than for LUC while the opposite occurred in the case of peach. The choice of transient transformation method for analysis of promoter region in fruit depends therefore from the availability of optimized species specific protocol. In some cases similar results were obtained using agroinfiltration or biolistic method (Augias et al., 2005), however these correspondence should be proved for each species.

II AIMS OF THE THESIS

Apple ripening is influenced by many environmental factors but the genotype is the most important in its determination. Therefore, the knowledge of the mechanisms underlying ripening in each cultivar has to be considered essential to improve growing and commercial handling practices.

Apple is a typical climateric fruit, whose ripening is associated with an upsurge in the rate of respiration and ethylene production. The rates of ethylene biosynthesis in apple fruits differ considerably among cultivars and this variability is reported to be at the base of their storage capability.

Ethylene synthesis is catalyzed by two enzymes, *ACS* (1-aminocyclopropane-1-carboxylate synthase) and *ACO* (1-aminocyclopropane-1-carboxylic acid oxidase) both encoded by multi-gene families. *ACS* is considered to catalyzes the rate-limiting step of ethylene production.

Most studies have focused on the climacteric *MdACS1* gene to study the role of ethylene in apple fruit ripening. Two different alleles (*MdACS1-1* and *MdACS1-2*) of this gene have been identified. Most of the cultivars that are homozygous for *MdACS1-2* allele have a very pronounced reduction in ethylene production and this is well correlated with the increase of firmness and with a longer shelf-life in respect to the cultivar carrying the *MdACS1-1* allele. However, it is also reported that apple cultivars with the same *MdACS1 2-2* genotype show different patterns of firmness loss.

The *MdACS1-2* allele is reported to have a very low expression. An insertion of a retrotransposon-like sequence in the promoter region of the *MdACS1-2* allele has been found and proposed as the responsible for the reduction of transcription in fruits and for their consequent low ethylene evolution.

However, the specific expression of each *MdACS1* allele has never been reported in literature as well as any in vivo analysis of the 5'-flanking region of *MdACS1*.

The aim of this thesis was to increase the knowledge on the molecular mechanisms responsible for the different ethylene production in apple cultivars. Considering the main role of *MdACS1* gene in apple ethylene biosynthesis, the characterization of this gene was the main task of our research. The differential expression of each *MdACS1* allele was evaluated by specific qPCR expression analysis and with an *in vivo* analysis of their 5'-flanking regions by transient transformation assay. Since the only one protocol for transient transformation

analysis in apple fruit reported in literature has never been applied in research studies a second task of the thesis was its evaluation and optimization for promoter analysis.

Moreover, since the *MdACS1* genotype cannot alone explained all the storage variability between some apple cultivars an assessment of ethylene production on two apple cultivars (Fuji and Mondial Gala) with the same *MdACS1* genotype but different ripening behaviour was planned. To better investigate the genetic bases of ethylene production in these two genotypes, a segregating progeny derived from the cross between Fuji and Mondial Gala was also assessed.

III MATERIALS AND METHODS

1. ISOLATION AND ANALYSIS OF *MdACS1* ALLELES SEQUENCES

1.1. Plant material and DNA extraction

Genomic DNA (gDNA) was isolated using the CTAB method (Doyle and Doyle, 1987) from leaf tissue of the following apple cultivars growing in Department of Fruit Tree and Woody Plant Sciences (University of Bologna) fields: Florina, Durello di Forlì, Mondial Gala, Fuji, Jersejmac, GoldRush and McIntosh.

1.2. Assessment of apple cultivars *MdACS1* allelotype

The *MdACS1* allelotype of different apple cultivars (Florina, Durello di Forlì, Mondial Gala, Fuji, Jersejmac, GoldRush and McIntosh) was assessed with primers specific for the promoter region of *MdACS1* (Harada et al., 2000, sequences in table 1) by PCR. The PCR amplifications were performed in a 17,5 µl of volume containing 50 ng of DNA, 0,1 µM primers, 1,5mM $MgCl_2$, 100µM dNTPsS, 0,5 Unit DNA Polymerase (Fisher Molecular Biology, Hampton, NH, USA) and 1X reaction buffer. The thermal cycler performed the following thermal profile: 94 °C for 2 min and 30 sec, 35 cycles of 60 °C for 45 s, 72 °C for 2 min, 94 °C for 45 s followed by a final anneling and extension at respectively 60 °C for 45 s and 72 °C for 10 min. The amplicons were visualized on an Image Station 440 CF (Kodak, Rochester, N.Y., USA) after electrophoresis in 1,5% (w/v) agarose gel and ethidium bromide staining.

Region	Primer name	Sequence 5' → 3'
Promoter	ACS1-5' for	AGAGAGATGCCATTTTTGTTCGTAC
	ACS1-5'rev	CCTACAAACTTGCGTGGGGATTATAAGTGT
Gene	ACSS For	GGTGCAACTTCAGCGAATGAG
	ACS Rev	CAGGTTCCGTGCAATGACAAGA

Table 1. Primer pairs used for the PCR-based BAC screening.

1.3. Apple BAC library

A BAC library from the cultivar Florina (Vinatzer et al., 1998) already available at the Department of Fruit Tree and Woody Plant Sciences (University of Bologna) was used. The library consists of 36,864 BAC clones with an average insert size of 120 kb, representing

approximately 5 X apple haploid genome equivalents. BAC screening was performed on the bi-dimensional pooled samples as reported by Cova (2008). The horizontal pool consisted of 96 samples (plate pool) each containing all the BAC clones from a single 384-wells plate. The vertical pool consisted in 4 X 96 samples prepared by bulking the clones of a specific position (i.e. A1, A2, ecc...) from all the original 384-wells plates (96 clones/well) and plasmids from the BAC clone pools were extracted using the alkaline extraction procedure (Birnboim and Doly, 1979).

1.4. PCR-based screening of the BAC library

The BAC library was screened for *MdACS1* promoter and gene sequences by PCR. Specific primers (ACS1-5' for and rev, described in Harada et al., 2000) able to distinguish between the two *MdACS1* alleles were used for promoter screening and ACSSF-ACSR (sequences in table 1) for the gene with the same PCR thermal profile reported for the assessment of *MdACS1* allelotype in apple cultivars. The positive BAC clones identified with the screening were picked up from the library, singularized and tested by colony PCR with the same primers used for the screening. The PCR amplifications were performed in a 17,5 µl of volume containing 200 ng of DNA from BAC library pools, 0,1 µM gene/promoter-specific primers, 1,5mM $MgCl_2$, 100µM dNTPsS, 0,5Unit DNA Polymerase (Fisher Molecular Biology, Hampton, NH, USA) and 1X reaction buffer. The amplicons were visualized on an Image Station 440 CF (Kodak, Rochester, N.Y., USA) after electrophoresis in 1,5% (w/v) agarose gel and ethidium bromide staining.

1.5. Analyses of positive BAC clones

Plasmid DNA from each positive BAC clone was extracted by alkaline lysis/PEG treatment protocol (http://csb.wfu.edu/brf/plasmidprep.pdf). Approximately 20 µg of DNA from each plasmid were digested with 5U *Eco*RI overnight at 37°C. Digested fragments were loaded onto 1% agarose gel and electrophoresed at 35 V overnight. Profiles of *Eco*RI-digested DNA fragments of positive BAC clones were used to identify overlapping BAC clones. Two BAC clones, one for each *MdACS1* allele, positive for both promoter and gene were chosen and sequenced by primer walking (Bio-Fab Research srl Pomezia, Italy) with a set of specific walker primers designed with the Primer3 software (http://frodo.wi.mit.edu/primer3/) to cover the full-length sequence of *MdACS1* gene and promoter region. Final assembly was carry out manually with the CAP3 software (Huang and Madan 1999).

1.6. *In-silico* analysis

The two sequences obtained by primer walking (*MdACS1-1* and *MdACS1-2*) were compared to each other and with two other Gene Bank accessions (no. U89156 and no. AB010102), both genomic sequences of *Md-ACS1* gene from cultivar 'Golden Delicious'. All sequence alignments were made using ClustalW2 at http://www.ebi.ac.uk/Tools/clustalw2/. The sequences were also screened for the presence of putative *cis*-acting elements using PLACE (Higo et al. 1999) at http://www.dna.affrc.go.jp/PLACE/

2. EXPRESSION ANALYSIS OF *MdACS1* ALLELES

2.1. Primer design

Alignment of the coding sequences of Florina *MdACS1-1* and *MdACS1-2* was performed with the software ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html) looking for SNPs among *MdACS1* alleles coding sequences. Specific primers pair were designed in SNPs containing regions with the software Primer3 (http://frodo.wi.mit.edu/primer3/). To ensure high specificity and efficiency during qPCR amplification the following set of criteria were used for primers design: the primer lengths of 18-24 nucleotides, a guanine-cytosine content of 20-80% and PCR amplicon lengths of 70-200 base pairs. Each primer pairs was also tested with the software PrimerSelect® v8.0- MegAlign for the formation of primer homo and heterodimers.

2.2. Specificity validation of primer

Designed primers were first tested on genomic DNA (gDNA) of cvs Florina, Durello di Forlì and Mondial Gala (extracted as previously reported in paragraph 1.1) for their ability to produce an amplicon. The allele specificity of primers was validated checking their ability to give amplification only on the corresponding BAC plasmid DNA. Each primer pair was tested on two different BAC plasmids: one containing the specific allele targeted by the primer pair (positive control) and one containing the other *MdACS1* allele (negative control). Where amplification was detected also in negative control, the conditions were further optimized in order to increase gene specificity by adjusting primer concentration (from 100 to 60 nM) and annealing temperature (from 60 to 64°C). All the amplifications were performed in a 17.5 ul volume containing 50 ng of DNA, 60-100 nM allele specific primers, 1.5 mM $MgCl_2$, 100uM dNTPss, 0.5 Unit AmpliTaq Gold® DNA Polymerase (Applied Biosystem, Foster City, CA, USA) and 1X reaction buffer. A gradient PCR was used to assess the right anneling temperature for specific amplification. The reaction included an initial 2 min denaturation at 94°C, followed by 38 PCR cycles (30 s at 94 °C, 30 s at 60-64 °C and 45 s at 72 °C) followed by 30 s at 94 °C, 30 s at 60 °C and a final extension of 2 min at 72 °C. The amplicons were visualized on an Image Station 440 CF (Kodak, Roachester, N.Y., USA) after electrophoresis on 2 % (w/v) agarose gel and ethidium bromide staining.

To further validate the specificity of our primers the two Florina gDNA amplicons obtained with each primer pair and the PCR products of the amplification of Florina flesh

cDNA with primers specific for *MdACS1 1-1* allele were sequenced (BIO-FAB Research). Retrieved sequences were compared with that of BAC clones.

2.3. Plant material, RNA extraction and cDNA synthesis

Apple fruits were collected at the Cadriano Experimental Station, Bologna University, Italy, from Florina, Gala, Fuji and Durello di Forlì trees at different stages of fruit development (T2 = one month before ripening; T3 = at ripening). Apple skin and flesh were separately frozen in liquid nitrogen and stored at -80 °C. Fruits RNA extractions were carried out according to Pagliarani et al. (2009) starting from 6-8 g of frozen tissue.

The expression of the two *MdACS1* alleles was also investigated in cotyledon, leaf, stem and root of a Florina seedling heterozygous for *MdACS1* gene and in Florina flower. The plant materials were frozen in liquid nitrogen and stored at -80°C until RNA extraction. The RNA was extracted from 0.1 g of leaves with the Spectrum^{TM} Plant Total RNA Kit (Sigma-Aldrich) and quantified using a Nanodrop^{TM} ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). First-strand cDNA was synthesized according to Paris et al. (2009) starting from 1ug DNA-free RNA. The cDNA was diluted 1:9 and its quality was verified by the amplification with *actin* specific primers (Paris et al., 2009).

2.4. Conditions of qPCR reaction

The qPCR reactions were performed in triplicate in a final volume of 10 µl containing 5ul of Power SYBR® Green Master Mix 1X, 60nM of each primer, PCR-grade water and 1:9 cDNA from apple skin and flesh or vegetative tissues. Reactions were incubated at 50 °C for 2 min and 95 °C for 10 min to activate the AmpliTaq Gold® DNA Polymerase, followed by 40 cycles at 95 °C for 15 sec and 63 °C for 1min. The gene expression was evaluated as presence or absence according to the detectability of the raw dye fluorescence by the qPCR machine. Where amplification was detected also in negative control the conditions were further optimized in order to increase gene specificity by adjusting DNA or primer concentrations. To ensure the absence of unspecific PCR products and primer dimers, an heat dissociation protocol (from 60 °C to 95 °C) was also performed and a dissociation curve for each samples was generated. The StepOne Software version 2.1 (Applied Biosystem) was used to analyse the fluorescence data. The qPCR raw data were analysed with the standard curve method, and with *actin* as reference gene. Primers used for this reference genes were: MdActF/MdActR reported in Paris et al. (2009). In order to minimize the influence of PCR conditions on gene expression values, in each plate the *actin*

amplification was performed under the same conditions as the relative *MdACS1* primer pair. Amplicons obtained with each specific primer pair and with primers for *actin* have been used for the preparation of standard curves which consisted in a ten-fold dilution series of the amplicons over six dilution points. These standard curve samples were used as reference for qPCR amplifications with the specific primer pairs. The optimal threshold was chosen automatically by the StepOne Software version 2.1 (Applied Biosystem) and was used to calculate the threshold cycles (Ct) value for each standard curve point. Ct values in each dilution were measured in duplicate and were plotted against the logarithm of their initial template concentration. Each standard curve was generated by a correlation coefficient (R^2) of the plotted points. The final results represent the transcript amount levels of *MdACS1* alleles normalized with the transcript amount levels of *actin*, so they can be defined also as relative expression levels. They are expressed as Arbitrary Unit (A.U.).

3. CLONING OF PROMOTER SEQUENCES

3.1 Primer design for promoter fragments amplification

A series of forward and reverse primers, including a tail with *Hind*III and *Nco*I restriction site respectively, were designed to amplify the promoter fragments for deletion analysis, gain of function experiments and site-directed mutagenesis experiments.

3.1.1. Reverse primers

Two different reverse primers, each specific for one *MdACS1* allele, were designed starting directly upstream from the ATG start codon in a promoter sequence in which the two alleles differ for 2 bp: PromS for the *MdACS1-1* allele and PromL for the *MdACS1-2* allele (Table 2).

3.1.2. Forward primers for deletion analysis

Specific forward primers (Table 2) were designed all along the promoter sequences to produce nested deletion constructs. C1 For, C2-3 For, C4-5 For, C6-7 For and C6-7noere primers were designed in sequence regions identical for the two alleles and where used in PCR with DNA of the BAC clones resulted positive for the presence of the two *MdACS1* alleles; C6not For and C7not For primers were designed specifically for *MdACS1-1* and *MdACS1-2* respectively.

3.1.3. Forward primers for gain of function experiments

Three specific forward primers (Table 2) were designed to have an *Hind*III tail for cloning, the sequence of the element to be tested and the anneling sequence of the C1 For primer. The C1tx1 For and C1-gt1 For primers were used to amplify with the reverse primer PromL the plasmid DNA with *MdACS1-2* allele and C1tx2 For to amplify with PromS rev the plasmid DNA with *MdACS1-1* allele.

3.1.4. Forward primers for site-directed mutagenesis experiments

Four forward primers, C6tx2 For, C6tx2snp For, C7tx1 For, C7tx1snp For (Table 2) containing the desired mutation (Table 3) were designed to specifically modify the promoter regions of the two *MdACS1* alleles. They were used with the appropriate reverse primer and DNA template (primers sequences and primers/DNA combination in table 3) in a two steps PCR process.

To specifically remove a retrotransposon element from the promoter region of *MdACS1-2* allele two 5′-phosphate-labelled PCR primers, C7sine-P For and Rev (Table 2), were designed just upstream and downstream the region to be deleted.

Reverse Primer	**Sequence 5' → 3'**
Prom L	agtgcgtgcc**ccatgg**TTTGGTTAATTTTCTACTGTATGGA
Prom S	agtgcgtgcc**ccatgg**TTTGGTTAATTTTCTTGTGTATGGA
	tail - ***NcoI***
C7sine-P Rev	P-ACAAGAATTAATACTCGGGTCTAGTTT
Forward Primer	
Deletion fragments	
C1 For	gctaaccgat**aagctt**CAGTGTGACGTGTCATTCCT
C2-3 For	acggattcag**aagctt**CCCCACGCAAGTTTGTAG
C4-5 For	gctaaccgat**aagctt**TCGCTCTTGGCATTTTCTA
C6-7 For	gctaaccgat**aagctt**CCAAATTCTCCTCTAAATGAACG
C6not For	gctaaccgat**aagctt**CGCAAAACTCTCGGTACTGTT
C7not For	gctaaccgat**aagctt**CGCAAAACTCTTGGTACTGTT
C6-7noere For	gctaaccgat**aagctt**GTTCGTACCGGATTTTCGAG
Gain of function experiments	
C1tx1 For	gctaaccgat**aagctt**GGTTAACAAAAAGCAGTGTGACGTGTCATTCCT
C1tx2 For	gctaaccgat**aagctt**GGTTAACAAAAAGGGTTAACAGTGTGACGTGTCATTCCT
C1-gt1 For	gctaaccgat**aagctt**GGTTAACAGTGTGACGTGTCATTCCT
	tail - ***HindIII***
Site-directed mutagenesis	
C6tx2 For	CCAAATTCTCCTCTAAATGAACGGTTAACAAAAAGGGTTAACAAAAAGTAAACTTTAACG
C7tx1 For	CCAAATTCTCCTCTAAATGAACGGTTAACAAAAAGGAAACTTTAACG
C6tx2snp For	CCAAATTCTCCTCTAAATGAACGGTTAACAAAAAGGGTTAACAAAAAGGAAACTTTAACG
C7tx1snp For	CCAAATTCTCCTCTAAATGAACGGTTAACAAAAAGTAAACTTTAACG
C7sine-P For	P-CACGATTAATGCTTCTATGTACACTT

Table 2. Sequences of the primers used for the amplification of promoter fragments. Restriction site used for cloning the PCR products into the binary vector pCAMBIA0305.1 are indicated in bold. The underlined bases represent the sequence to be introduced or modified in the site-directed mutagenesis experiments.

3.2 Strategies and conditions of PCR reaction to generate *MdACS1* promoter fragments

Different amplifications strategies were used to specifically amplify each kind of promoter fragments. All the fragments were amplify by Herculase II Fusion DNA

Polymerase (Agilent Technologies, Inc.). The PCR amplifications, if not differently indicated, were performed according to the manufacturers instruction in a final volume of 50 µl, under the following conditions: denaturation 94 °C for 3 min; 35 cycles: 94 °C for 1 min, 58 °C for 1 min, 72 °C for 2 min and 30 sec; extension 72 °C for 10 min. Prior to ligation the PCR products were double digested overnight with *Hind*III and *Nco*I (Fermentas) according to the double digestion reaction in manufacturer instruction and gel purified.

The C1, C2-3, C4-5, C6-7, C6-7noERE and C6-7noT fragments were generated with a single PCR reaction at the above conditions.

For the generation of C6TX2, C6TX2SNP, C7TX1 and C7TX1SNP mutated promoter fragments a two steps PCR was performered. Specific forward primers described above were used with the appropiate reverse primer in a first PCR using 50 ng of BACs DNA. One µl of the amplification product of this PCR, a mutated promoter fragment with a *Nco*I restriction site at one end, became the template for a second PCR reaction with C6For/Prom S and C7For/Prom L primer pairs to allow the addition of the *Hind*III tail.

The deletion of the retrotransposon sequence from the promoter region of the *MdACS1-2* allele was performed with a polymerase chain reaction based on amplification of the circular plasmid DNA sequence that excludes the fragment to be deleted (Pinera et al., 2006). The expression vector containing the C7 fragment was used as template. The amplification condition were the following: denaturation 95 °C x 4 min; 15 cycles: 92 °C for 20 min, 56 °C per 20 s, 68 °C for 15 min; 16 cycles: 92 °C for 20 sec, 56 °C for 20 sec, 68 °C for extension time increased of 40 sec each 2 cycles; 4 cycles: 92 °C for 20 min, 56 °C per 20 s, 68 °C for 21 min; final extension 68 °C for 8 min). Different plasmid DNA amounts (1ng, 5ng, 10ng) were tested as template. The amplification product, after gel purification was treated with *DpnI* (Promega) according to the manufacturer's instructions and gel purified again prior to a self blunt end ligase reaction.

Construct	Forward Primer	Reverse Primer	Allele DNA Template	Mutation	Mutated Sequence	Position (a)
C6TX2	C6tx2	Prom S	1-1	Insertion	GGTTAACAAAAAG	-1605
C6TX2SNP	C6tx2snp	Prom S	1-1	Insertion	GGTTAACAAAAAG	-1605
				Single base change	T→G	-1603
C6SNP	C7tx1	Prom S	1-1	Single base change	T→G	-1603
C7TX1	C7tx1	Prom L	1-2	Deletion	GGTTAACAAAAAG	-1754 to -1743
C7TX1SNP	C7tx1snp	Prom L	1-2	Deletion	GGTTAACAAAAAG	-1754 to -1743
				Single base change	G→T	-1742
C7SNP	C6tx2	Prom L	1-2	Single base change	G→T	-1742

Table 3. Primers sequence and primers/DNA combination used for site directed mutagenesis **(**a). All the position are calculated in respect of ATG codon on the respective *MdACS1* allele sequences.

3.3. Preparation of plasmid DNA

3.3.1 pCAMBIA0305.1 vector

To clone all promoter fragments a pCAMBIA0305.1 vector (Figure 3) was chosen between the huge number available for free at CAMBIA (Canberra, Australia). This vector, a derivative of pCAMBIA 1305.1 (AF354045), is a compact binary vector (9661 bp) with the pBR322 *ori* and *bom* sites for high copy replication in *E. coli* and transmission by mating. It contains the broad host range pVS1 *ori* for low copy, stable replication in *A. tumefaciens.* The expression cassette between T-DNA border sequences is composed of a GUS*Plus* reporter gene with an intron from the castor bean catalase gene to prevent expression by bacteria and ensure detection of plant-expressed glucuronidase activity. The expression of the GUS gene is driven by a CaMV35S promoter. Just upstream this promoter region, a truncated *lacZ alpha* (functional for blu/white screening) containing a multi-cloning site (MCS) is present. A kanamycin resistence gene driven by CaMV35S promoter allows selection in bacteria. No selectable markers for selection in plant are present.

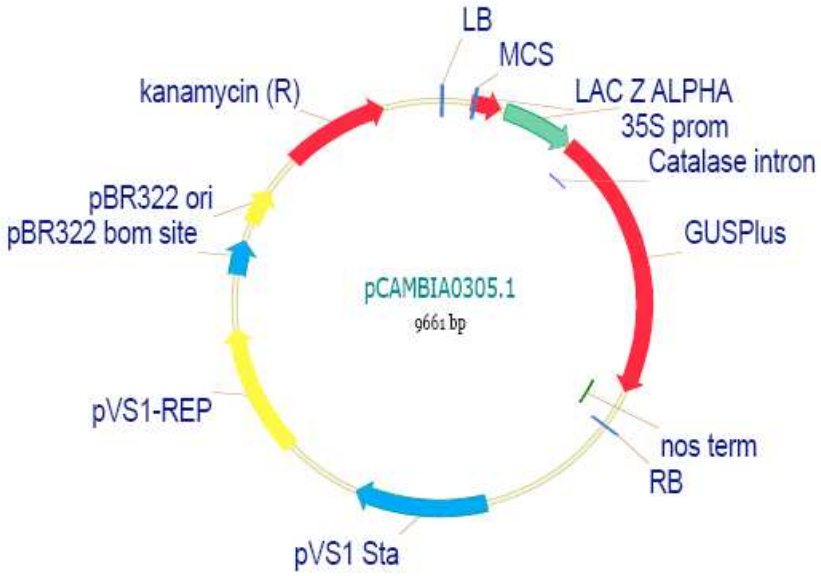


Figure. 3 Schematic map of the pCAMBIA0305.1 vector

3.3.2. Modification of pCAMBIA0305.1 vector

The original binary pCAMBIA0305.1 plasmid with the CaMV35S promoter in front of the GUS*Plus* gene was used as positive control.

This vector was instead modified to prepare the negative control and the vectors with the promoter fragments for the transient transformation assay. A restriction analysis of the entire plasmid sequence was performed with ReBase software (Roberts et al., 2010).

The binary pCAMBIA0305.1 plasmid was transferred to DH5α competent *Escherichia coli* cells by freeze and thaw method and extracted with alkaline lysis/PEG treatment. The CaMV35S promoter in front of the GUS*Plus* was eliminated with an overnight double digestion at 37 °C of 5µg of the extracted pCAMBIA0305.1 with 1U of *Hind*III and 0,5 U of *Nco*I restriction enzymes (Fermentas). The digested plasmid was loaded onto 1% agarose gel and electrophoreted at 35V overnight to separate the linearized vector from the CaMV35S fragment. The vector band was cleaned with the Wizard sv gel and PCR clean-up system (Promega) according to the manufacturer instructions.

This modified vector was used to obtain a promoter-less vector (negative control – pCAMBIA0305.1-35S) after filling-in the 5'-overhangs with Klenow enzyme (Promega) according to the manufacturer instructions and a 3 hours blunt end ligation (200 ng vector DNA, 2U Ligase, 0,1 µg/µl BSA, 1X ligase buffer in a final volume of 10 µl) at 16 °C to re-circularize the vector.

3.4. Cloning of promoter fragments in the expression vector

The open modified vector pCAMBIA0305.1-35S was used to clone promoters fragments. The promoter sequences were inserted before the GUS*Plus* reporter gene after the digestion with the proper restriction enzymes. The insert-to-vector molar ratio for ligation was fixed at 3:1 and the DNA amount of promoter fragment used in the ligation reaction was calculated according to the following equation:

$$X = \frac{3 \times insert\ lenght\ (bp) \times 200}{9661\, bp\ (vector)}$$

where *X* is the amount ng of promoter fragment required for a 3:1 insert to vector molar ratio when 200 ng of the vector pCAMBIA0305.1-35S are used. The ligation mix with 2 Unit T_4-DNA ligase was incubated overnight at 16 °C and used immediately or stored at 4°C.

3.4.1. Transformation of competent *E. coli* cells

The pCAMBIA0305.1-35S negative control and the pCAMBIA0305.1-35S vectors containing promoter::*GUSPlus* fusion constructs were transformed into either JM109 (Promega) or DH5α *E. coli* competent cells prepared as follow. A dilution 1/100 of an *E. coli* overnight 2XYT (Appendix A) culture was performed and the bacteria were grown at 37 °C until the suspension reached an OD_{550} of 0,6. After 10 min of incubation on ice, the bacteria were collected by centrifugation at 5000 rpm for 10 min at 4 °C. The *E. coli* were then resuspended in 5ml 100mM $CaCl_2$ with 14% glycerol and stored at -80 °C.

For *E.coli* transformation 3 µl of ligase mix were added to 100 µl of defrosted competent cells, the samples were incubated for 30 min on ice, then warmed for 45 sec at 42 °C in a water bath and chilled 2 min on ice. The bacteria were diluted with 950 µl SOC medium (Appendix A) and incubated for 1 hour and 30 min at 37 °C with shaking. An aliquot of the resulting bacterial suspension (120 µl) was plated on LB medium (Appendix A) with kanamycin 50 mg/l.

3.4.2. Cloning verification and sequencing

Bacterial colonies were selected and analysed for insert presence by colony PCR amplification with specific primers designed on the vector sequence (pc0305.1 For TTTATGCTTCCGGCTCGTAT, pc0305.1 Rev GAGAAAAGGGTCCTAACCAAGAA) or with ACS1-5' for and rev primers (Table 1). The PCR amplifications were performed in a 17,5 µl of volume containing bacterial cells as DNA template, 0,1 µM primers, 1,5mM $MgCl_2$, 100µM dNTPsS, 0,5Unit DNA Polymerase (Fisher Molecular Biology, Hampton,

NH, USA) and 1X reaction buffer under the following conditions: initial denaturation 94 °C for 2 min and 30 s; 35 cycles: denaturation 94 °C for 45 sec, annealing 60 °C for 45 sec, polymerization 72 °C for 2 min; final extension 72 °C for 10 min. The plasmid DNA was isolated from positive colonies with the alkalin-method.

Promoter fragments sequences were verified by DNA sequencing (BIOFAB Research - Pomezia) using the pc0305.1 F/R primers. For constructs larger then 600 bp, further sequencing reactions were performed using internal primers specifically designed in order to cover the entire sequence. The single sequences were assembled by CAP3 (Huang and Madan, 1999) and compared with the original ones by ClustalW.

4. TRANSFORMATION OF *Agrobacterium tumefaciens*

The *Agrobacterium tumefaciens* strain EHA105, harbouring a resistence gene for rifampicin, was chosen as vector for transient transformation assay. The pCAMBIA0305.1 modified vectors containing the different promoter::*GUSPlus* fusion constructs as well as the positive and negative control vectors were transferred to competent *Agrobacterium tumefaciens* strain EHA105 by freeze/thaw shock transformation method. In order to produce *A. tumefaciens* competent cell, a single colony was inoculated in 5ml YEP broth pH 7.2 (Appendix A) supplemented with rifampicin (50mg/l) and grown at 28 °C overnight at 130 rpm. 100 ml fresh YEP broth pH 7.2 were inoculated with 4 ml of the O/N culture and placed on shaker at 28°C until the suspension reached an OD_{600} of 0.8. The bacteria were chilled on ice and collected by centrifugation at 3900 g for 5 min (4 °C). Agrobacteria were finally resuspended in 2 ml $CaCl_2$ (20mM), divided in 0.2 ml aliquots into pre-chilled microfuge tubes, freezed rapidly in liquid nitrogen and stored at -80 °C. For transformation 2 μg of plasmid DNA were added to 100 μl of agrobacteria defrosted competent cells, the samples were incubated for 5 minutes on ice, then submerged in liquid nitrogen for 5 minutes and thawed in 37°C water bath for other 5 min. After, 1 ml of LB broth was added and the bacteria were shaken for 4 h at 28 °C. Then 100 μl were plated on YEP medium containing the proper antibiotics (rifampicin 50mg/l and kanamycin 50mg/l) and incubated at 28 °C for 48 hours. In order to verify the effective transformation, single colonies were dissolved in 10 μl of NaOH 20 mM and warmed at 37 °C for 5 minutes. A 2 μl aliquot of this bacterial solution was used as template for a colony PCR amplification with the same primers and under the same reaction conditions used for the verification of the of *E. coli* transformation. Bacterial colonies that provided a positive signal were grown for two days in selective liquid YEP medium containing 50 mg/l kanamycin and 50 mg/l rifampicin and prepared for storage at -80 °C by mixing 1ml bacterial culture with 0.5 ml sterile glycerol (60%).

5. APPLE AGROINFILTRATION

5.1 Plant material

Commercially ripe apples (*Malus* x *domestica*) were purchased at a market in Bologna or collected in DCA fields. Some immature apple fruits were also used. Different apple cultivars (Florina, Golden Delicious, Annurca, Topaz, Gala, Fuji and Pink Lady) were tested. All the fruits were first washed in water added with Tween 20 (0,05%) and then rinsed with autoclaved distilled water before infection. The pH of the apple flesh was assessed by litmus paper.

5.2 Preparation of agrobacterium suspension

Agrobacterium cultures (5ml) from individual colonies were grown for two days at 28 °C in liquid YEP medium pH 7,2 plus selective antibiotics at 130 rpm. 50 ml of YEP medium pH 5,6 plus kanamycin (50 mg/l), 1 mM proline L, 20 µM acetosyringone and 10 mM MES were inoculated with the saturated culture until OD_{600} 0.02 and incubate at 28 °C at 130 rpm. The cells were grown to log phase (OD_{600} 0.8) and recovered by centrifugation (10 minutes at 3200 rpm). The cells were then re-suspended in infiltration medium (Appendix A) at the desired concentration and incubated for 4 hours at room temperature with gentle agitation before infection.

5.3 Infection methods

Different infection methods were assayed. All experiments were carried out three times. More then five explants were infected and three injection per fruit were done for every promoter construct tested.

5.3.1 Agroinfiltration of intact fruit

The *Agrobacterium* suspension was evenly injected throughout the whole apple intact fruit by means of a sterile 1 ml hypodermic syringe after Spolaore et al. (2001) agroinfiltration protocol or apple slices were submerged in an agrobacteria solution stirred for 15 minutes or kept under vacuum condition for 10 minutes. Different agrobacteria concentrations were tested: OD_{600} 0.9, 1.5 or 2.5.

5.3.2 Sonication-assisted-agrobacterium transformation (SAAT) and vacuum infiltration treatments

For SAAT, apple flesh pieces of about 0.5 cm or half apple seeds were immersed in 2 ml Eppendorf tubes containing 1.5 ml of positive and negative control *Agrobacterium* suspension (OD_{600} 1,5) or in infiltration medium pH 5,6 without bacteria. The tubes were placed in the middle of a bath-type sonicator (Starsonic90, LIARRE) and subjected to ultrasound at 28-35 kHZ. The treatments differed as to sonication duration (5, 15, 30, 60 and 80 sec). After sonication the explants were maintained under vacuum condition (water vacuum pump) or submerged in *Agrobacterium* solution for further 10 min.

5.4 Co-cultivation period

The outside of the injected intact fruits was dried and the fruits were placed at 20-26 °C for 2 days or 6 days for the co-cultivation period until GUS staining assay. The sliced fruit were dried on sterile filter paper to remove the excess of bacteria and stored in 90 mm disposable Petri plates with filter paper under the lid wet with sterile water at the same condition of the infected intact fruits.

5.5 Histochemical detection of GUS activity

For the histochemical GUS assay the injected tissues were sampled and immersed into 2 mmol/L X-GlcA (5-bromo-4-chloro-3-indolyl β-D-glucoronide), 200mM or 100mM phosphate buffer pH 7, 0.1%(v/v) Triton X-100, 0.5 mmol/L $K_3Fe(CN)_6$, 0.5mol/L $K_4Fe(CN)_6$, 10mmol/L EDTA and 20% methanol. After 10 minutes under vacuum treatment the immersed tissues were kept overnight in the dark at 37°C. The tissues were rinsed in ethanol 96% prior to be evaluated. The samples were dried on filter paper and images were taken with the Image Station 440 CF (Kodak, Rochester, N.Y., USA). The pH of X-GlcA solution prior and after the introduction of apple flesh pieces was verify by litmus paper.

6. TRANSFORMATION OF LETTUCE AND IN VITRO APPLE LEAVES

6.1. Plant material

Iceberg variety of lettuce was purchased at a market in Bologna. The outermost leaves were discarded and the adjacent inner four leaves were used for the transformation assay. All the material was first washed in water added with Tween 20 (0,05%), rinsed with autoclaved distilled water before infection and dried on sterile filter paper. Leaves detached from in vitro-cultured shoots of cultivar Gala were used in apple leaf experiments.

6.2. Preparation of agrobacterium suspension

The agrobacteria suspension used for the infection was prepared as described for apple fruit transient transformation.

6.3. Infection, co-cultivation and histochemical detection of GUS activity

Lettuce or apple leaves were wounded with a sterile blade in different point. Then, they were incubated 10 minutes with stirring in an agrobacteria solution at OD_{600} 1.5 or OD_{600} 2.5. After infection, the leaves were blotted dry on sterile Whatman filter paper and placed on Petri plates with filter paper under the lid wet with sterile water at the same condition of the infected fruits. After the co-cultivation period the leaves were cut and an histochemical GUS assay was conducted at the same condition of the fruit transformation experiment. All experiment were carried out three times. More then five explants were infected for every promoter construct.

7. ASSESSMENT OF ETHYLENE PRODUCTION BY GAS-CHROMATOGRAPHY ANALYSIS

Apple fruits of Mondial Gala and Fuji cultivars were harvested at ripening stage and stored at 20-25 °C for all the duration of the experiment. Measures of ethylene were done at harvest and every 7 days until 28 days after harvest on 12 fruits/cultivar. A single apple was sealed for 60 min in a single 1,7 l glass jar with air-tight screw metal caps equipped with rubber septa for gas sampling. For ethylene measurement 10 ml headspace samples were taken and analysed with gas chromatography (Dani DS 86.01carrier nitrogen and helium).

A segregating progeny of 121 individual genotypes derived from the cross of Fuji x Mondial Gala available at the Experimental station of the of Fruit Tree and Woody Plant Sciences Department (University of Bologna) was used. Fruits at harvest physiological maturity stage were collected from each seedlings and stored at 20-25 °C for ethylene evolution analysis at 21 days after harvest. A single analysis for genotype was performed on 3-4 fruits/jar at the same conditions of the Fuji and Mondial Gala parents. The data were analysed with the following formula:

$$X = (ppm / hours \times gram) \times Volume\ of\ the\ jar \times 1000$$

where X is the amount of ethylene expressed in $\mu l\ g^{-1}\ h^{-1}$.

IV RESULTS

1. ISOLATION AND SEQUENCE CHARACTERIZATION OF *MDACS1* ALLELES

1.1. *MdACS1* allelotype of different apple cultivars

The preliminary PCR analysis with primer specific for the 5' flanking region of *MdACS1* gene of different apple cultivars worked very well. The results demonstrated that cultivar Florina and GoldRush are heterozygous for the *MdACS1* gene promoter; Durello di Forlì, Jersejmac and McIntosh are homozygous for *MdACS1-1*; Fuji and Mondial Gala are homozygous for *MdACS1-2* (Figure 4).

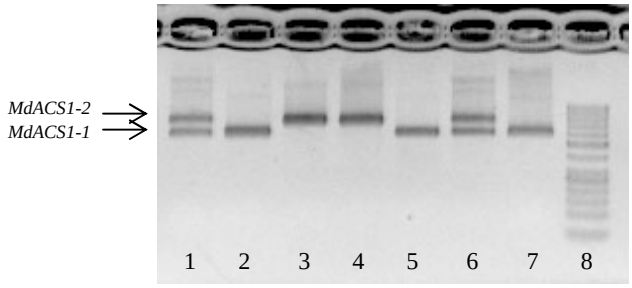


Figure 4. *MdACS1* allelic variation in different apple cultivars. Lanes: 1 - Florina; 2 - Durello di Forlì; 3 - Fuji; 4 - Mondial Gala; 5 – Jerseymac; 6 – GoldRush; 7 – McIntosh; 8 – Ladder

1.2. BAC library screening

The PCR screening of the pooled BAC library samples with primer specific for the promoter region of *MdACS1* gene revealed three clones carrying the long *MdACS1-2* allele and ten clones carrying the short *MdACS1-1* allele (Figure 5). Ten of these clones resulted positive also for the screening with primer specific for the coding region and overlap among the BAC clones was assessed according to their digestion profiles (Figure 5). BAC 96N17 and BAC 22F23 clones, carrying both the gene and promoter region, were chosen respectively for *MdACS1-2* and *MdACS1-1* further analysis.

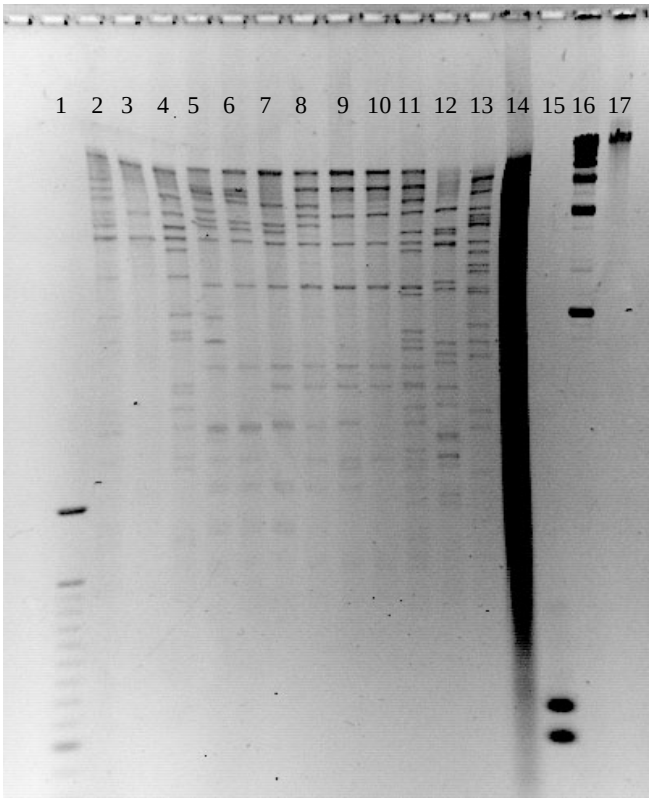


Figure 5: Profile of *Eco*RI-digested BAC clones. Lane 1, 100bp ladder; lanes 2-4, BACs with *MdACS1-2* allele; lanes 5-13, BACs with *MdACS1-1* allele; lane 14, *Eco*RI-digestion of Florina gDNA; lane 15, Forina *MdACS1* amplicon; 5Kb ladder; not digested BAC clone.

To sequence the full sequences of *MdACS1* alleles 10 primers were designed all along the sequence (Table 4). The primer walking allowed the assembly of unique full-length *MdACS1* sequences of 3985 bp for BAC 96N17 and 3752 bp for BAC 22F23.

Primer name	Sequence 5' → 3'	Position on	
		BAC 22F23	BAC 96N17
HaradaOUT	TCCGGTACGAACAAAAATGG	-1341	-1479
HaradaIN	ATCCCCACGCAAGTTTGTAG	-793	-853
Harada-1814	CAGTGTGACGTGTCATTCC	-225	-225
Harada-1521	GATCAATGTCTTTAGATGG	-515	-515
Harada-2237	GCATATATGTTACCATATGTAG	+214	+214
Harada-2618	GATCCCAACCACTTAGTGC	+577	+577
Harada-3168	CTCCGGCACAGCTTTTAGC	+1125	+1125
Haradaoutnew	CGCACAGGTTTTTATGTCTGC	-1206	-1403
Harada For	AGAGAGATGCCATTTTTGTTCGTAC	-350	-1488
Harada Rev	CCTACAAACTTGCGTGGGGATTATAAGTGT	-862	-862

Table 4. Primers used for primer walking on BAC clones.

The length of the 5' flanking region (until ATG codon) was approximately 1903 bp and 2137 bp for the *MdACS1-1* and *MdACS1-2* alleles respectively, and it is assumed to include the 5' untranslated region (UTR). The size of both *MdACS1* coding sequences was 1422 bp corresponding to a protein of 473 amino acids. The homology between the two *MdACS1* gene sequences from cultivar Florina was very high (ClustalW score = 99). Only seven nucleotide substitution were found within the coding sequence but due to the redundancy of the genetic code, six out of seven SNPs, were not causing any amino acid change. Only a substitution of guanine with adenine at position 3964 of the *MdACS1-2* gene is able to determine a change in the predicted amino acid sequence from Gly to Ser at position 468. Two more SNPs were detected inside the first and second intron. More differences were found between the two 5' flanking region of *MdACS1* of cultivar Florina (Appendix B). Using the nucleic acid comparison program CLUSTALW, the homology among the promoter regions of the two *MdACS1* gene was assessed. The overall identity between the two 5' flanking regions is quite high (ClustalW score = 98) but the local alignments between different promoter regions (Figure 6) showed a wide variation. The two 5' flanking sequences could so be divided into four regions according to their similarity degree (Figure 6). The position on the sequence of the different promoter regions is indicated in respect of the ATG codon. The first regions from -1 to -225 of the two alleles have an identity of 99% with only two nucleotide changes at -16 and -17. The identity of the second regions (-851 to -225) is of 98% and 7 SNPs were found. The third regions, from -851 to -985 on *MdACS1-1* and from -851 to -1123 on *MdACS1-2*, with a 60% identity are that with the bigger differences as already reported in Sunako et al. (1999). In more detail, a short interspersed nuclear element (SINE retro-transposon) of 162 bp (from -1045 to -884) is present only in *MdACS1-2* sequence while in the corresponding region of *MdACS1-1* sequence a 24 bp insertion from -908 to -884 was found. The forth regions (from -985 to -1639 on *MdACS1-1* and -1790 to -1123 on *MdACS1-2*) present 16 single nucleotide changes and a tandem repeat insertion of 13 bp from -1768 to -1743 in the *MdACS1-2* sequence. The identity between the two alleles in this region is of 97%.

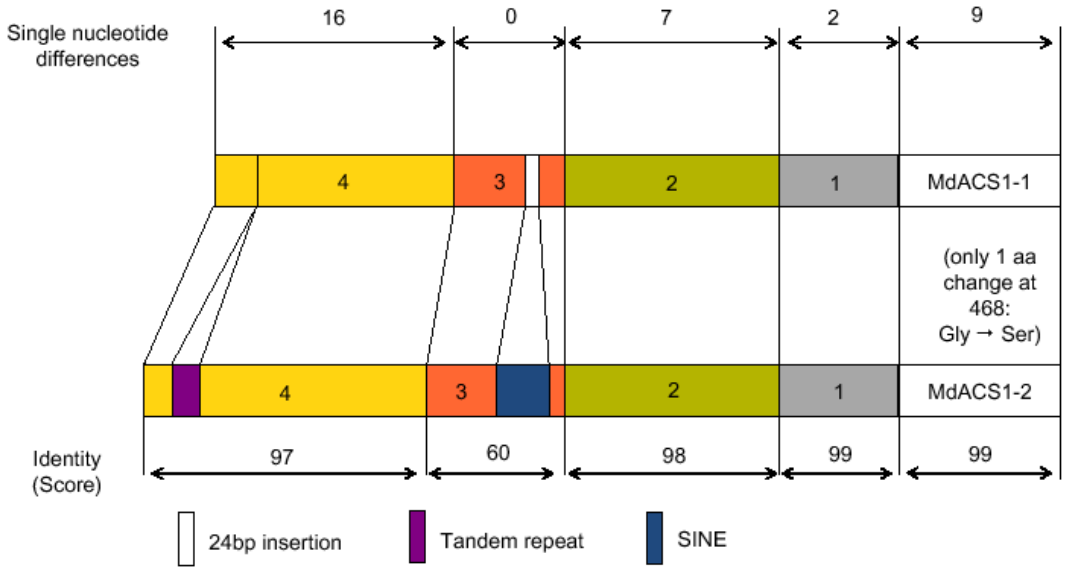


Figure 6. Schematic representation of the promoter region of the two *MdACS1* alleles . The 1-2-3-4- numbers correspond to the four regions identified on the base of the similarity analysis.

Alignment of our sequences with that in gene bank (5676 bp, accession no.AB010102 and 5526 bp accession no. U89156) showed no differences between the two *MdACS1-1* and two *MdACS1-2* coding sequences of Golden Delicious and Florina (Appendix B). A few SNPs were found between the Florina and Golden Delicious 5' flanking region sequences: two nucleotide substitution at 220 and 858 position on *MdACS1-2* Florina sequence and 7 SNPs (461, 478, 499, 549, 555, 763, 1887, 1888 sequence positions) on *MdACS1-1* of Florina.

1.3. *In silico* analysis of the *MdACS1* promoters

The screening of the two promoter regions of *MdACS1* alleles against PLACE database gave as output a lot of putative *cis*-acting elements all along the two sequences. The results were evaluated singularly for each promoter and making a comparison between the two alleles to search for interesting differences. A first analysis was done looking at the four regions in which the promoters were divided on the basis of similarity analysis. A very high number of *cis*-elements of light regulated genes, as GT1, I-BOX and GATA motifs were found. They were arranged along all the promoter sequences and 5' UTR region while they where not found in the region from -226 to -100 from ATG. In particular a dimerization of the GT1 element was found in the 16 bp tandem repeat in the 5’ flanking region of *MdACS1-2*. Also different transcriptional activator element (ARRIAT), gibberellin

responsive element (GARE) and DNA motif involved in cold induced response (MYCCONSENSUSAT) were found. Only one Ethylene Responsive Element (ERE - AATTCAAA) was localized in both the promoter regions (-1398 to – 1391 on *MdACS1-1*; -1626 to -1619 on *MdACS1-2*) in a zone with a very high concentration of other putative DNA binding elements.

While several putative *cis*-acting elements were present in both promoters, a few were specific for the *MdACS1-1* or *MdACS1-2* alleles. Between these some of particular interest are reported in table 5. The CCA1ATLHCB1 is related to regulation by phytochrome, it was found in the promoter region of an *Lhcb* gene in *Arabidopsis* (Wang et al., 1997). ELRECOREPCRP1 is an elicitor responsive element found in parsley, it's reported to bind WRKY transcription factors. SORLIP are sequences over-represented in the phyA-induced promoters (Hudson and Quail, 2003) while EF2consensus are involved in cell cycle regulation.

Allele	Factor	Site *	Strand	Motif	Species
MdACS1-1	EF2 CONSESUS	323	-	TTGCCCC	Tobacco
MdACS1-2	SORLIPAT2	910	-	GGGCC	Arabidopsis
MdACS1-2	ELRECOREPCRP1	920	-	TTGACC	Parsley
MdACS1-1	CCA1ATLHCB1	1200	+	AAMAATCT	Arabidopsis

Table 5. Putative *cis*-acting element specific for each *MdACS1* allele
*Distance from ATG codon.

2. EXPRESSION PROFILES OF *MdACS1-1* AND *MdACS1-2* ALLELES

2.1. *MdACS1* alleles primer design and validation

The sequences of the two *MdACS1* alleles derived from BAC clones sequencing were aligned (Figure 7). Due to the high homology of their coding sequences the choice for the primer design was constrained by the position of a few SNPs. Three SNPs were exploited to design two specific set of primer for each allele. It was possible to design only one reverse primer for each allele while two different forward primers for each allele were designed exploiting two more SNPs (Table 6).

Figure 7. Alignment of a portion of the coding region of *MdACS1* alleles. SNPs between the two alleles are indicated in gray. The qPCR primer sequences and position specific for *MdACS1-1* or *MdACS1-2* are reported in red and green respectively.

The different primer pairs were first tested by conventional PCR on gDNA of Florina (*MdACS1 1-2*), Durello di Forlì (*MdACS1 1-1*), Mondial Gala (*MdACS1 2-2*) and on plasmid DNA of 22F23 and 96N17 BAC clones. Many efforts were spent to avoid the generation of unspecific amplicons by optimising PCR conditions. The annealing temperature and the primers concentration for amplifications were adjusted for each primer pair in order to obtain an amplification signal only in the positive controls represented by the BAC clone containing the *MdACS1* allele under study. The results of gradient PCR experiments suggested 63°C as optimal anneling temperature and 60nM as optimal primer

concentration (data not shown). Under these conditions amplification signal was obtained for all the specific templates (Table 6) validating their ability to produce an amplicon. No unspecific signal was observed in the water control. The rtACS1-1aF primer wasn't able to amplify the Durello di Forlì gDNA while the rtACS1-2aF gave unspecific amplification with gDNA of cultivar Durello di Forlì and DNA of BAC 22F23, so these primers were rejected. The other two primer pairs, rtACS1-1bF/1R and rtACS1-2bF/2R, gave amplifications only with the expected templates (Table 6) so were used for further analysis with real time PCR.

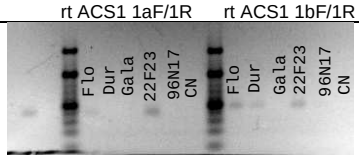
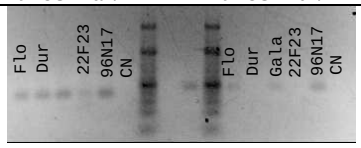
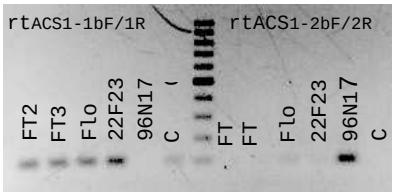
Allele	Primer name	Primer sequence 5' → 3'	
MdACS1-1	RT-ACS1-1aF	ACTC**A**AGAAGACAGTCGCTCAC**A**	rt ACS1 1aF/1R rt ACS1 1bF/1R 
	RT-ACS1-1bF	GCAGCCATTTAAGCCACTC**A**	
	RT-ACS1-1R	TTCATCTACCGGGAATAGGAC**C**	
			rt ACS1 2aF/2R rt ACS1 2bF/2R
MdACS1-2	RT-ACS1-2aF	CGAGAAGACAGTCGCTCAC**G**	
	RT-ACS1-2bF	CAGCCATTTAAGCCACTC**G**	
	RT-ACS1-2R	TTCATCTACCGGGAATAGGAC**T**	

Table 6. Sequence of *MdACS1* allele-specific primers for real time PCR and amplifications on DNA of different apple cultivars and the two BAC clones 22F23 and 96N17. Bold nucleotides in the primer sequences indicate SNPs between the two *MdACS1* alleles. Genotypes: Flo- Florina; Dur – Durello di Forlì; Gala- Mondial Gala. CN – negative control.

One more corroboration for the primers specificity was obtained by direct sequencing of the two products of the amplification of Florina gDNA with the two allele specific primer pairs and that of Florina flesh (T3) cDNA obtained by the amplification with *MdACS1 1-1* specific primer pair at the optimized conditions (Figure 8).



a)

```
Flo-ACS1 1/1  ctcaagaagacagtcgctcacaaagtgggttt------------------ 56
Flo-ACS1 1/2  ctcgagaagacagtcgctcacgaagtgggttt------------------ 56
cDNAFloT3     ctcaagaagacagtcgctcacaaagtgggttt------------------ 53
BAC22F23      ctcaagaagacagtcgctcacaaagtgggttt------------------ 240
BAC96N17      ctcgagaagacagtcgctcacgaagtgggttt------- ---------- 212
              *** ***************** **********
```

b)

Figure 8. a) Specific amplification of *MdACS1-1* (rtACS1-1bF/1R) and *MdACS1-2* (rtACS1-2bF/2R) on Florina flesh cDNA (T2 and T3 indicate the harvesting time, one month before ripening and at ripening respectively. Florina gDNA (Flo), negative control (C), BAC clones 22F23 and 96N17. b) Alignment of the sequences of Florina gDNA, Florina flesh (T3) cDNA amplicons, obtained with the two allele specific primers, and BAC 22F23 and 96N17 sequences. The SNPs between the two *MdACS1* alleles are highlighted

As shown in figure 8 the alignment of the products of sequencing with the two BAC clones confirmed the expected single target sequence and so the ability of the designed primers to discriminate between the two alleles. These primers were then used in real time PCR experiments to study the expression pattern of each allele in different tissues and stages of development. These primers are suitable for qPCR gene expression analysis with the SYBR-Green chemistry. In fact, they amplify short amplicons (from 75 to 93 bp) to ensure the efficiency of the Taq polymerase processivity. The analysis of the amplicon melting curves obtained with each primer pair provided a further validation regarding the primers. In fact, at the optimized conditions, single peaks in the heat dissociation curves were obtained indicating also the absence of primer dimers.

2.2. Expression levels of *MdACS1* alleles in the fruits of different genotypes

The expression levels of the two *MdACS1* alleles were evaluated firstly in flesh collected at ripening stage of different apple cultivars. The *MdACS1-2* allele was found to be not expressed in any cultivars even if the specific primers were amplifying a band of the expected size with genomic DNA as reported above (Figure 8a). The expression of *MdACS1-1* allele was not detected in the flesh of the two *MdACS1-2* homozygous cultivars Mondial Gala and Fuji while it was found to be expressed in both the heterozygous cultivar Florina and the homozygous cultivar Durello di Forlì. The expression level in Florina was

however more than double than that of Durello di Forlì as shown in figure 9. qPCR analysis on gDNA of all these cultivars was also performed with primer pair for both the *MdACS1* alleles. Slightly differences for the cycle treshold (Ct) value were found: *actin* Ct around 25 for all the cultivars; *MdACS1-2* allele Ct of 27-28 for Fuji and Gala respectively and *MdACS1-1* Ct of 28 or 25 for Durello di Forlì and Florina respectively.

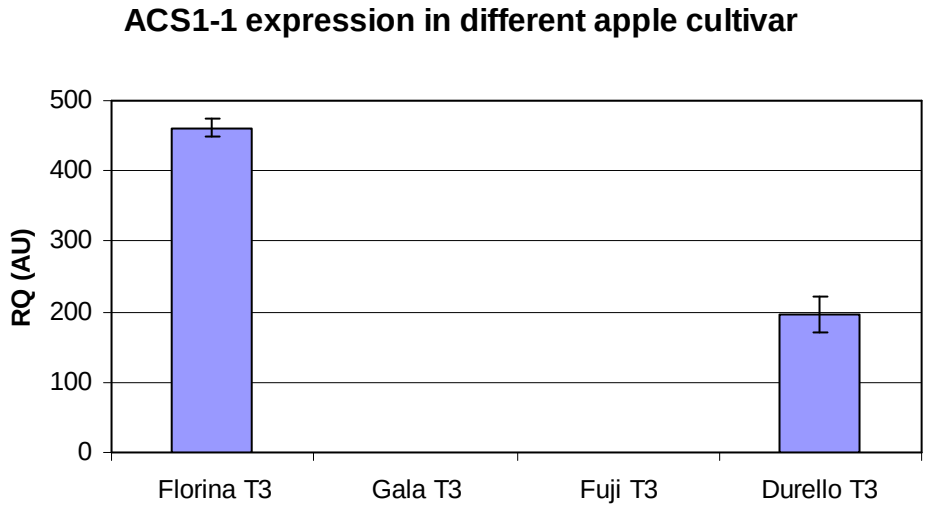


Figure 9. Relative *MdACS1 1-1* expression in different apple cultivars with different *ACS1* composition: Florina (heterozygous ACS1-1/ACS1-2), Gala and Fuji (homozygous for ACS1-2 allele) and Durello di Forlì (homozygous for ACS1-1 allele). T2 and T3 indicate the harvesting time, one month before ripening and at ripening respectively. Data are reported in arbitrary units (AU) as mean normalised expression. Bars represent the standard errors.

2.3 Expression levels of *MdACS1* alleles in different apple tissues at different developmental stages

The expression levels of the two *MdACS1* alleles were then evaluated in different apple tissues and in skin and flesh of fruits of the cultivar Florina at different developmental stages. Also in this case for *MdACS1-2* allele no detectable amplification was found in any of the analyzed tissues. Very low or undetectable level of expression was found for *MdACS1-1* allele in vegetative tissues. In particular, it was not expressed in stem and root, and only a weak expression was observed in cotyledon, leaf and flower. Almost undetectable was the expression in Florina skin at T2 stage while no amplification was found in Florina flesh at T2 stage. An accentuated differential expression of this allele has been found between skin and flesh of apple at ripening stage, with very low expression in skin and a very high abundance of transcripts in apple flesh (Figure 10).

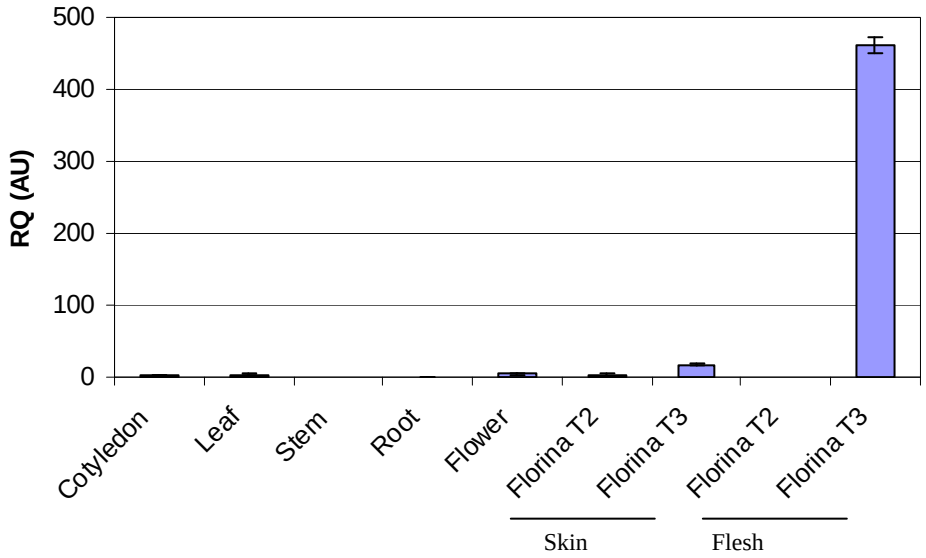


Figure 10. Relative *MdACS1-1* expression in different apple tissues and stages of development. T2 and T3 indicate the harvesting time, one month before ripening and at ripening respectively. Data are reported in arbitrary units (AU) as mean normalised expression versus fruit flesh at T2. Bars represent the standard errors.

3. *In vivo* ANALYSIS OF *MdACS1* PROMOTER

In order to reveal the causes of the lack of *MdACS1-2* expression in ripening fruits and to determine important regulatory regions, that drive the expression of the *MdACS1* gene, a detailed promoter analysis is required. The study of the activity of the promoters directly in apple fruit is the most desirable option. It could be done preparing chimeric constructs with the promoter or parts of it fused to a reporter gene and introducing them into plant cell. The *Agrobacterium*-transient transformation assay allows the simultaneous analysis of different promoter::reporter constructs in short time so this methodology was chosen for our experiments.

3.1. Optimization of transient transformation assay

Some preliminary experiments were done to verify the efficiency of a protocol for agroinfiltration of apple fruit reported in literature and to optimize an assay to test all the chimeric promoter constructs produced in this work. The optimization experiments were performed with two control constructs: a positive control in which the reporter gene GUS*Plus* is under control of the constitutive CaMV35S promoter and a negative promoterless control.

3.1.1. Positive and negative control constructs

In the pCAMBIA0305.1 vector the *GUSPlus* reporter gene is under control of a CaMV35S promoter so it is just ready to be used as positive control construct instead, it was necessary to remove by double digestion reaction the CaMV35S promoter to prepare the negative control (promoterless construct). On the basis of plasmid restriction analysis results *Hind*III and *Nco*I enzymes were chosen for the digestion because the sequences that they cleave (AAGCTT and CCATGG respectively) are present only once in the plasmid sequence. *Hind*III recognition sequence is in the multi-cloning site (MCS) while that of *Nco*I is very close to the CaMV35S promoter sequence to be excised (Figure 11). However the cut with these enzymes produced also the removal of part of the *LacZ* gene sequence resulting in a loss of gene functionality.

Figure 11. Detail of T-DNA region of pCAMBIA0305.1 vector. In bold letters the sequence of pc0305.1 primers

The product of the double digested pCAMBIA0305.1 vector loaded on agarose gel gave two bands of expected size, one of 762 bp corresponding to the CaMV35S promoter and part of the *LacZ* gene and the other of almost 8899 bp corresponding to the linearized vector (Figure 12). The digestion reaction produced a vector with two overhangs end.

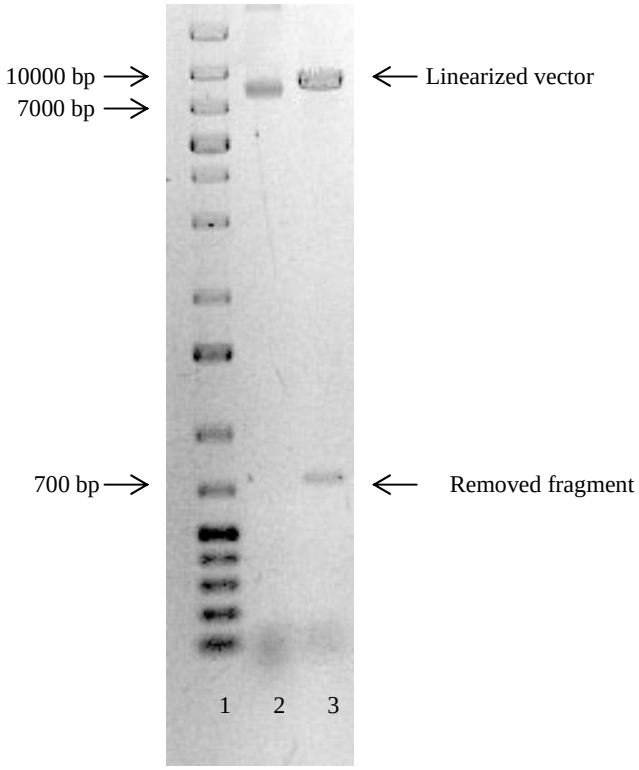


Figure 12. Double digestion of pCAMBIA0305.1 with *Hind*III and *Nco*I enzymes. Lanes: 1 - 1Kb ladder; 2 - not digested pCAMBIA 0305.1; 3 – digested pCAMBIA 0305.1

The gel-purified linearized vector itself was later used for the preparation of chimeric promoter fragments::*GUSPlus* construct while it was necessary to re-circularize it to prepare a negative control promoter-less construct. The double digestion reaction generated two not compatible overhang ends so it was necessary their filling with Klenow enzyme to generate blunt ends for the re-circularization of the vector by a blunt ligation reaction (Figure 13).

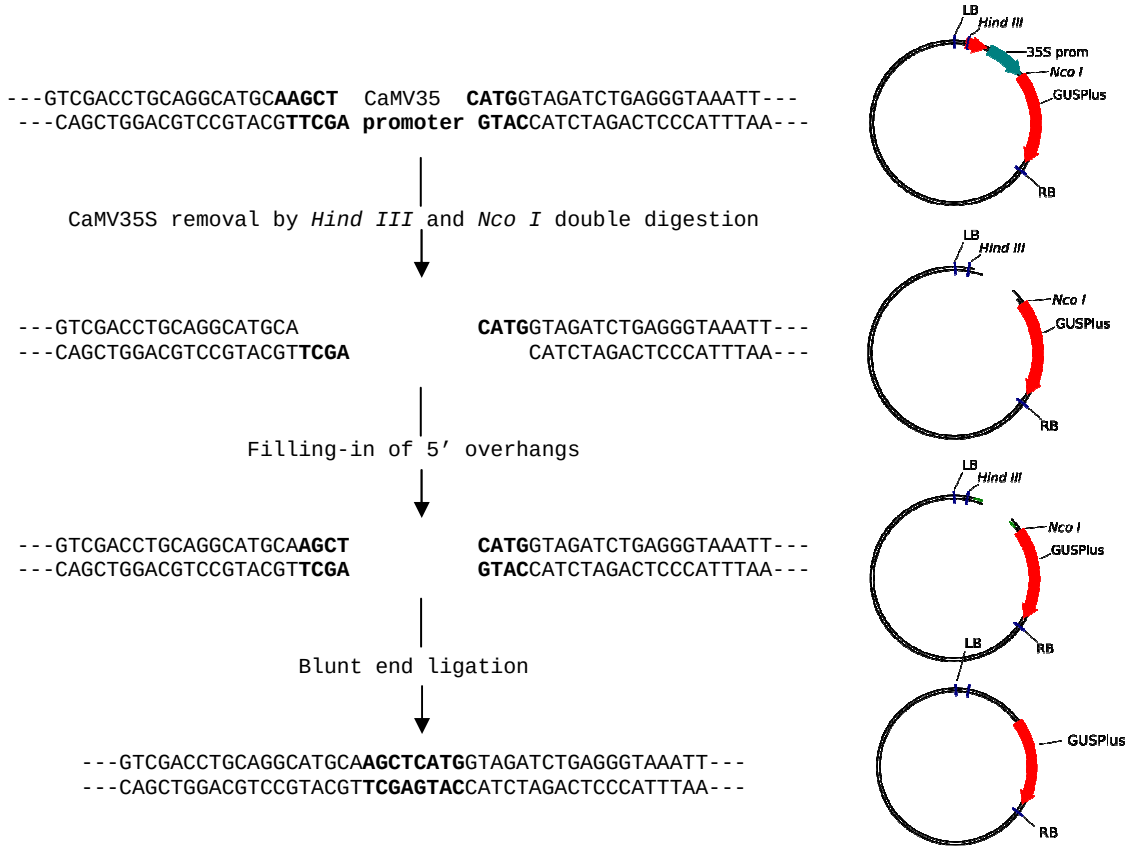


Figure 13. Schematic representation of the negative control, pCAMBIA0305.1-35S, preparation process.

The product of the filling-in and ligation reaction was cloned in *E. coli*. The transformed colonies couldn't be distinguished from the not transformed ones by blue/white screening because the *LacZ* gene was not more functioning. It was so necessary to design specific primer pair to test the colonies by PCR.

The primers (pc0305.1 For TTTATGCTTCCGGCTCGTAT, pc0305.1 Rev GAGAAAAGGGTCCTAACCAAGAA) were designed on plasmid DNA sequence just upstream and downstream the removed region (Figure 11) and used to test transformed colonies. The PCR product was of the expected size. To verify that no unexpected base change has been occurred during the vector modification process the DNA extracted from the colonies resulted positive with the PCR assay was sequenced in the modified region. The output of the sequencing reaction confirmed that the Klenow reaction reconstituted the recognition sites of the two restriction enzyme in the right way (Figure 14) so that the prepared negative control pCAMBIA0305.1-35S vector was identical to the original one except for the elimination of the 35S promoter.

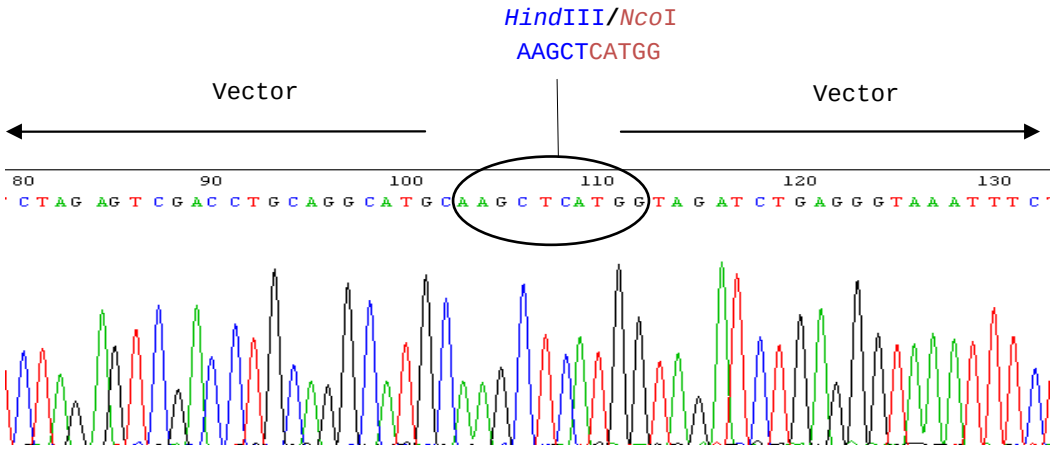


Figure 14. Detail of the sequence of pCAMBIA0305.1-35S negative control at the *Hind*III and *Nco*I junction.

3.1.3 Transient transformation of apple fruits

Experiments of agroinjection of apple fruit conducted according to Spolaore et al. (2001) protocol with CaMV35S:GUS (positive control) and promoter-less (negative control) constructs resulted with very faint blue staining observed on fruits transformed with both constructs (Figure 15). To verify if this patterns was due to plant GUS unspecific activity, the pH of X-GlcA solution after the introduction of infected fruits as well as the pH of apple fruits were assayed by litmus paper. The Pink Lady flesh showed a pH of 3.5 and when immersed in the substrate solution it was able to change the pH of X-GlcA from 7 to 5. At this pH value plant unspecific GUS expression can occur. So we increased the phosphate buffer in X-GlcA solution from 100mM to 200mM. The following transformation experiments, in which this modified solution was used for GUS histochemical assay, showed no GUS staining in apple infected with negative control construct and very well defined dark blue spot on apple flesh transformed with *CaMV35S:GusA* (Figure 15). So this optimized solution was used for all our GUS histochemical assays.

The transformation efficiency of the agroinjection of apple intact fruits was not very high. A consistent variation of transient expression was observed within a series of transformations performed in the same apple fruit and the reproducibility of different experiments was quite low.

In order to increase the transformation efficiency, we tried to facilitate the bacteria penetration into the tissues by using different infection approaches.

The immersion of flesh apple pieces in the infection solution at different bacteria concentration, with or without vacuum treatment, was tested. Some GUS staining was

observed in the experiments with positive control while no blue was visible with negative control construct independently from the vacuum treatment. The blue pattern was slightly different from that obtained with agroinjection method: huge dark blue areas rather than single blue spots. However, also with this approach we were not able to reach very consistent results. Furthermore, the fruit slices decay faster then the tissues from intact fruits and also present more brown areas.

Different concentrations of bacteria solution were also tested to increase the number of transformed explants. The highest concentration tested, OD_{600} 2.5, gave slightly better results in experiments with intact fruits while no differences were found in term of transformation efficiency in experiments with apple slices (Figure 15).

Sonication-assisted Agrobacterium-mediated transformation resulted even less efficient than the other methods because only an apple seed resulted transformed with the CaMV35S:*GUSPlus* construct (Figure 15). No one of apple flesh SAAT infection experiments at any sonication duration, with or without the post sonication vacuum treatment, worked.

To test if apple cultivars or ripening stages could have any influence on transformation efficiency, different apple cultivars at different developmental stages were used in our preliminary transient transformation experiments. Annurca, Topaz, Gala and Fuji never showed GUS expression while Golden Delicious, Pink Lady and Florina gave some blue spots. Golden Delicious was the cultivar with the highest GUS expression in term of number of transformed explants and number of blue spots/explants. No differences were observed between apple at commercial ripening stage or immature ones.

Different co-cultivation parameters were tested to increase transformation efficiency. Relevant differences were observed in term of number of transformed explants and number of blue foci when the co-cultivation temperature was changed. Experiments in which infected tissues were kept at 20 °C gave higher efficiency than that with 26 °C. No effects were instead seen when the co-cultivation time was extended from 48 hours to six days in agroinjection of intact fruit experiment.

3.1.3 Transformation of leaf from lettuce and in vitro apple plants

Transformation of apple leaves from *in vitro* culture resulted in very small blue spots when they were transformed with positive control and no blue staining with negative control (Figure 15). However the efficiency was very low and the blue spot were very difficult to analyze due to the green background color of the leaves.

Lettuce leaves were transiently transformed with agrobacteria solutions with positive and negative control. No GUS activity was found in any of the experiments for the negative control while a very strong GUS staining was obtained when leaves were infected with the CaMV35S::*GusPlus* construct. The staining was not restricted to few blue spots, but the majority of the leaf was stained with a very dark blue color (Figure 15). No considerable differences were found when different concentration agrobacteria suspensions were tested.

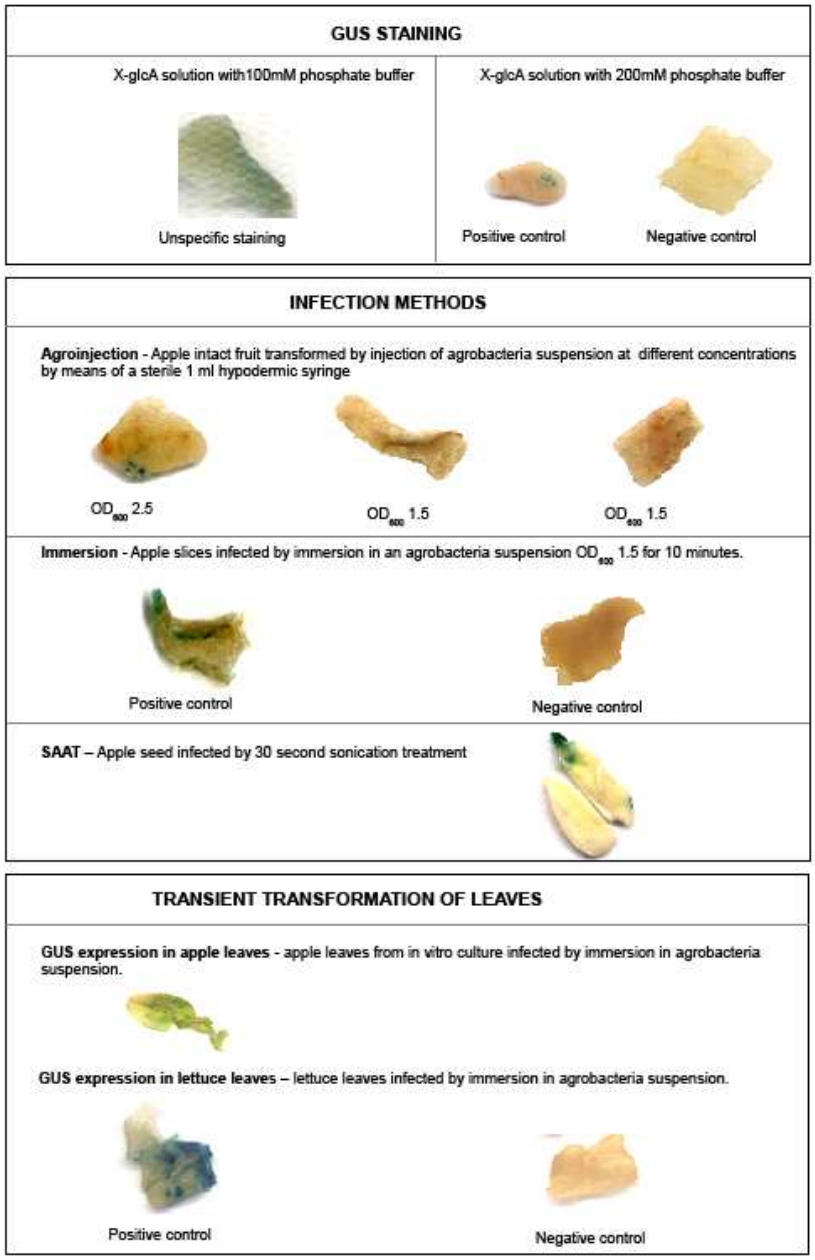


Figure 15. Optimization of *Agrobacterium*-transient transformation assay. For all the transformation experiments, except where differently indicated, was used a suspension of agrobacteria with pCAMBIA0305.1 binary vector (positive control).

3.2 Analysis of *MdACS1* promoters by transient transformation assay

When a reliable protocol for the *in vivo* study of *MdACS1* 5' flanking regions was set, different chimeric promoter and promoter fragments::*GUSPlus* construct were prepared and tested after their transfer to the *Agrobacterium tumefaciens* strain for *in vivo* analysis.

The linearized vector pCAMBIA0305.1 without the CaMV35S promoter (see negative control preparation) was used for the preparation of chimeric promoter fragment::*GUSPlus* constructs. The promoter fragments were generated via PCR so specific forward and reverse primers were designed for their amplification. To allow the cloning of the promoter fragments into the vector, the primers were designed with three distinct regions: an anneling zone, with sequence designed to recognize the specific promoter sequence; a recognition sequence for the proper restriction enzyme (*Hind*III or *Nco*I*)* and an extension of some generic bases to allow to the enzyme to properly cut (Table 2 at page 31). The primer designed with these criteria where used in amplification reactions as described in the materials and methods section. The PCR products were double digested with *Hind*III and *Nco*I and ligated in the expression vector. Thanks to the two different overhangs ends generated by the restriction enzyme the promoter fragments could be inserted in the vector only in the right orientation. The chimeric plasmid were then transferred into *Agrobacterioum tumefaciens* and used in transient transformation experiments.

3.2.1 Analysis of the four identified promoter regions with deletion constructs

The first experiments were directed to analyze the role in the transcriptional process of the four different 5' flanking regions identified with the similarity analysis. So the position of the deletion fragments was decided on the bases of these regions as well as taking into account the bioinformatic results. The fragments were thought to be nested into each other so that the longest one comprise the sequences of all the others (Figure 16). Seven different promoter deletion fragments called C1 (-225 to -1), C2 (-851 to -1), C3 (-851 to -1), C4 (-985 to -1), C5 (-1123 to -1), C6 (-1639 to -1) and C7 (-1790 to -1) were prepared. Even and odd numbers where used respectively for fragments of *MdACS1-1* or *MdACS1-2* promoter region. The 5' flanking region upstream the C6 and C7 fragments, the remaining 264 and 346 bases at the 5' of the BAC22F23 and BAC96N17 respectively, were not considered in our experiments.

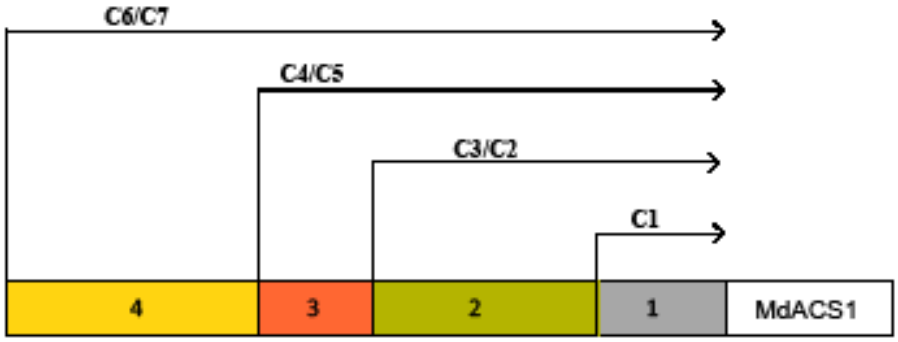


Figure 16. Schematic representation of promoter deletion fragments design. The 1-2-3-4 numbers correspond to the four promoter regions identified on the base of the similarity analysis.

All the primers prepared for the amplification of the deletion fragments worked and the PCR products were of the expected size: C1 225 bp, C2 and C3 851 bp, C4 985bp, C5 1123 bp, C6 1639 bp, C7 1790 bp, (Figure 17).

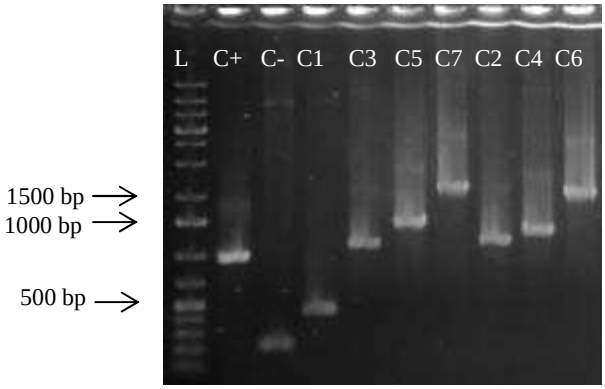


Figure 17. PCR amplification, with primer designed on vector (pc0305.1 F/R), of the full-length and 5' deletion fragments *of MdACS1-1* (even number) and *MdACS1-2* (odd number) promoters. C+, pCAMBIA0305.1 positive control. C-, pCAMBIA-35S negative control.

The purified amplification products were digested and ligated into the binary vector in front of the *GUSPlus* gene. To verify if the ligation occurred, all the ligated product were used as template in a PCR analysis with primers designed on the vector (pc0305.F/R). Amplification bands of the expected sizes were obtained for all the constructs also confirming that the insert-to-vector molar ratio of 3:1 chosen for the ligation reaction was suitable for the reaction.

The chimeric promoter::*GUSPlus* constructs were then transferred to *E. coli.* and different colonies for each chimeric constructs were obtained. The majority of them resulted positive to the PCR screening with the pc0305.1 F/R primers and with primers specific for

each fragment. To further corroborate the accuracy of the cloning process, DNAs extracted from the positive colonies were sequenced. The full sequences of C1-C2-C3 fragments were covered just with the two primers designed on the plasmid DNA; for C4 and C5 an extra step with HaradaIN primer was done; for C6 and C7 fragments one more additional step with primer Harada For was necessary to cover the full sequence. The sequencing of each construct showed no difference from the original sequences obtained from primer walking on the two BACs. All the constructs were successfully transferred into *Agrobacterium tumefaciens*. The transformation efficiency was not very high and for some constructs only one colony was obtained. Also in this case the colonies were tested for the presence of the right plasmid by PCR and at least one positive colony for all the constructs was obtained.

Transformation in apple

This deletion constructs were used in transient transformation assay of apple Golden Delicious slices to verify the functionality of our constructs in their native contest. As expected we didn't obtain a complete reproducibility and the transformation efficiency was very low but in some experiments clear blue staining was obtained and not only in the tissues infected with the positive control but also in that with C6:*:GUSPlus* construct (Figure 18). The pattern and intensity of GUS staining with this construct was comparable to that obtained with CaMV35S*::GUSPlus* construct. Any GUS staining was found in the tissues infected with the promoterless and the other deletion constructs.

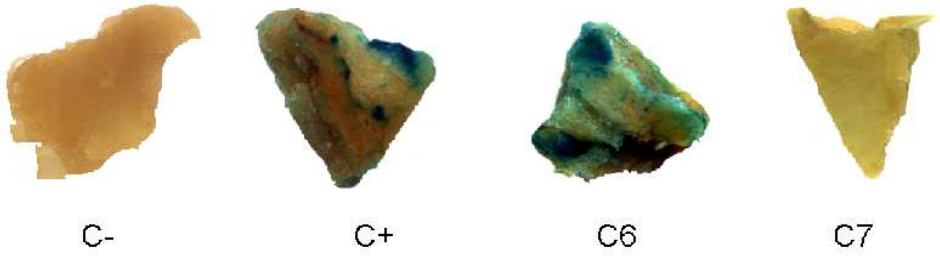


Figure 18. GUS histochemical assay of apple slices infected with suspension of agrobacteria harboring pCAMBIA0305.1 positive control (C+), pCAMBIA-35S negative control (C-), pCAMBIA-C6 and pCAMBIA-C7 *MdACS1* promoter constructs.

To have a more reliable and clear results we adopted the 'lettuce leaf system' to test all the prepared promoter constructs. All the transformation experiments were done under the same transformation conditions used in our preliminary transient transformation experiments of lettuce leaves. Transformations with the positive and negative constructs were performed in all the experiments.

Transformation in lettuce

Very high level of histochemical GUS activity was observed transforming with the positive control (CaMV35S::*GUSPlus*) and no staining for the promoter-less construct, as expected (data not shown).When the promoter deletion constructs C1, C2, C3, C4, C5, C6 and C7 were used to agro-infect lettuce leaves GUS activity was observed in transformed tissues with all the constructs, except the C7 construct, also if at different levels (Figure 19). The highest activity was observed in leaves infected with the C6::*GUSPlus* construct that showed blue areas comparable with that of CaMV35S::*GUSPlus* infected tissues. All the other constructs (from C1 to C5) showed similar results with only a few spots. The C7::*GUSPlus* construct showed a lack of expression comparable to that observed for the promoterless construct. Since the level of GUS expression increases dramatically only with the C6 construct and the *MdACS1-2* promoter stop to work when the forth region is added, further transformation experiments with specific constructs were assessed to study more in deep this distal region.

3.2.2 Analysis of the forth promoter region

As resulted from the *in-silico* analysis, this region is characterised by 16 SNPs, the ERE element and the tandem repeat in the *MdACS1-2* promoter with a dimerization of the GT1 element (Figure 20). The effect on GUS expression level of these elements was evaluated in transformation assay experiments with specific constructs: C6-7noT, C6TX2, C6TX2snp, C6snp, C7TX1, C7TX1snp, C7snp for the analysis of the tandem repeat region; C1-GT1, C1-TX1 and C1-TX2 for the gain of function analysis of GT1 putative c*is*-acting element; C6noERE and C7noERE for the analysis of the ethylene responsive element (ERE). The accuracy of all these new constructs was verified by sequencing with the same primers used for the C6 and C7 constructs.

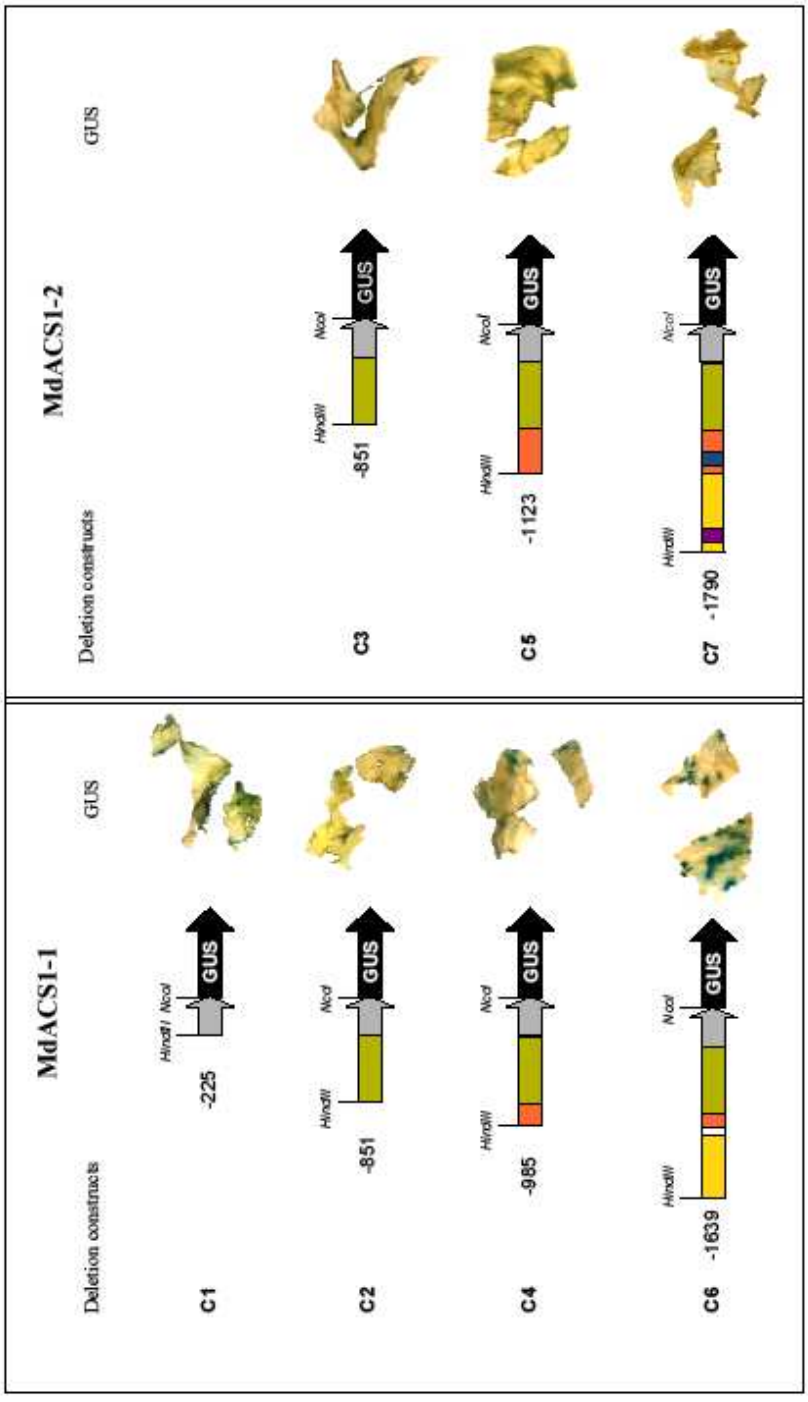


Figure 19. Schematic illustrations of the deletion promoter constructs of the two *MdACS1* alleles and results of the GUS histochemical assay on lettuce leaves. In yellow, orange, green and gray are indicated the forth, third, second and first regions of the promoters identified on the basis of the similarity analysis.

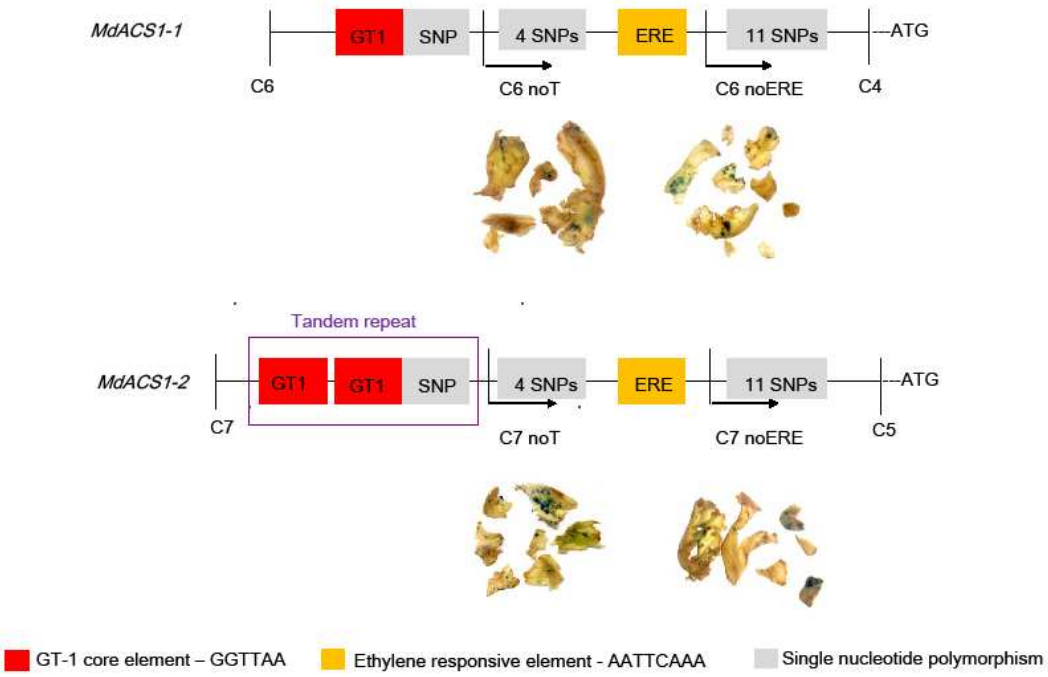


Figure 20. Schematic representation of the forth *MdACS1-1* and *MdACS1-2* promoter regions and histochemical analysis of the C6not, C6noERE, C7not and C7noERE promoter deletion constructs in lettuce leaves.

Tandem repeat analysis

To evaluate the role of the tandem repeat region (comprising the two GT1 elements and a SNP in *MdACS1-2*) two deletion constructs, the C7noT::*GUSPlus* and C6noT::*GUSPlus* in which this region is not present at all were prepared (Figure 20).

The amplifications on the two BAC clones DNA with primer designed just downstream the tandem region gave products of expected size, 1733bp for C7noT and 1594 bp for C6noT. This amplified fragments were successfully digested and ligated into the expression vector. Both the cloning in *E.coli* and in *Agrobacterium tumefaciens* worked well and it was possible to obtain different transformed colonies. The results of the sequencing process confirmed the accurancy of the process. The *Agrobacterium tumefaciens* with these chimeric constructs were used in transformation experiments. GUS expression was observed not only in tissues transformed with C6noT::*GUSPlus* but also with C7noT::*GUSPlus*. The level of expression induced by C7noT::*GUSPlus* was comparable with that of C6noT::*GUSPlus* or positive control, and higher of that produced by the closer chimeric constructs C4 and C5 (Figure 20). Any expression of the reporter gene was ever found with the negative control. These results suggested that this region is actually very important in

the transcription control, so the effect of GT1 element and the SNP close to it were singularly evaluated with specific chimeric constructs.

It's reported that a single GT element cloned upstream of a minimal promoter enhance the reporter gene activity, but the introduction of second GT element in near proximity leads to a reduction in activation of the minimal promoter. So the presence of the GT1 element could explain both the increase of the reporter activity with the full promoter construct of the *MdACS1-1* allele and the inactivation of the *MdACS1-2* allele. To test this hypothesis a dimerization of GT1 element was introduced in the original *MdACS1-1* and one copy of this element was eliminated from *MdACS1-2* original promoter by a two steps PCR process as described in material and methods section. Both the primer pair for the first and the second amplification worked very well and bands of the expected size were obtained. Also the cloning in the expression vector was concluded with success bringing to different *E.coli* colonies positive for the presence of the C6TX2::*GUSPlus* and C7TX1::*GUSPlus* constructs. The DNA sequencing confirmed that one GT1 element was added to C6 in the C6TX2 and one was eliminated from C7, generating C7TX1, without introducing any other unwanted modification. The constructs were successfully transferred to *Agrobacterium tumefaciens* and used to infect lettuce leaves. The tissue infected with C7TX1:: *GUSPlus* constructs showed also in this case GUS activity, a result that supported our hypothesis, however also the leaves infected with C6TX2::*GUSPlus* construct where characterized by very extended blue areas with a slight increase of GUS activity observed in respect of the original C6 construct (Figure 21).

Gain of function experiments

To further test the GT1 element also gain of function experiments were done. The putative GT1 *cis*-element of *MdACS1-2* promoter, from -1768 to -1763 (GGTTAA), was fused alone, with its flanking region CAAAAAG or in its dimeric form (GGTTAACAAAAAGGGTTAA) with C1 shortest fragment to generate respectively C1-GT1, C1TX1 and C1TX2 fragments (Figure 22). The amplifications with the specific prepared primers gave three fragments of expected size (C1TX1 265 bp, C1TX2 271 bp and C1-GT1 258 bp) that were successfully double digested and ligated in the modified pCAMBIA0305.1-35S vector. The chimeric C1-GT1::*GUSPlus*, C1TX1::*GUSPlus* and C1TX2::*GUSPlus* resulting constructs were cloned first in E.Coli and then in Agrobacterium cell successfully. The sequencing of each construct confirmed also in this case the accuracy of the obtained sequences. The ability of these sequences to increase o

decrease the GUS expression driven by the C1 promoter fragment was tested by lettuce transformations experiments in which CaMV35S:GUSPlus, promoterless and C1 constructs were used as controls. None of the tested constructs was found to inhibit the C1 activity while they seem to induce a slight increase of GUS expression in respect of C1 construct (Figure 22). None of them was able to enhance the strength of C1 promoter fragment at the same level induced by 35SCaMV::GUSPlus construct.

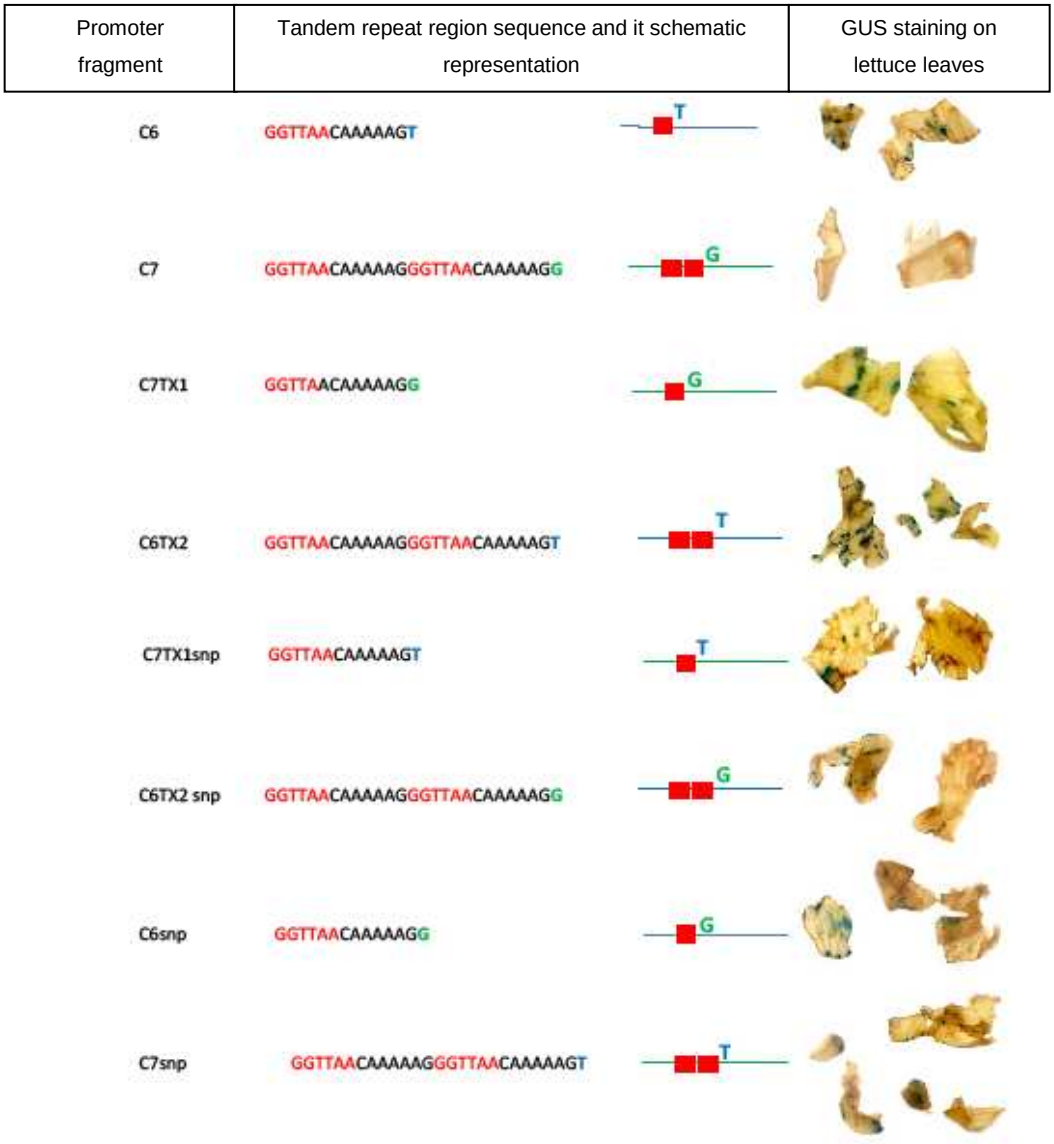


Figure 21. Analysis of the tandem repeat region. C6 and C7 are the longest deletion fragments with original *MdACS1-1* and *MdACS1-2* promoter sequences. All the other reported promoter fragments derive from C6 and C7 specific mutations in the tandem region. The red characters and squares indicate the GT1 element. In blue and green are indicated the *MdACS1-1* and *MdACS1-2* promoter sequences respectively.

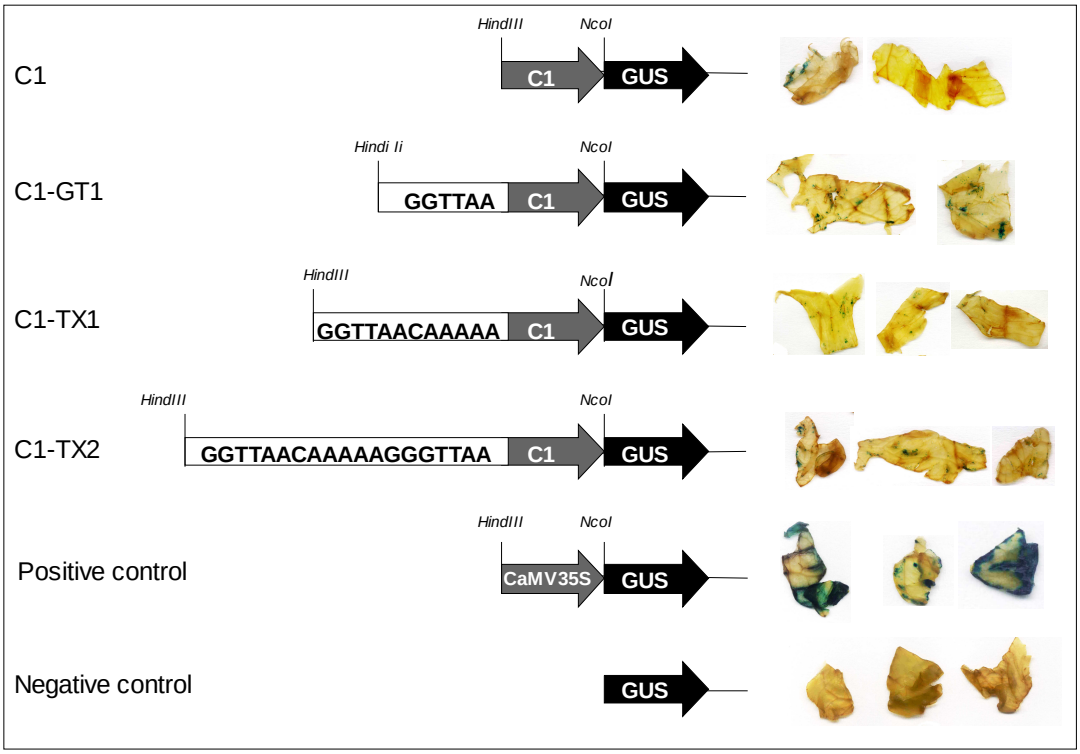


Figure 22. Schematic representation of chimeric constructs for GT1 element (GGTTAA) gain of function experiments and results of GUS histochemical assay on lettuce leaves.

The two promoters differ in the tandem region not only for the presence of the GT1 element but also for a single nucleotide change. To test if this SNP could play an important role in the transcription control other four different promoter constructs were produced: the C7TX1SNP::*GUSPlus* and C6TX2SNP::*GUSPlus* with both GT1 and SNP mutated; C6SNP::*GUSPlus* and C7SNP::*GUSPlus* with only one base changed. All the promoter mutated fragments were successfully amplified and transferred to the expression vector. The sequencing of DNA of C7TX1SNP::*GUSPlus* and C6TX2SNP::*GUSPlus* containing colonies confirmed that they were identical to C7 and C6 constructs except for the desired mutation: the base change G→T at -1742 and the deletion of the GGTTAACAAAAAG region from -1754 to -1743 of C7; the T→G base change at -1603 and the insertion of the GGTTAACAAAAAG sequence at -1605of in C6.

The results of the sequencing of C7SNP::*GUSPlus* confirmed that a promoter fragment identical to C7 except for only one base change was generated. The C6SNP::*GUSPlus* sequence on the other hand revealed two single nucleotide changes in original promoter construct C6, one as expected is the T→G substitution at -1603 and the other is a G→T single base change at -1604 is probably due to a base change in the synthesized primer. All

the chimeric constructs were in any case transferred successfully in *Agrobacterium tumefaciens* and used for transformation experiments.

All the constructs induced the expression of the reporter gene, even the C7SNP with only one nucleotide change in respect of the C7 construct that instead never showed GUS activity. The GUS expression induced with the C6SNP was slightly lower than that of C6 original construct. The blue area obtained in tissues transformed with the constructs C7TX1SNP::*GUSPlus* and C6TX2SNP::*GUSPlus* were not dissimilar to that induces by constructs with single mutated element (Figure 21).

Ethylene responsive element evaluation

To test the putative effect of the ERE motif two more deletion constructs C7noERE (-1471 to -1) and C6noERE (-1332 to -1) were designed to have the 5' end almost 150 bp downstream the ERE element. All the primers prepared for the amplification of the deletion fragments worked and the PCR products were of the expected size: C7noERE 1470 bp, C6noERE 1332 bp. The sequencing of the cloned fragments confirmed the cloning of the right sequences. Also in this case the level of reporter expression in tissues transformed with these constructs was higher than that obtained with C4 and C5 deletion constructs (Figure 20) by which differ for approximately 359 bp at the 5' end.

3.2.3 Deletion of SINE insertion

A “site directed mutagenesis of the whole plasmid” approach was tried to delete the SINE insertions from C7 construct. The position of the two designed primers in respect of the SINE insertion is showed in figure 23 a. Using the Herculase II Fusion DNA Polymerase and a PCR amplification program with very long extension time and high cycles number we were able to obtain PCR product of the expected size even when the smallest amount of plasmid DNA was used as template.

a)

```
                                                    C7sine-P Rev
MdACS1-2 ATGATACTTGTTGTCGGTAAGGTTTTGTAAACAAAACTAGACCCGAGTATTAATTCTTGT 1091
MdACS-1  ATGATACTTGTTGTCGGTAAGGTTTTGTAAACAAAACTAGACCCGAGTATTAATTCTTGT 996
         ************************************************************

MdACS1-2 TTCACTAGTACATAACAGCATATTTACCACATCAATTCCATCACGGGCATCAACAGCTCG 1151
MdACS-1  TTCTTTGTT-----------TTTTT---------------------------------- 1010
         ***  *  *           * ***

                                      SINE INSERTION
MdACS1-2 TGGTAGAAACTATTCAATTACCACGGTTACATGTAGATCGTGGTAGATAACATTAATTCA 1211
MdACS-1  ------------TTCAATTAC------------------------------------- 1019
                     *********

                                                    C7sine-Pfor
MdACS1-2 CCACGGTCAAATTCTGGCCCGTTGTTAAAAGTAAATAATTCACCACGATTAATGCTTCTA 1271
MdACS1-1   ------------------------------------ AAGCCGATTAATGCTTCTA 1038
                                                 ***************

MdACS1-2 TGTACACTTATAATCCCCACGCAAGTTTGTAGGTAATGCCAGGTAATGGTGAACGCCCTA 1331
MdACS1-  TGTACACTTATAATCCCCACGCAAGTTTGTAGGTAATGCCAGGTAATGGTGAACGCCCTA 1098
         ************************************************************
```

b)

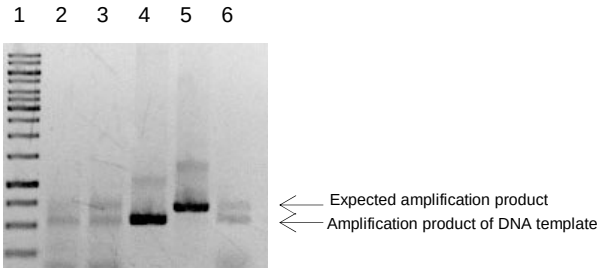


Figure 23. a) Primers sequence and position for SINE deletion. b) amplification products with pc0305.1 primers from the ligase reaction product and *E. coli* transformed colonies with this ligation product. Lanes: 1, 1 Kb ladder; 2-3, amplification of ligase products; 4-5, control amplification of pCAMBIA0305.1-35S::C7 and pCAMBIA0305.1-35S::C6 not modified vectors; 6, transformed *E. coli* colony.

These products were treated with *DpnI* enzyme that specifically cleaves fully methylated $G^{Me6}ATC$ sequences, so it should chews up the bacterially generated DNA used as template but not the PCR product. The resulting fragment with blunt and phosphorylated 5' and 3' ends were purified and used for a self-ligation reaction to circularize the plasmid. The products of the ligation reaction were transferred to *E. coli* and different colonies were obtained. However when the colonies were assayed for the presence of the right construct,

two different bands were found, one corresponding to expected product and the other ascribable to the amplification of original DNA template (Figure 23 b). To completely eliminate this contaminating DNA, a small amount of the product of the fist amplification was used as template for a new amplification reaction and the product of this reaction was digested and ligated. However when the product of the ligase reaction was assayed via PCR, again we found the unexpected band. So it was not possible to prepare the C7-SINE construct.

4. ETHYLENE PRODUCTION IN APPLE FRUITS

4.1. Ethylene production on Mondial Gala and Fuji

As expected, a marked difference for ethylene evolution was found between Fuji and Mondial Gala. The first cultivar is unable to produce ethylene while in Mondial Gala there is a strong ethylene burst starting at 7 days after harvest (dah) and reaching the maximum from 14 to 28 dha (Figure 24). On the base of this data we decided to assess ethylene evolution at 21 days in the segregating progeny.

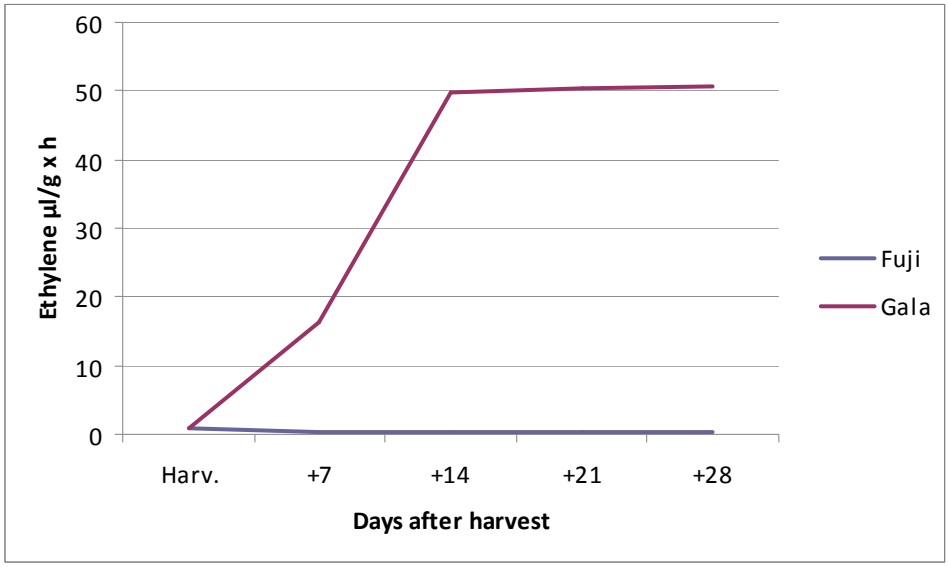


Figure 24. Ethylene evolution in the two parental cultivars during a 28-day period after

4.2. Ethylene production on the progeny Fuji x Mondial Gala

The seedlings showed a large variation in ethylene production from 0 to 182,4 µl/g h. The data summarized in figure 25 show a bimodal distribution. About 37% of the seedlings produce a very little amount of ethylene as the Fuji parent, the 43% an intermediate ethylene value between the two parents and 8% produce ethylene in higher amount then Mondial Gala.

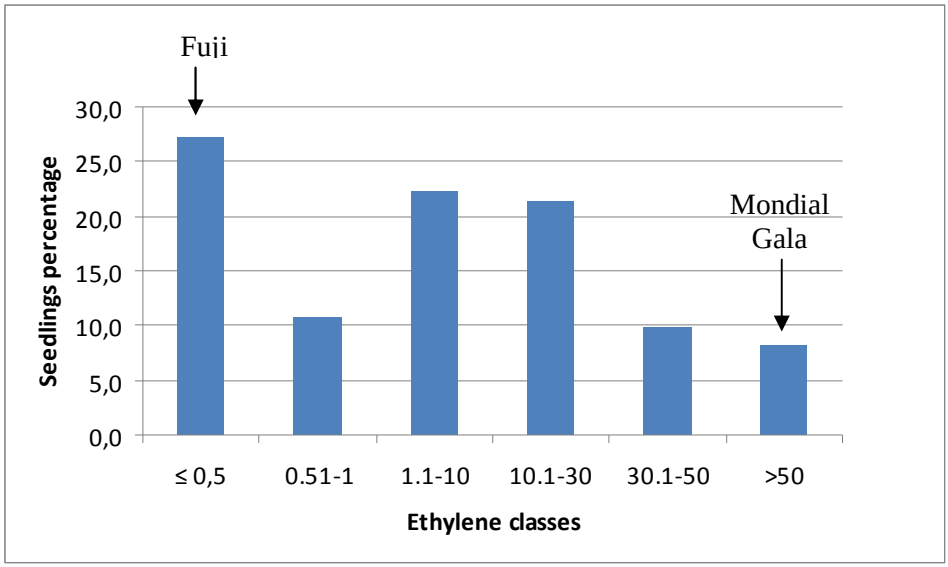


Figure 25. Ethylene production in 121 genotypes of Fuji x Mondia Gala population at 21 days after harvest.

In order to further analyze these data, the 121 genotypes of Fuji x Mondial Gala population were then classified in respect of ripening date in three groups: 35 early (13- 27 August), 60 middle (17 September) and 26 late (1 October) ripening plants. When the ethylene data were evaluated in function of these three groups a different distribution was found in respect of the ripening date. The early-ripening genotypes produce more ethylene than the middle- and late-plants. The 73% of the late-ripening seedlings produce less then 1µl/g h and none of them showed an ethylene value major than 50 µl/g h (Table 7).

	Ripening classes		
Ethylene classes	**early**	**middle**	**late**
<0,5	8,6	28,3	50,0
0.5 a 1	5,7	8,3	23,1
1.1 a 10	42,9	16,7	7,7
10.1 a 30	25,7	23,3	11,5
30.1 a 50	2,9	15,0	7,7
>50	14,3	8,3	0,0

Table 7. Distribution of Fuji x Mondial Gala progeny in function of ripening date and ethylene production.

We knew, from our previous research, the allelic composition of 58 of the 121 seedlings used for the ethylene production analysis: 28 seedlings are homozygous for *MdACO1-1* while 30 are heterozygous for this gene.

This information was used to elaborate the ethylene data of the Fuji x Mondial Gala population in function of *MdACO1* allelotype. In the classes with higher ethylene production there are mainly seedlings heterozygous for *MdACO1* (Figure 26).

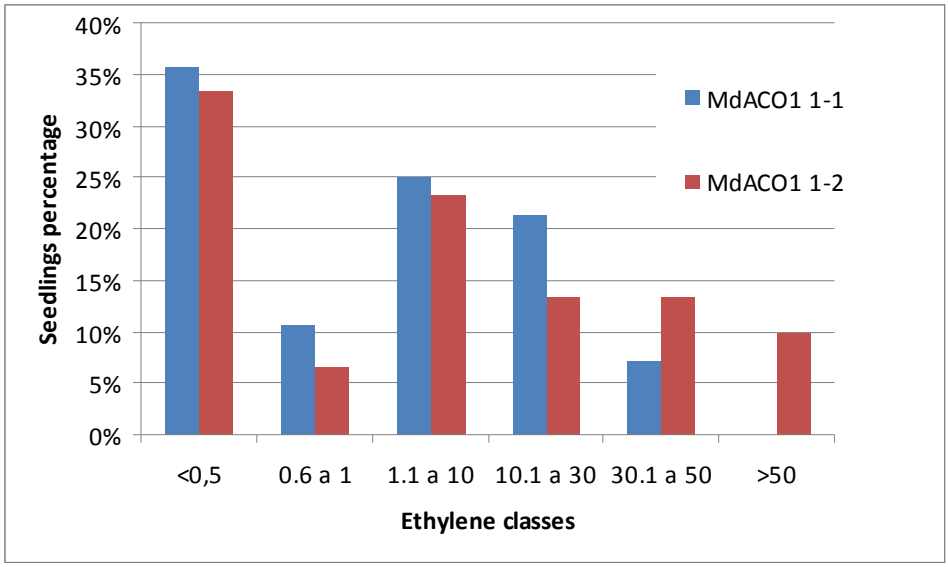


Figure 26. Distribution of 58 genotypes of Fuji x Mondia Gala population in function of *MdACO1* allelotype and ethylene production.

Also this 58 genotypes of Fuji x Mondia Gala population were organized in respect of ripening date in three groups: 20 early- (13- 27 August), 23 middle- (17 September) and 15 late- (1 October) ripening plants. The seedlings so grouped were analysed in function of *MdACO1* allelic composition and ethylene production (Figure 27). All the seedlings homozygous for *MdACO1* produce always low level of ethylene irrespective of the ripening date. The early ripening seedlings that are heterozygous for *MdACO1* produce more ethylene than the middle- and late-ripening seedlings.

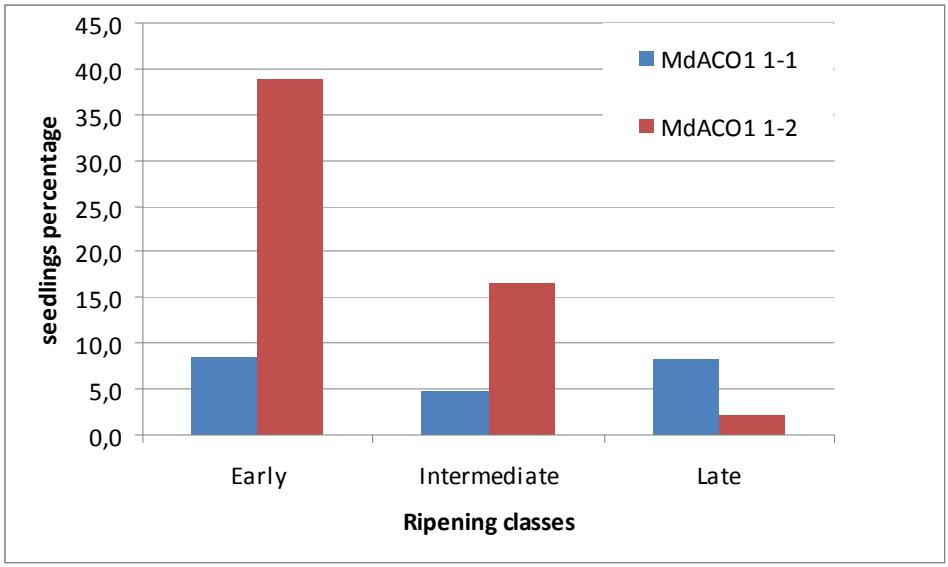


Figure 27. Distribution of 58 genotypes of Fuji X Mondia Gala population in function of date of ripening, *MdACO1* allelotype and average value of ethylene production.

V DISCUSSION

1. STRATEGIES FOR APPLE PROMOTER ANALYSIS

The sequencing of the apple genome and its publication (Velasco et al., 2010) allow scientists to more rapidly identify which genes provide desirable traits that could be incorporated into new varieties, including fruit quality feature desired by consumer. So more genes are expected to be characterized in the near future.

The full characterization of a gene comprise the functional analysis of it's 5' flanking region that is responsible for its specific expression pattern. For this reason the setting-up of a reliable protocol for *in vivo* promoter analysis is an essential step to fully exploit all the gene information derived from the availability of the apple genome sequence.

As discussed in the introduction, for promoter analysis a transient transformation assay is preferable, in respect of generation of stable transformed plants, due to the high number of constructs to be tested. In our study, for example, only for the characterization of the *MdACS1* promoter region a total of 20 different promoter constructs were prepared. Moreover, since regulatory network can differ between different species and tissues the study of promoters in their native contest is desirable. *MdACS1* is a ripening-related gene so apple ripe fruit is the better tissue in which to study its expression.

The only available protocol for apple fruit transient transformation in literature is that for the agroinjection of intact fruits reported by Spolaore et al. (2001), but it has never been effectively used in apple studies since its publication. Another group of researchers tried to optimize the biolistic methods for transformation of apple fruit tissue (Biricolti personal communication) but with no success. So we decided to adopted the *Agrobacterium* transient transformation assay for our research.

1.1 pCAMBIA 0305.1 and its derivative binary vectors

The use of an *Agrobacterium tumefaciens*-mediated genetic transformation to express heterologous DNA in plant tissues entail the preparation of a particular kind of expression vector, a T-DNA binary vector, that is then transferred to an appropriate *Agrobacterium* strain containing the virulence genes required for T-DNA processing and transfer (Lee and Gelvin, 2008). Different kind of binary vectors are available. We decided to use a free of charge pCAMBIA vector that is based on restriction/ligase strategy for the fusion of promoter sequence in front of the reporter gene. The choice of the vector type, between the big number of different pCAMBIA vectors available, was driven by the following criteria:

small dimension, for an ease handling; a reporter gene already successfully tested in apple and the presence of a strong promoter upstream the reporter that could also be easily removed to insert our promoter fragments. The vector that better corresponded to this description is the pCAMBIA 0305.1 which contain a GUS reporter gene, already used by Spolaore et al. (2001) in apple fruit, driven by a CaMV35S. The absence in this vector of a gene for selection in plants allow to have a vector of small dimension without affecting our analysis since we opted for a transient assay in which the regeneration and consequent selection of transformed plant tissue are not necessary. Moreover the chimeric promoter::reporter constructs generated with this vector could be anyway used for later generation of stable transgenic plants by a co-transformation strategy. The presence of the CaMV35S promoter upstream the GUS sequence allowed its use as positive control without any other modification, while the constitutive promoter CaMV35S was removed to insert our promoter fragments. The linearization of the vector and its purification from the removed promoter required some work and time but not particular problems were encountered. More difficulties were found in the ligation of the promoter fragments, especially the longest ones, but anyway all the prepared fragments were successfully fused in the binary vector confirming the choice of a small vector as helpful in the different steps of the cloning process. The major drawback was instead the loss of the *LacZ* gene functionality due to the removal of CaMV35S promoter. Indeed, the loss of the blue/white screening methods required the screening of the transformed bacteria colonies by PCR to identify the positive ones. The open vector without the constitutive promoter was also re-circularized to generate a negative control to evaluate the level of background in our experiment. Although some work and time were needed to prepare the vectors, this new generated promoterless binary vector, pCAMBIA0305.1-35S, as well as the linearized pCAMBIA0305.1 vector without the constitutive promoter could be used also in future transformation experiments to assay promoters of other plant genes.

1.2 Agrobacterium-mediated transient transformation of apple fruit

The results of our preliminary experiments to test the agroinjection method of apple fruit highlighted some problems. At the same experimental condition reported by Spolaore et al. (2001) for GUS histochemical assay, we observed that apple fruits infected with both positive and negative control constructs gave an evenly distributed blue staining with very faint colour on all the flesh area and for all the tested explants. This high GUS staining background in not desirable because it can make difficult the interpretation of the results of

analysis. Since the reporter gene in our expression vector is interrupted by an intron from the castor bean catalase gene the blue staining observed with the negative control could not be due to bacterial expression. One of the reasons of the huge application of *GUS* as reporter gene in plant transformation experiments since its introduction to the scientific community in 1987 (Jefferson et al. 1987), was the firm belief that plants lack intrinsic GUS activity. On the basis of this opinion and when an interrupted *GUS* gene is used, all the GUS expression observed in the transformed tissue is imputable only to the construct under study. However more recently it has been reported that at pH 4 *GUS* activity could be detected in all the plants and in almost all the organs (Sudan et al., 2006). The S*taphylococcus* sp-derived *GUS* gene that we used in our constructs, as well as the most widely used *E.coli*-derived glucuronidase, has optimum activity at pH 7.0. However, as we demonstrated in our experiments, the pH of the apple fruits ranged from 3.5 to 4 and when immerse for 12 h in the standard GUS assay buffer, with 100mM phosphate buffer, they induced the change of the pH of the solution from 7.0 to 4.0-5.0. So, we concluded that the observed GUS staining obtained with negative control construct was the result of unspecific plant endogenous GUS activity. The histochemical GUS assay protocol was successfully optimized by increasing the buffering capacity of the phosphate buffer. This change was sufficient to avoid the unspecific staining of apple flesh transformed with negative control and to obtain a lot of small blue foci or very dark blue area when apple tissues were transformed with positive control allowing robust comparison between the constructs.

Despite of the optimization of the GUS hystochemical assay, the application of the Spolaore et al. protocol for our research experiments resulted very problematic. A very high variability in GUS staining strength and frequency between different apple samples and experiments was observed. Even though apple transformed with the negative control never showed any blue spot, not all the apple pieces infected with positive control in a single or in different experiments resulted blue stained. These results are clearly due to differences in the transformation rate.

The efficiency of *Agrobacterium*-mediated transformation is influenced by experimental variables that affect the virulence of *A. tumefaciens* and the plant's physiological condition. Also the compatibility between plant and bacterium, regarding the ability of the bacteria to reach and attack plant cells and the availability of an efficient T-DNA transfer machinery encoded by plant cells, could influence the result of a transformation experiment (Wroblewski et al., 2005). Therefore, to reach an efficient transformation rate the optimization of *Agrobacterium tumefaciens* – plant interaction is probably the most

important aspect to be considered. For this reason we tried to make same adjustments to the transformation protocol to reach a greater repeatability and reliability of the results.

The most relevant improvements in term of number of transformed explants and number of blue foci was obtained when the infection and co-cultivation temperature was kept at 20 °C. This results is in agreement with the study of Dillen at al. (1997) that reported as optimum for *Agrobacterium* infection a range of co-cultivation temperature between 19 and 22 °C. They also found in their experiments that the transient expression of reporter gene markedly decreased when the temperature was increased from 22 °C to 25°C, and expression was low at 27 °C and undetectable at 29 °C.

The increasing of the length of co-cultivation period from 48 hours to six days didn't resulted in significative change in GUS expression. Indicating that 48h are already sufficient to induce transient expression as already reported by Spolaore et al. (2001). The experiments with infection solution at different bacteria concentration confirmed that an higher concentration (OD_{600} 2,5) is useful when intact fruit are infected (Tittarelli et al., 2003), while no differences were observed when sliced fruits were used. Probably the hypothetic greater transformation efficiency deriving from a more concentrated bacteria suspension is clouded in sliced fruit by their faster decay. Also two different staining patterns were observed when intact apple fruit or apple slices were transformed by agroinjection or immersion in agrobacteria solution, respectively. This could be explained by a different agrobacteria diffusion in apple flesh due to the different techniques applied. Although high variability between fruit at different stages of development has been reported in agroinjection of mature and immature fruits of rough lemon (Ahamad and Mirza, 2005) we didn't find any difference between apple at commercial ripening stage or immature ones. Some differences were found using apple fruits of different cultivars, however due to the reduced repeatability of the experiments, we cannot certainty assert that the transformation rate is genotype dependent and not rather due to differences in the physiological condition of the single fruits.

Since adjustments in the main parameters of the agroinjection protocol didn't allowed to obtain good results, we also tested the sonication assisted *Agrobacterium* transformation (SAAT) and vacuum-infiltration methods to try to increase the transformation rate. By SAAT the plant tissues, subjected to a brief period of ultrasound in the presence of *Agrobacterium s*how a large number of small and uniform wounds that are expected to produce an easier agrobacteria access into the target plant cells or tissues. In addition, the use of vacuum infiltration after sonication may provide additional entry sites for bacteria.

Indeed, the intercellular spaces of mature apple tissue are largely filled with air that could be replaced by agrobacteria solution thanks to the positive differential pressure which results when the atmospheric pressure conditions are restored. Different tissues respond in different ways to SAAT so the best treatment for each tissue needs to be empirically determined (Trick and Finer, 1997). We tried different sonication times covering the range of that usually reported in literature for different kind of tissues but none of them was able to produce apple fruit transformation.

This transformation method is reported to work well in various tissues of soybean, wheat and maize as well as in epicotyl segments of sweet orange (Trick and Finer 1997; De Oliveira et al., 2009) but only one report was found with the application of this methodology for transient transformation of fruit tissues (Matsumoto et al., 2009). Moreover, the attempt of this researchers to increase *Agrobacterium* transient transformation of immature banana fruits by SAAT failed and rather they observed negative influence on transformation efficiency in some transformation experiments. Probably, sliced fruit tissues are already sufficiently wounded and the cells in ripening fruit are very frail so that even the shortest sonication time could be sufficient to induce irreversible damages to the cells.

So, even if *Agrobacterium* transient transformation of fleshy fruit has been applied with success in different species such as tomato (Orzaez et al., 2006), peach (Spolaore et al., 2003; Tittarelli et al., 2009), strawberry (Hoffmann et al., 2006) and rough lemon (Ahamad and Mirza, 2005), it was not possible yet to obtain an optimized protocol for apple fruit. In conclusion, by using the best optimized conditions for agroinfiltration, only the stronger promoters showed a rather stable and strong GUS expression (35S and C6) while no expression was detected for all the other deletion constructs. An alternative to the promoter analysis in native contest is a transient or stable functional analysis in an heterologous system. For the analysis of genes in fruit system, tomato has usually been used. Indeed, common regulatory mechanisms are conserved at the molecular level among widely different species that exhibit climacteric ripening of fleshly fruit. Tomato allow a quick generation of transformed fruit and a continue analysis during the different stage of fruit development. This model plant has already been used with success for the analysis of promoter region of apple *ACO* and *PG* genes (Atkinson et al., 1998). Another system that guarantee a fast screening is the transient expression in lettuce leaves. Lettuce was found to readily express the recombinant protein β-glucuronidase (GUS) following agroinfiltration and to be a good and reliable system for the study of promoter sequences (Wroblewski et al. 2005). For these reasons and for the easy availability, we decided to perform our

preliminary promoter constructs screening in this system. As expected the GUS assay of lettuce leaves infected with our positive and negative control showed highly reproducible results with a very strong staining induced by the constitutive CaMV35S promoter and no blue spot for tissue infected with promoterless construct.

Also if a conserved heterologous regulation between different species has been in some case demonstrated (Tittarelli et al. 2009), the expression levels can vary drastically and regulation often does not work properly in an heterologous system (Fütterer et al.,1995) so the results should be carefully interpreted and possibly validated in homologous contest by a stable transformation approach. Stable transformation of apple, for which reliable protocol are already available, is a feasible choice to evaluate few of the most interesting promoter constructs in an homologous system. Very recently, an interesting alternative to study apple promoters in their native contest is the use of "early flowering" transgenic apple. These plants were developed by Flachowsky et al. (2007), to shorten apple juvenile phase, using the *BpMADS4* gene of silver birch, and are able to flower in the first year after sowing. Transforming these plant again with the promoter constructs of interest a quick characterization of apple regulatory region in their own contest could become possible.

2. *MdACS1* CHARACTERIZATION

ACS gene is generally considered the rate-limiting enzyme in the ethylene biosynthetic pathway (Kende 1993) and so the *MdACS1* gene, one member of the apple multigene family predominantly expressed in apple fruit, has been considered directly involved in the different apple ripening behaviour. Although, as reported in the introduction, different studies on *MdACS1* have been published a functional analysis of the *MdACS1* gene has never been performed. To better understand the role of this gene in the control of apple ripening we decided to analyze more in deep its expression through qPCR analysis and *in vivo* characterization of the promoter regions of the two alleles.

2.1 *MdACS1* gene of apple cultivar Florina

The first step in the characterization of a gene is its identification and isolation. A preliminary *MdACS1* genotyping analysis of different apple cultivars showed that Florina is heterozygous for this gene. This result along with the availability of a BAC library of apple cultivar Florina (Vinatzer at al., 1998) allowed us to isolate the sequence of the coding region and 5' flanking region of both the *MdACS1* alleles. The alignment of the sequences of the two BAC clones containing each a specific *MdACS1* alleles, with that of cultivar Golden Delicious from gene bank, showed as expected only few base change differences between the two cultivars while the structural characteristics of the gene were corresponding to that described in Sunako et al. (1999). A typical *ACS* gene organization consisting of four exons and three intron (Jakubowicz and Sadowiski, 2002) was found. The alignment of the promoter regions of the two alleles showed a very high identity of the two sequence and confirmed the presence of the retrotransposon insertion and a tandem repeat in *MdACS1-2*, and of the 24bp insertion in *MdACS1-1* allele as previously reported (Sunako et. al 1999). A total of 25 single nucleotide changes between the two alleles were also found.

Since transcription is a finely regulated process in which interaction between transcription factor proteins and specific DNA motif can modulate the onset of the process and the level of expression, each of the found differences in the sequences of the two alleles could be hypothetically responsible for an alteration in a protein/DNA recognition step (modifying the DNA recognition site or the DNA fold) and led as consequence to a change in the transcription level.

2.2 *MdACS1* allele differential expression

The expression of the *MdACS1* gene in apple has been studied all along the ripening process in different tissues. While all the studies concord on *MdACS1* expression in cultivar homozygous or heterozygous for *MdACS1-1*, contrasting expression data have been found in literature regarding the *MdACS1* gene expression studied in cultivars homozygous for the *MdACS1-2* allele, so that it was not clear if the *MdACS1-2* allele is transcribed or not.

To try to resolve this brain-teaser we decided to set up a qPCR analysis. qPCR is a precise and quick method for measuring gene expression (Larionov et al., 2005) especially to distinguish among highly similar genes. When this methodology was applied for the studies of *MdACS1* allele in literature, the primer pairs were designed in sequence region identical between the two alleles so they were not able to distinguish between them. To have more information about the transcriptional behaviour of the two *MdACS1* alleles we performed an accurate set up of qPCR experiments specific for each allele. Our sequencing and alignment of the coding region of the two Florina *MdACS1* alleles confirmed the high homology found by Sunako et al. (1999), with only nine base changes between the two sequences. The design of primer pairs was therefore conditioned by the SNPs position and it was possible to obtain only two primer pairs specific for each allele with one or two SNPs in the forward primers and one SNP at 3' end for the reverse one. To reach reliable results the primer should be validated for their specificity. We evaluated the primer specificity with different methods. A specific amplification on the two BAC clones carrying the *MdACS1* alleles was obtained also if to guarantee the specificity of the amplification it was necessary to apply very stringent conditions with the risk of reducing the efficiency of amplification and so the final expression value. The specificity was also tested with a melting curve analysis in which the presence of only a single peaks for a sample assured that the amplicon was allele specific and not a mix of different amplicons. Finally the amplicons sequencing confirmed without any doubt the allele specificity of the tested primers because a single SNP was included within the amplified sequence. On the basis of the results two primer pairs for qPCR, each able to recognize and amplify only one of the two alleles of *MdACS1* gene were efficiently designed. These primer pairs were used to evaluate the expression of the two allele in different apple cultivars, tissue and stages of development. For the data processing the standard curve method was preferred at the comparative Ct method. Although the standard curve method requires the addition of the standard samples in all the plates and for all the tested genes, including reference gene, increasing the time and cost of analysis, however it simplifies calculations and avoids problems currently associated with

PCR efficiency assessment. In this way the comparisons among results obtained with different primer pairs and in different qPCR plates are more reliable.

In all our experiments amplification signal for *MdACS1-2* was never detectable. Since we were sure that the primers specific for this allele function, they were indeed able to amplify genomic DNA of apple cultivars homozygous for *MdACS1-2* allele, we concluded that this allele is never expressed. Since *MdACS1* is reported to be involved in system 2 ethylene this finding indicate that this system might be absent in fruit homozygous for *MdACS1-2* or controlled by other genes.

Our results are in contrast with that of Sunako et al. (1999). With their RT-PCR and restriction analysis on poly(A+)RNA fractions extracted from climateric fruit of cultivars Fuij (*MdACS1 2-2*) and Golden Delicious (*MdACS1 1-2*), they found that the *MdACS1-2* is not expressed in the heterozygous Golden Delicious, in which the mRNA is transcribed exclusively from *MdACS1-1* allele, while the *MdACS1* transcript in cv Fuji was derived from *ACS1-2* allele. Also in their Rna gel-blot analysis with a probe in the 3' UTR *MdACS1* region the signal in Fuji was very faint. So they concluded that the promoter of the *MdACS1-2* allele still functions. Tan et al. (2012), on the other end, in their real time PCR experiments on Red Fuji, a cultivar homozygous for *MdACS1-2*, found that the *MdACS1* was not expressed and hypothesized that the small amount of ethylene detected in these fruit could be the results of system 1.

Our qPCR analysis showed that the *MdACS1* gene is expressed in flesh fruit only at ripening stage, indeed no expression at all was found in Florina flesh collected one month before ripening while a very high level of expression was detected in flesh at full ripening stage. These results were expected on the basis of literature data, which reported that *MdACS1* expression in fruit is not detected until the ripening stage. As ripening progress there is a 100-1000 fold increase in *MdACS1* expression (Wiersma et al. 2007) that reach maximum abundance on 10 day of storage at room temperature (Tan et al., 2012).

The analysis in different apple tissues confirmed the data reported in literature of a *MdACS1* predominant expression in flesh fruit. A small amount of expression was found in Florina skin only at pre-climateric stage. Similar results were obtained by Kondo et al. (2009) when *MdACS1* expression was studied in skin of pre-climateric fruits. The very low expression in our case could also be ascribable to a small amount of flesh remained on the skin. We also found very low expression in cotyledon, leaf and root of florina seedling and in flower. This result is in contrast with Wakasa et al. (2006) studies in which no expression was found in young expanding leaves, in vitro-cultured shoots, roots emerged from seed and receptacles

and pistills of full-bloom flowers of Golden Delicious. However, also Wiersma et al. (2007) found a small amount of *MdACS1* expression in apple leaf. This predominant gene expression in fruit compared with ones in vegetative tissues after all support the role of the *MdACS1* gene in ripening process. However, also if very low, the presence of *MdACS1* expression in tissues other than fruit prevent its employment as fruit-specific promoter for tissue specific transgene expression in fruit crop as hypothesized by James et al. (2001).

2.3 Transcription regulatory elements in *MdACS1* promoter

Transcriptional inducibility of different members of multigene *ACS* family is the main regulatory step of their activity. Obviously given that this is a multigene family that operate in different tissues and developmental stages and that it's induced by different stimuli, an heterogeneous group of regulatory elements has been found as reported by Jakubowicz and Sadowiski (2002).

Our *in silico* analysis of the promoter regions of the two *MdACS1* alleles gave back a huge amount of putative *cis*-acting elements potentially involved in the regulation of this gene. However, a great number of them are unlikely to play a role in the studied *MdACS1* promoter considering their function in the promoter where they were identified. *MdACS1* gene is one of the main actors in fruit ripening process. Ripening related genes can have different regulatory modules that control their specific expression, as discussed in the introduction. The knowledge of the spatial and temporal expression profile of *MdACS1* as well as of the internal and external stimuli that induce its expression can help in data interpretation. Our results of qPCR analysis revealed a developmentally-regulate expression of the *MdACS1* gene. Similar result are reported in literature. Expression analysis of *MdACS1* gene in fruit of trees located in two localities differing in elevation and season day degree sum (Dal Cin et al., 2007) demonstrated that the increase in *MdACS1* transcript at 100 days after bloom precede the onset of ethylene evolution especially in fruit from low elevation site. At this stage the gene expression appeared so to be developmentally regulated. In the late stage of ripening when the system 2 of ethylene biosynthesis is largely established, the *MdACS1* trancription level correlate with ethylene evolution indicating that it is ethylene related. *MdACS1* seems also to be sensitive to temperature regime: the transcript accumulation was observed earlier at low elevation site in which daily average temperature is constantly 2° C higher than in the other localities. The involvement of ethylene in the regulation of *MdACS1* expression has also been demonstrated by experiments with 1-methylcyclopropene (1-MCP), an ethylene antagonist, and ethephon, an

ethylene releaser, as well as by characterization of transgenic plants with reduced ethylene production. *MdACS1* was blocked completely by 1-MCP and enhanced significantly by ethephon treatment (Tan et al., 2012). Moreover fruit obtained from apple plants silenced for either *ACS* or *ACO* genes didn't displayed autocatalytic ethylene production suggesting that ethylene is required for sustaining the steady state level of ACS mRNA (Dandekar et al., 2004). An other plant hormone, *n*-propyl dihydrojasmonate (PDJ), is reported to greatly increase the transcript level of *MdACS1* (Kondo at al., 2009). Finally the differential expression of *MdACS1* in different apple tissues resulting from our expression analysis, suggests also a not fruit-specific but tissue-differential regulation. So *MdACS1* gene expression appears to be developmentally regulated, ethylene induced, tissue and temperature controlled. The screening of our sequence with PLACE database to search for *cis*-acting element reported in other gene similarly regulated however gave as output only an ethylene responsive element (ERE) that is reported to confer ethylene induction of *E4* tomato gene. This results is probably due to the fact that despite a great number of ripening related genes has been isolated, very few ripen-specific *cis*-acting elements and transcription factors has been identified in fruit species and recorded in plant CARE database.

Instead a huge number of *cis*-acting elements originating from light-regulated genes (GT; I box; GATA) have been found. However, until now there is no evidence for light or circadian clock regulation of Md*ACS1* in fruit and the light greatest impact during fruit development has been reported on pigmentation with apparently little effect on additional ripening phenomena.

Some additional information could be derived by comparison of *MdACS1* promoter region with that of homologous genes in distinct species. This gene, called orthologs, usually retain very similar functions in distinct species therefore their promoter regions probably contain similar regulatory motifs. The phylogenetic relationship of *ACS* sequences calculate by El Sharkawy et al. (2004) placed each of the apple *ACS* sequences very closely in branch with putative orthologs from *Pyrus communis* and *Pyrus pyrifolia*. Also a BLAST analysis that we performed with our *MdACS1* promoter sequences (data not showen) resulted in a very high omology with promoter region of this species. However only very short promoter sequence of the *PcACS1a*, the ortholog of *MdACS1*, is reported prevening a comparison of the most important regulatory region usually located between the – 200 and -1000 bp in respect of the transcription starting site.

2.4. Functional regions of *MdACS1* promoter

The spatial/temporal expression of genes is the results of precise regulation networks that involve the interaction between DNA motifs and their cognate transcription factors. Therefore, it is expected that the *MdACS1* promoter presents defined regions able to drive its specific expression mainly in fruit at ripening stage. Moreover the completely absence of *MdACS1-2* allele transcript suggests that some of the identified differences between the two *MdACS1* alleles could be responsible for the loss of its transcriptional activity.

A common strategy to identify important regions in promoters is the analysis of the promoter and its progressive deletion fragments in an *in vivo* system. For this reason we prepared promoter deletion constructs for both *MdACS1* alleles, taking into account the four regions identified in the promoter on the basis of similarity analysis, and analyzed them by *Agrobacterium*-mediated transient transformation of lettuce and apple tissues.

The transient transformation with full promoter sequences of both the *MdACS1* alleles (C6 and C7 constructs) confirmed the results of our qPCR expression analysis in apple fruit. The C6 construct containing 1639 bp of the 5' flanking region of *MdACS1-1* allele was able to induce GUS expression in infected lettuce leaves at level similar to that of the CaMV35S promoter. This result confirmed that this promoter fragment is fully functioning and able to drive transcription of *MdACS1-1* allele, whose transcript was effectively detected in apple fruit by qPCR. The reporter expression was detected not only in lettuce but also in the apple flesh at levels similar to that of the CaMV35S. Despite the limited repeatability observed in apple system, this result is very important because suggests that our promoter is functioning in its native contest as well as in an heterologous ones and that the expression in the two systems is comparable.

qPCR analysis for *MdACS1-1* allele showed a predominantly expression of this allele in ripe fruit while very low expression was found in skin of immature fruit, cotyledon and leaf. However when tested by transient expression assay, the chimeric C6::GUS fusion construct allowed gene expression in both apple fruits and lettuce leaves at comparable level. This discrepancy could be imputable to the use of an heterologous system but we cannot exclude also that the promoter sequence used in our studies, whose length was arbitrarily chosen, doesn't contain regulatory region for the modulation of expression in different tissues. Yin et al. (2009) for example found that a TCCAAAA motif is necessary for fruit-specific expression in watermelon by inhibiting gene expression in leaves.

The C7 construct, representing 1790 bp of the *MdACS1-2* promoter region, wasn't able to activate transcription of GUS*Plus* reporter gene as expected on the basis of our real time PCR results in which the expression of the *MdACS1-2* allele was never observed.

When 667 bp and 654 bp (the forth regions) were eliminated starting from the 5' end of C7 and C6 constructs, to generate respectively constructs C5 and C4, a change in the GUS expression was observed for both promoters. The level of expression decreased dramatically in C4 infected tissue in respect of that transformed with C6 suggesting that these 654 bp should contain enhancer elements. Similar results are reported in literature in which a particularly high efficiency was obtained when the promoter fragment changed from about 1 Kb to about 2 kb and plant 5' fragment of about 3 Kb showed a strength higher than that of the constitutive CaMV35S promoter (Spolaore et al. 2003). So we could not excluded that in the 5' flanking region upstream the C6 and C7 fragments, that we didn't consider in our promoter analysis, are present more regulatory elements. Moreover we didn't considered in the design of our promoter fragments the 3' UTR region, but it is known that in this un-transcribed region can be located further regulatory elements able to control transcriptional activity.

An important role of the forth region in the control of *MdACS1* transcription was suggested from the activation of the *MdACS1*-2 promoter with its elimination from the C7 construct. Indeed, lettuce leaves infected with this shorter construct (C5) showed GUS activity unlike that inoculated with C7. The level of expression was similar to that obtained with the corresponding construct C4 of the *MdACS1-1* allele. C4 and C5 constructs were designed comprising the most variable promoter region (the third) between the two alleles. This region included the SINE element in the C5 construct and the 24 bp insertion in the C4 region. The comparable *GUS* activity obtained with the two constructs (C4 and C5) and the re-activation of *MdACS1-2* promoter with C5 construct both suggest that the SINE element could not be alone responsible for the loss of promoter activity as was hypothesized by Harada et al. (2000). At the same time these results suggested a role of the forth region of C7 fragments in the loss of transcriptional activation.

Reducing further the sequence of the two promoters didn't change the level of GUS expression as observed in lettuce leaves transformed with C2 and C3 constructs (comprising only the first and the second promoter region) that showed blue staining areas comparable to that induced by C5 and C4 constructs. Only a slightly lower GUS activity was found in experiments with the shortest C1 fragment demonstrating that this 225 bp are sufficient for accurate onset of transcription. Our findings are in agreement with that reported in other studies in which GUS activity just above the background started to be detected with

promoter fragment of about 200bp (Spolaore et al. 2003, E. Silfverberg-Dilworth et. al 2005). However many studies on plant promoters report a modulation in GUS strength with promoter fragments of different sizes (Spolaore et al., 2003; Yamagata et al., 2002; Yin et al., 2009) and it is also known that *cis*-acting element with positive or negative function are usually localized in a region of almost 1000 bp in plant promoters. So, some variability in the induction of GUS staining between our deletion constructs was expected. Without a quantitative analysis however it is not possible to distinguish clearly if the similar GUS staining results observed in tissues infected with C1-C2-C3-C4-C5 constructs correspond effectively to similar GUS activity or if significant, even though rare, differences occur. It could also be hypothesized that the heterologous lettuce system lack specific apple transcription factors able to interact with these promoter regions. When C1-C2-C3-C4-C5 constructs were used in transient transformation of apple fruit however we didn't found any *GUS* expression. Since the transient transformation in lettuce confirmed that they are functioning, and so no mistakes in construct preparation were done, the absence of reporter expression in fruit could be due to an inefficient transformation rate in apple system that make difficult to detect the reporter gene activity driven by promoter fragments with very low strength. Indeed, also in lettuce the activity of this deletion constructs was very low in respect of that of the longest promoter construct C6 that was the only one that showed GUS staining in apple. We also didn't prepared promoter fragment shorter than C1, so we can not ruled out if the observed GUS expression in tissues infected with this construct is effectively the basal level generated only by the binding of the RNA polymerase complex or if C1 include already some binding sites for proteins that interact with this complex and enhance transcription. All the evidences suggested to investigate more in detail the almost 600 bp in the distal region of promoters that seem to be responsible both for the highest expression found in the *MdACS1-1* promoter and for the loss of functionality of the *MdACS1-2* promoter.

2.5 Regulation of *MdACS1* transcription

The forth regions of the C6 an C7 fragments differ for the presence of 16 single base changes and for the insertion of a 16 bp tandem repeat in the *MdACS1-2* promoter. The *in silico* analysis for CAREs in these zones revealed that a dimerization of a GT1 core *cis*-acting elements is present in the 16 bp tandem repeat insertion of the *MdACS1-2* promoter region while only one GT1 element was found at the same position in the *MdACS1-1* promoter. GT elements are regulatory DNA sequences usually found in tandem repeats in the promoter region of many different plant genes and depending on promoter structure they

can have a positive or negative transcription function (Zhou, 1999). The transcription factors that recognize this element are transcriptional activator that can act directly through stabilization of the pre-initiation complex (Kaplan-Levy et al., 2012). They can bind to DNA either as a dimer or bind two GT elements at the same time. Modification and dimerization between GT-factors as well as their interaction with other transcription factors appear to play a major role in producing a distinct regulatory function (Zhou, 1999).

It's reported that a single GT1 element cloned upstream of a minimal promoter enhance the reporter gene activity, but the introduction of a second GT element in near proximity leads to a reduction in activation of the minimal promoter (Mehrotra and Panwar, 2009). So the presence of the GT1 element in the forth region of the promoters could potentially explain both the increase of the reporter activity found with the C6 promoter construct of *MdACS1-1* in respect of C4 and the inactivation with C7 construct of *MdACS1-2* allele.

To test this hypothesis a dimerization of GT-1 element was introduced in the C6 fragment (C6TX2 construct) and one copy of this element was eliminated from C7 fragment (C7TX1 construct). The removal of one element from the C7 fragment as hypothesized determined the activation of the *MdACS1-2* allele but the expression level was lower than that of the full *MdACS1-1* promoter construct C6. However the introduced GT dimerization in C6 doesn't caused the failure of transcription as expected, but rather a slightly increase of GUS activity was found. Also the gain of function experiment with GT element in both dimeric form or as individual element didn't showed a clear regulatory activity of this putative CARE. These results seem to suggest that the increase in GUS expression observed in C6 in respect of C4 infected tissues and the loss of GUS expression observed when the more distal region is added to C7 are due to different events.

2.5.1 The enhancer region of *MdACS1* promoter

When the tandem repeat zone and other adjacent 9 bases where eliminated in both promoters the GUS activity, induced by the transformation with the resulting C6-7noT constructs, was detected at similar levels for both the promoters and comparable to that of C6. This result allow us to assert that in both promoters the almost 600bp between C4/C5 and C6/7noT constructs contain an enhancer-like region for high-level gene expression. This region is responsible for the substantial difference in GUS activity between the basal level found with C1-C2-C3-C4-C5 deletion constructs and the highest one observed with C6 and C6-7noT construct. When these 600 bp were scanned against plant CARE database a very high concentration of putative DNA binding elements was found. In particular different

transcriptional activator element (ARRIAT) and an Ethylene Responsive Element (ERE) was found in both the promoter regions. Since the *MdACS1* gene control the ethylene production in the autocatalytic system 2 the role of ERE as *cis*-acting element was evaluate with specific promoter deletion constructs that start just downstream this sequence. The GUS activity obtained with these constructs was very similar to that observed with constructs that include this element so it seems that it is not directly involved in an increasing of promoter activity. Moreover, this result allow to further delimitate the region with the enhancer module to 261 bp between -1732 to -1471 and -1593 to -1332 in *MdACS1-2* and *MdACS1-1* promoter respectively.

2.5.2 The loss of transcriptional activity of *MdACS1-2* allele

The final 57 bp at 5' of C7 construct eliminated in the generation of C7not construct resulted in some way responsible for the loss of C7 activity. Further analysis with constructs that taking into account the SNP just downstream to the tandem repeat (C6snp and C7snp constructs) or both the SNP and the tandem repeat (C6TX2snp and C7TX1snp constructs) showed that even the single base change alone can determine the activation of transcription in *MdACS1-2* allele. The remark that the promoter of *MdACS1-1* remain active also when the exact distal region of *MdACS1-2* is reconstituted in its sequence allow us to conclude that the 57 bp sequence are not essential for the control of *MdACS1-1* transcription and that C7 distal region cannot be responsible alone for the inactivation of the allele but probably a joint action with other promoter regions with distinct features between the two promoters is involved. It's difficult on the basis of our data clarify the precise mechanism by which repression occurs. We cannot exclude neither change in recognition site for specific transcription factor proteins nor more complex changes in secondary DNA structure as responsible for the failure of the assembly of a functioning transcription complex.

In any case these new findings call attention again on the SINE element, that is the remarkable difference between the promoter region of the two alleles, no more as unique responsible but as co-actor in the *MdACS1-2* lack of transcription activity. However, we cannot also excluded the involvement of the 24 SNPs scattered along the whole promoter sequences.

To verify the role of the retrotransposon insertion we started to prepare a modified C7 construct without the SINE element. The most common approach to delete DNA sequences in plasmids uses naturally occurring restriction site followed by ligation of the plasmid (Allemoandou et al., 2003). However, in our sequence the available sites for restriction enzyme didn't allowed the precise elimination of the SINE insertion. For this reason we

tried a PCR-based technique that takes advantages of the circularity of plasmid DNA to amplify the entire plasmid except for the region to be deleted (Pinera et al., 2006). Although the amplification of the pCAMBIA0305.1-C7 plasmid, with specific primers designed just upstream and downstream the SINE, worked and the ligation process of the amplification product was successful, it was not possible to eliminate plasmid DNA template. All the transformed *E. coli* colonies were found to contain both the original and the mutated plasmids. The DNA template contamination is the main drawback of this technique and not even the suggested treatment with *DpnI* enzyme (Pinera et al., 2006), that is able to cleaves specifically the plasmid DNA template, was successful in our case. Therefore, the lack of the availability of this construct doesn't allowed to ruled out the role of the SINE element in the loss of *MdACS1-2* transcriptional activity.

3. GENETIC CONTROL OF ETHYLENE BIOSINTHESIS IN APPLE FRUIT

In general there is a good correlation between *MdACS1* genotype, ethylene production and apple fruit storability (Harada et al., 2000; Costa et al., 2005). However, sometimes the *MdACS1* allelic form was not correlating with storage competency (Oraguzie et al., 2004). Moreover, Zhu and Barrit (2008) found that apple cultivars with the same *MdACS1 2-2* genotype showed different patterns of firmness loss. Fuji and Mondial Gala are two apple cultivars homozygous for *MdACS1-2* allele, that are expected to have both a good storability on the base of the *ACS* genotype. However, Mondial Gala, a summer apple that in Italy ripens in the second decade of August, is characterized by a poor storage quality becoming soon overripe while Fuji, a late-ripening cultivar, show no firmness loss for at least a month, even at room temperature (Gussman et al., 1993). Our ethylene measurement in apple fruits of these two cultivars showed that they have a markedly different production of this hormone: only a trace amount of ethylene was detected in Fuji fruit during storage at room temperature while a higher amount is produced by Mondial Gala apple fruits.

As consequence of this experimental evidence, the ethylene production rate observed in different apple cultivars cannot be explained only by the *MdACS1* allelic genotype but other ripening related genes should be involved in its control. The bimodal-like distribution observed in the progeny Fuji x Mondial Gala could confirm that other genes are involved in ethylene production. When a threshold of 1µl/g h is used to divide the seedlings more similar to Fuji from the others, the observed segregation is about 38% and 62% respectively. Therefore, a system of at least two genes has to be hypothesized to explain the observed variability.

The storability of harvested fruits is also considered to be influenced by the association between the *MdACS1* and *MdACO1* allelotypes: the influence of *MdACO1* genotypes on fruit firmness is significant when combined with *MdACS1 2-2* but not with *MdACS1 1-2* (Zhu and Barrit, 2008) so that homozygousity for *MdACO1-1* further reduces ethylene level within an homozygous *MdACS1-2* background (Costa et al., 2005). Our finding of a slight shift of the distribution of Fuji x Mondial Gala seedlings homozygous for *MdACO1-1* toward the classes with lower ethylene production could support this hypothesis.

The analysis of the ethylene production in genotypes of Fuji x Mondia Gala population showed that the early-maturing seedlings produce more ethylene than the middle and late ones but only for genotypes heterozygous for *MdACO1*. Instead, all the seedlings homozygous for *MdACO1* produce always low level of ethylene irrespective of the ripening

date. Therefore genotypes homozygous for *MdACO1-1* seems to be not influenced by ripening date that seem instead to have a role in the control of ethylene production in heterozygous genotypes.

In the light of our results one of the other genes, in addition to *MdACS1*, responsible for the bimodal distribution observed in our experiments could be *MdACO*. The different rate of ethylene production in Fuji and Mondial Gala, both homozygous for *MdACS1-2*, could so be explained by the different *MdACO1* allelic composition and the different ripening date. Mondial Gala that has a functional allele of *MdACO1* and is an early-ripening cultivar produce more ethylene than the late-maturing cultivar Fuji that has both the *MdACO1* alleles not functioning. However, only this gene is not able to explain all the ethylene variability found in the Fuji x Mondial Gala progeny so at least another gene should be involved.

In tomato it has found that different *ACS* genes are involved in the ripening process. Four of the nine *ACS* genes reported, *LeACS1A, LeACS2, LeACS4* and *LeACS6,* are expressed in ripening fruit (Barry et al. 2000). *LeACS1A* and *LeACS6* were assigned to the system 1 ethylene while *LeACS2* and *LeACS4* are involved in the system 2. Also arabidopsis has eleven *ACS* genes and one pseudogene suggesting the possibility of more family members in apple, as well. The only other *MdACS* gene known to be expressed in fruit is the *MdACS3*. Only recently a crucial role of this gene in the control of the ethylene biosynthesis in apple fruit has been proposed. This gene is expressed prior to *MdACS1* and is suppressed after the burst of ethylene production suggesting a function in system 1 ethylene biosynthesis in apple fruit (Tan et al., 2012). *MdACS3a-G289V*, a null allele of *MdACS3a*, is reported to be responsible for low ethylene production in apple fruit (Wang et al., 2009). A relationship with date of ripening is also been proposed. Early- and middle maturing cultivars may easily enter the system 2 stage regardless of their *MdACS3a* allelotype while the null genotype is considered to influence the initiation of ripening of only later maturing cultivars (Bai et al., 2012). *MdACS3* genotype alone however cannot explain the full spectrum of ethylene production by which ripening progression is controlled (Bai et al., 2012).

Therefore probably the ethylene production in apple fruit is regulated by at least these three genes, *MdACS1*, *MdACO1* and *MdACS3* with a supporting role of environmental signal related to the ripening date.

On the basis of our results we can formulate an hypothesis for a possible genetic control of ethylene regulation in apple fruit.

The *MdACS1* doesn't segregate in Fuji and Mondial Gala as reported in table 7.

	ACS1	ACO1	ACS3
Mondial Gala	- / -	+ / -	+ / -
Fuji	- / -	- / -	+ / -

Table 7. Allelic composition of Mondial Gala and Fuji apple cultivars for *ACS1*, *ACO* and *ACS3* genes; + indicate a functional allele; - indicate a not functional allele.

Fuji does not produce ethylene because of the lack of the functionality of *ACO1* that catalyze the last step in ethylene biosynthesis (even if a functional copy of the *ACS3* is present). Mondial Gala fruits are able to produce ethylene because of the presence of a functional copy of both *ACS3* and *ACO1* genes. The expected segregations is showed in table 8.

		Mondial Gala			
		ACS3 +		ACS3 -	
		ACO1 +	ACO1 -	ACO1 +	ACO1-
Fuji	ACS3 +	12,5%	12,5%	12,5%	12,5%
	ACS3 -	12,5%	12,5%	12,5%	12,5%

Table 8. Expected segregation of *ACO1* and *ACS3* genes in Fuji x Mondial Gala population; + indicate a functional allele; -indicate a not functional allele.

On the bases of the combination between the different alleles of the two genes, the expected ethylene production in the progeny plants is reported in table 9.

a	Mondial Gala		Fuji	b
1	ACS3 -	ACO1 -	ACS3 -	12,5%
2	ACS3 -	ACO1 -	ACS3 +	12,5%
3	ACS3 +	ACO1 -	ACS3 -	12,5%
4	ACS3 +	ACO1 -	ACS3 +	12,5%
5	ACS3 -	ACO1 +	ACS3 -	12,5%
6	ACS3 -	ACO1 +	ACS3 +	12,5%
7	ACS3 +	ACO1 +	ACS3 -	12,5%
8	ACS3 +	ACO1 +	ACS3 +	12,5%

Table 9. Expected genotypes for *MdACS3* and *MdACO* in Fuji x Mondial Gala progeny. *a)* expected allelic combinations *b)* expected frequency;, + indicate a functional allele; - indicate a not functional allele, in gray are indicated the not functional allelic combinations.

The first four combinations should originate seedlings with fruits not able to produce ethylene as Fuji due to the impossibility to complete the ethylene biosynthesis for the lack of a functioning *MdACO1* gene. All the other allelic (6,7 and 8) combinations are supposed

to guarantee ethylene production because at least one allele of *MdACS3* and *MdACO1* are expected to be functional.

However, we cannot exclude that other genes are involved in the fine regulation of ethylene production in apple fruit. Three other *ACS* genes expressed differentially during apple fruit ripening have recently been found, also if not yet characterized (Tan et al., 2012), and more information could came from the recent availability of apple genome (Velasco et al., 2010).

VI CONCLUSIONS

The expression analysis by qPCR and the *in vivo* analysis by Agrobacterium-mediated transformation approaches allowed to characterize more in depth the *MdACS1* gene.

For the first time, a set of allele specific primers for *MdACS1* gene and a protocol for qPCR using SYBR Green chemistry have been developed and optimized. Their application to assess the expression of the two alleles of the *MdACS1* gene in different apple tissues and stages of development has demonstrated that:

- *MdACS1-1* expression is ripening-related, confirming the predominant role of this gene in the ripening process;
- *MdACS1-2* allele is never expressed in any apple tissue or stage of development. Since *MdACS1* is reported to be involved in system 2 of ethylene production, this finding indicate that this system is not functional in fruits homozygous for *MdACS1-2*. Therefore, ethylene production in these genotypes must be controlled by other genes;
- MdACS1-1 allele is expressed predominantly but not exclusively in the apple fruit. The presence of *MdACS1* expression in tissues other than fruit prevent its employment as fruit-specific promoter for tissue specific transgene expression in fruit crops.

The only protocol available in literature for transient transformation of apple fruits was evaluated and further optimized. In particular, some adjustments in the GUS histochemical assay of apple transformed fruit allowed to eliminate all the unspecific background staining, so that now a reliable evaluation of the transformation results is possible.

This optimized protocol allowed us to verify the expression of the functional *MdACS1-1* allele directly in apple fruits by using a chimeric construct with 1639 bp of its promoter region. The scored GUS activity has revealed an expression comparable to that of the constitutive promoter CaMV35S. However, this protocol seems not suitable for the analysis of promoters with a very low strength as well as when promoter constructs with very similar strength should be compared. The comparable expression obtained with this construct in both apple fruit and lettuce leaves showed that our promoter is functioning in its native contest as well as in an heterologous system and that the expression in the two systems is comparable. This result allowed to evaluate our promoter in that heterologous system with the awareness that the data should be carefully interpreted.

A promoter-less binary vector and a linearized vector without any promoter but ready with sticky ends for the easy ligation of DNA fragments have been generated starting from pCAMBIA 0305.1 vector and are now available for the promoter analysis of other plant genes.

Our work based on the Agrobacterium-mediated transformation of lettuce leaves with different promoter fragments confirmed the results of qPCR expression analysis and allowed the identification of important regulatory regions in the promoters of the two MdACS1 allele. In particular it was found that:

- the first 1639 bp starting from the ATG codon in the 5' flanking region of *MdACS1-1* are sufficient to drive an expression comparable to that of CaMV35, demonstrating to contain important elements for its regulation;
- the first 225 bp contain all the elements necessary for the onset of transcription;
- the first 985 and 1123 bp of the 5' flanking region of *MdACS1-1* and *MdACS1-2*, respectively, showed a comparable and basal level of expression. This proved that the SINE insertion in *MdACS1-2*, the 24 bp insertion in *MdACS1-1* as well as all the single base changes between the two sequences in these regions are not able alone to induce a change in the expression between the two alleles. Moreover, it suggests that in this region are not present cis-acting element able to modulate significantly the expression level.

The in vivo analysis also allowed to identify:

- a region of 261 bp between -1732 to -1471 and -1593 to -1332 in *MdACS1-2* and *MdACS1-1* promoter respectively that contains an enhancer-like zone for high-level gene expression;
- a region of 57 bp between -1790 to -1733 in *MdACS1-2* certainly responsible also if not alone in the inactivation of the *MdACS1-2* allele.

On the bases of our results we can hypothesize that the inactivation of the *MdACS1-2* allele is the result of an interaction between the identified 57 bp region and other promoter regions with distinct features between the two alleles. Although by now we cannot explain which specific repression mechanism occurs, our finding will help in the design of further specific experiments to determine if alterations in the recognition site for specific transcription factor proteins or more complex changes in secondary DNA structures are involved. The already started experiment for the removal of the SINE insertion, the main difference between the promoter region of the two alleles, is expected to better highlight the process. Moreover, further studies addressed to identify specific *cis*-acting element in the

delimitated enhancer region identified in this study and their cognate transcription factors will help to complete the knowledge on the regulation of the *MdACS1* gene.

Furthermore, an increase in the knowledge on the molecular mechanism responsible for different ethylene production in apple cultivars has been effectively addressed.

We demonstrated that at least two other genes in addition to *MdACS1* may be involved in apple fruit ethylene production and that genetic and environmental factors related to the ripening date are able to further regulate the ethylene rate production. An hypothesis that could explain the difference between Fuji and Mondial Gala have been proposed and in the next months a coordinated action is already planned to unravel the genetic bases of ethylene production in these two genotypes. In particular, in the frame of the EU project Fruit Breedomics, an Illumina SNP chip of 20Kb recently developed (December 2012) by FEM (S. Michele all'Adige, TN) will be used to genotype the whole progeny. So, we expect to built a highly detailed molecular map to be used for QTL analysis and to correlate the *MdACS3* and *MdACO* allelic composition of each seedlings with its fruit ethylene production.

References

Abel S, Theologis A. 1994. Transient transformation of Arabidopsis leaf protoplast: a versatile experimental system to study gene expression.

Ahamad M, Mirza B. 2005. An efficient protocol for transient transformation of intact fruit and transgene expression in citrus.

Allemoandou F, Nussberger J, Brunner HR, Brakch N. 2003. Rapid site-directed mutagenesis using two-PCR-generated DNA fragments reproducing the plasmid template. Journalof biomedicine and biotechnology, 3 202-207.

Atkinson RG, Bolitho KM, Wright MA, Inturrigagotia-Bueno T, Reid SJ, Ross GS. 1998. Apple ACC-oxidase and polygalacturonase ripening-specific gene expression and promoter analysis in transgenic tomato. Plant Molecular Biology 38: 449-460.

Augias F, Amaya I, Botella Ma, Valpuesta V. 2005. Functional analysis of homologous and heterologous promoters in strwberry fruits using transient expression. Journal of esperimenta botany. 56, 409 37-46.

Bai S, Wang A, Igarashi M, Kon T, Fukasawa-Akada T, Li T, Harada T, Hatsuyama Y. 2012. Distribution of MdACS3 null alleles in apple (Malus x domestica Borkh.) and its relevance to the fruit ripening characters. Breeding science 62: 46-52.

Barry CS, Llop-Tous MI, Grierson D. 2000. The Regulation of 1-Aminocyclopropane-1-Carboxylic Acid Synthase Gene Expression during the Transition from System-1 to System-2 Ethylene Synthesis in Tomato. Plant Physiology. 123, 979–986.

Barry CS, Giovannoni JJ. 2007. Ethylene and Fruit Ripening. J Plant Growth Regul. 26:143–159.

Birnboim HC and Doly J. 1979. A Rapid Alkaline Extraction Procedure for Screening Recombinant Plasmid DNA. Nucleic Acids Research 7:1513-1523.

Bouzayen M, Latché A, Nath P, Pech JC. 2010. Mechanism of Fruit Ripening - Chapter 16. In: Plant Developmental Biology - Biotechnological Perspectives vol. 1. Springer. ISBN 978-3-642-02300-2.

Capitani G, Hohenester E, Feng L, Storici P, Kirsch J. F, Jansonius J. N, 1999. Structure of 1-Aminocyclopropane-1-carboxylate synthase, a key enzyme in the biosynthesis of the plant hormone ethylene. J. Mol. Biol. 294, 745-756.

Castro-Giráldez M, Fito PJ, Chenoll C, Fito P. 2010. Development of a dielectric spectroscopy technique for the determination of apple (Granny Smith) maturity. Innovative Food Science & Emerging Technologies 11, 4, 749–754.

Costa F, Stella S, Van de Weg W. E, Guerra W, Cecchinel M, Dallavia 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.

Cova V. 2008. Isolamento di marcatori microsatelliti strettamente associati al gene di resistenza a ticchiolatura Vm in melo (PhD Thesis, Università di Bologna 2008, 114 pp).

Dal Cin V, Danesin M, Boschetti A, Dorigoni A, Ramina A, 2005 Ethylene biosynthesis and perception in apple fruitlet abscission (Malus domestica L. Bork). Journal of experimental botany 56, 421, 2995-3005.

Dal Cin V, Danesin M, Botton A, Boschetti A, Dorigoni A, Ramina A. 2007. Fruit load and elevation affect ethylene biosynthesis and action in apple fruit (Malus domestica L. Borkh) during development, maturation and ripening. Plant, Cell and Environment. 30, 1480–1485.

Dandekar AM, Teo G, Defilippi BG, Uratsu S L, Passey AJ,. Kader AA, Stow JR, Colgan R, James DJ . Effect of down-regulation of ethylene biosynthesis on fruit flavor complex in apple fruit. Transgenic Research. 13: 373–384.

Deikman J. 1997. Molecular mechanisms of ethylene regulation of gene transcription. Physiologia Plantarum100,561–566.

Dillen W, De clercq J, Kapila J, Zambre M, Van Montagu M, Angenon G. 1997. The effect of temperature on Agrobacterium Tumafaciens-mediated gene transfer to plants. The Plant Journal, 12 (6) 1459-1463.

Doyle JJ, Doyle JL. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 19:11-15.

El-Sharkawy I, Jones B, Gentzbittel L, Lelièvre JM, Pech JC, Latché A. 2004. Differential regulation of ACC synthase genes in cold-dependent and -independent ripening in pear fruit. Plant, Cell and Environment 27, 1197–1210.

Eulgem T, Rushton P, Robatzek S, Somssich I. E. 2000. The WRKY superfamily of plant transcription factors. Trends in plant science 5, 5 199-206.

Farrell R. 2007. The regulation of gene expression in plants and animals. Chapter 1. In: Regulation of gene expression in plants. The role of transcripts structure and processing. Springer.

Flachowsky H,. Peil A, Sopanen T, Elo A, Hanke V. 2007. Overexpression of BpMADS4 from silver birch (Betula pendula Roth.) induces early-flowering in apple (Malus · domestica Borkh.) Plant Breeding 126, 137—145.

Fütterer J. 1995. Genetics markers and expression signals. Expression signals and vectors. In: Gene transfer to plants (I. Potrykus & G. Spangenberg, eds). 311-324. Springer-Verlag, Berlin, Germany.

Gan C. 1989. Gene Gun Accelerates DNA-Coated Particles To Transform Intact Cells. The Scientist; Sep. 18, 1989, 3[18]:25.

Gaston K and Jayaraman PS, 2003. Transcriptional repression in eukaryotes: repressor and repression mechanisms. Cellular and molecular life science 60, 721-741.

Giovannoni JJ. 2001. Molecular biology of fruit maturation and ripening. Annu. Rev. Plant Physiol. Plant Mol. Biol. 52:725–49.

Giovannoni JJ. 2004. Genetic Regulation of Fruit Development and Ripening. The Plant Cell. 16, S170–S180.

Gussman CD, Goffreda JC, Gianfagna TJ (1993) Ethylene production and fruit-softening rates in several apple fruit ripening variants. HortScience 28:135–137.

Handa AK, Tiznado-Hernández ME, Mattoo AK. 2012. Fruit development and ripening: a molecular perspective. Biotechnology for improvement of yield and quality traits. 23:405-424

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 climateric fruits of some apple cultivars. Theor. Appl. Genet. 101:742-746.

Hartley JL, Temple GF, Brasch MA. 2000. DNAcloning usingin vitro site-specific recombination. Genome Res. 10, 1788–1795.

Hellens RP, Allan AC, Friel EN, Bolitho K, Grafton K, Templeton MD, Karunairetnam S, Gleave AP, Laing W. 2005. Transient expression vectors for functional genomics, quantification of promoter activity and RNA silencing in plants. Plant Methods 1, 1-14.

Hoffman T, Kalinowski G, Schwab W. 2006. Rnai-induced silencing of gene expression in strawberry fruit (Fragaria x ananassa) by agroinfiltration: a rapid assay for gene function analysis. The Plant Journal 48, 818-826.

Huang X, Madan A, 1999. CAP3: A DNA sequence assembly program. Genome Res. 9, 868-877.

Hudson M, Quail P. H, 2003. Identification of promoter motif involved in the network of phytochrome A-regulated gene expression byy combined analysis of genomic sequence and microarray data. Plant physiology 133, 1605-1616.

Jakubowicz M, Sadowiski J, 2002. 1-Aminocyclopropane-1-carboxylate synthase-genes an expression. Acta Physiologiae Plantarum 24, 4: 459-478.

James DJ, Gittings JR, Massiah AJ, Pellny TK, Hiles ER, Bricolti S, Passey AJ, Vaughan SP. 2001. Using heterologous and homologous promoter sto study tissue

specific transgene expression in fruit crops. Proc. IV IS on In Vitro Cult. & Hort. Breeding. Acta Hort 560 55-58.

Janssen BJ, Thodey K, Schaffer RJ, Alba R, Balakrishnan L, Bishop R, Bowen JH, Crowhurst RN, Gleave AP, Ledger S, McArtney S, Pichler FB, Snowden KC, Ward S. 2008. Global gene expression analysis of apple fruit development from the floral bud to ripe fruit. BMC Plant Biology, 8:16.

Jefferson RA, Kavanagh TA,Bevan MW. 1987. GUS fusions: ,B-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. The EMBO Journal 6 (13) 3901 -3907.

Jones HD, Doherty A, Sparks CA. 2009. Transient transformation of plants. Methods in molecular biology, Plant Genomics 513, 131:151.

Jones HD, Sparks CA. 2009. Stable transformation of plants. Methods in molecular biology, Plant genomics 513, 131-152.

Kaplan-Levy RN, Brewer PB, Quon T, Smyth DR. 2012. The trihelix family of transcription factors – light, stress and development. Trend in plant science. 17, 3, 163:171.

Kende H.1993. Ethylene biosynthesis. Annual Review of Plant Physiology and Plant Molecular Biology 44,283–307.

Kim WT, Silverstone A, Yip WK, Dong JG, Yang SF. 1992. Induction of 1-aminocyclopropane-1-carboxylate synthase mRNA by auxin in mung bean hypocotyls and cultured apple shoots. Plant Physiol. 98, 465-471.

Komarnysky S, Borisjuk N. 2003. Functional analysis of promoter elements in plants. Genetic engineering 25: 113-141.

Kondo S, Meemak S, Ban Y, Moriguchi T, Harada T, 2009. Effects of auxin and Jasmonates on 1-aminocyclopropane-1-carboxylate (ACC) synthase and ACC oxidase gene expression during ripening of apple fruit. Postharvest biology and technology 51, 281-284.

Larionov A, Krause A, Miller W. 2005. A standard curve based method for relative real time PCR data processing. BMC Bioinformatics. 6: 62.

Lee LY and Gelvin. 2008. T-DNA Binary Vectors and Systems. Plant Physiology 146, 325–332.

Lelièvre JM, Latchè A, Jones B, Bouzayen M, Pech JC. 1997. Ethylene and fruit ripening. Physiol. Plant. 101,727-739.

Matsumoto K, Cabral GB, Teixeira JB, de Castro Monte D. 2009. Agrobacterium-mediated transient expression system in banana immature fruits. African Journal of biotechnology. 8 (17), 4039-4042.

Mehrotra R, Kiran K, Chaturvedi CP, Ansari SA, Lodhi N, Sawant S, Tuli R. 2005. Effect of copy number and spacing of the ACGT and GTGGTTAG cis elements on transient expression of minimal promoters in plants. J. Genet. 84, 183-187.

Mehrotra R, Panwar J. 2009. Dimerization of GT element interferes negatively with gene activation. J. Genet. Publisched on web.

Montgomery J, Goldman S, Deikman J, Margossian L, Fisher RL. 1993. Identification of ethylene-responsive region in the promoter of a fruit ripening gene. Proc. Natl. Acad. Sci. U.S.A. 90, 5939-5943.

Nagaraja V, 1993. Control of transcription initiation. J.Biosci 18,1, 13-25.

Oraguzie NC, Iwanami H. Soejima J, Harada T, Hall A.. 2004. Inheritance of the Md-ACS1 gene and its relationship to fruit softening in apple (Malus×domestica Borkh.). Theor. Appl. Genet. 108, 1526–1533.

Orzaez D, Mirabel S, Wieland WH, Granell A. 2006. Agroinjection of tomato fruits. A tool for rapid functional analysis of transgenes directly in fruit. Plant phisiology 140, 3-11.

Pagliarani G, Paris R, Tartarini S, Sansavini S. Cloning and expression of the major allergen genes in apple fruit. JHSB ISAFRUIT Special Issue 176–181.

Palma JM, Corpas FJ, Del Río LA. 2011. Proteomics as an approach to the understanding of the molecular physiology of fruit development and ripening. Journal of proteomics 74, 1230-1243.

Paris R, Cova, V, Pagliarani, G, Tartarini, S, Komjanc, M. and Sansavini, S. 2009. Expression profiling in HcrVf2-transformed apple plants in response to Venturia inaequalis. Tree Genetics & Genomes, 5, 81–91.

Pech JC, Bouzayen M, Latche´ A, Sanmartin m, Aggelis A, Kanellis Ak. 2003. Physiological, biochemical, and Molecular aspects of ethylene Biosynthesis and action- Chapter 10. In Postharvest physiology and pathology of vegetables.

Peixoto de Oliveira ML, Febres VJ, Cardoso Costa MG, Moore GA, Otoni WC. 2009. High-efficiency Agrobacterium-mediated transformation of citrus via sonication and vacuum infiltration. Plant Cell Rep. 28:387–395.

Pinera PP, Gonzalez MM, Vega JA. 2006. Deletion of DNA sequences of using a polymerase chain reaction based approach. Electronic journal of biotechnology, 9, 5, 604-609.

Potenza C, Aleman L, Sengupta-Gopalan C, 2004. Invited review: targeting transgene expression in research, agricultural, and environmental applications: promoters used in plant transformation. In vitro cell. Dev. Biol. Plant 40:1-22.

Roberts RJ, Vincze T, Posfai J, Macelis D. 2010. REBASE--a database for DNA restriction and modification: enzymes, genes and genomes. Nucl. Acids Res. 38: D234-D236.2010.

Rombauts S, Florquin K, Lescot M, Marchal K, Rouzé P, Van de Peer Y. 2003. Computational approaches to identify promoters and cis-regulatory elements in plant genomes. Plant Physiology. 132, 1162-1176.

Sato T, Kudo T, Akada T. 2004. Allelotype of a ripening-specific 1-aminocyclopropane-1-carboxylate synthase gene defines the rate of fruit drop in apple. J. Amer. Soc. hort. Sci. 129 (1):32-36.

Silfverberg-Dilworth E, Besse S, Paris R, Belfanti E, Tartarini S, Sansavini S, Patocchi A, Gessler C. 2005. Identification of functional apple scab resistance gene promoters. Theor Appl Genet . 110: 1119–1126.

Sorkina A, Bardosh G, LiuYZ, Fridman I, Schlizman L, Zur N, Or E, Goldschmidt EE, Blumwald E, Sadka A. 2011. Isolation of a citrus promoter specific for reproductive organs and its functional analysis in isolated juice sacs and tomato. Plant Cell Report 30: 1627-1640.

Spolaore S, Trainotti L, Casadoro G. 2001. A simple protocol for transient gene expression in ripe fleshy fruit mediated by Agrobacterium..J Exp Bot. 52 (357):845-850.

Spolaore S, Trainotti L, Pavanello A, Casadoro G. 2003. Isolation and promoter analysis of two genes encoding different endo-β-1,4-glucanases in the non-climateric strawberry. Journal of experimental botany. 54 (381), 271-277.

Sudan C, Prakash S, Bhomkar P, Jain S, Bhalla-Sarin N. 2006. Ubiquitous presence of β-glucuronidase (GUS) in plants and its regulation in some model plants. Planta. 224, 4, 853-864.

Sunako T, Sakuraba W, Senda M, Akada S, Ishikawa R, Niizeki M, Harada T, 1999. An allele of the ripening-specific 1-aminocyclopropane-1-carboxylate synthase gene (ACS1) in apple fruit with a long storage life. Plant Physiology 119, 1297-1303.

Sunako T, Ishikawa R, Senda M, Akada S, Niizeki M, Harada T, 2000. MdACS-5A (accession no AB034992) and 5B (accession no AB034993), two wound-responsive genes encoding 1-aminocyclopropane-1-carboxylate synthase in apple. Plant Physiology 122, 620.

Tan D, Li T, Wang A, 2012. Apple 1-aminocyclopropane-1-carboxylic acid synthase genes, Md-ACS1 and Md-ACS3a are expressed in different system of ethylene biosynthesis. Plant Mol Biol Rep.

Tittarelli A, Santiago M, Morales A, Meisel LA, Silva H. 2009. Isolation and functional characterization of cold-regulated promoters, by digitally identifying peach fruit cold induced genes from a large EST dataset. BMC Plant Biology 9:21.

Trick HN, Finer JJ. 1997. SAAT: sonication-assisted Agrobacterium-mediated transformation. Transgenic Research 6, 329-336.

Velasco R, Zharkikh A, Affourtit J, Dhingra A, Cestaro A, Kalyanaraman A, Fontana P, Bhatnagar SK, Troggio M, Pruss D, Salvi S, Pindo M, Baldi P, Castelletti S, Cavaiuolo M, Coppola G, Costa F, Cova V, Dal Ri A, Goremykin V, Komjanc M, Longhi S, Magnago P, Malacarne G, Malnoy M, Micheletti D, Moretto M, Perazzolli M, Si-Ammour A, Vezzulli S, Zini E, Eldredge G, Fitzgerald LM, Gutin N, Lanchbury J, Macalma T, Mitchell JT, Reid J, Wardell B, Kodira C, Chen Z, Desany B, Niazi F, Palmer M, Koepke T, Jiwan D, Schaeffer S, Krishnan V, Wu C, Chu VT, King ST, Vick J, Tao Q, Mraz A, Stormo A, Stormo K, Bogden R, Ederle D, Stella A, Vecchietti A, Kater MM, Masiero S, Lasserre P, Lespinasse Y, Allan AC, Bus V, Chagné D, Crowhurst RN, Gleave AP, Lavezzo E, Fawcett JA, Proost S, Rouzé P, Sterck L, Toppo S, Lazzari B, Hellens RP, Durel CE, Gutin A, Bumgarner RE, Gardiner SE, Skolnick M, Egholm M, Van de Peer Y, Salamini F, Viola R. 2010. The genome of the domesticated apple (Malus × domestica Borkh.). Nat Genet. 42(10):833-9.

Vinatzer BA, Zhang HB, Sansavini S. 1998. Construction and characterization of a bacterial artificial chromosome library of apple. Theor Appl Genet, 97:1183-1190.

Wakasa Y, Kudo H, Ishikawa R, Akada S, Senda M, Niizeki M, Harada T. 2006. Low expression of an endopolygalacturonase gene in apple fruit whit long-term storage potential. Postharvest and technology 39, 193-198.

Wang A, Yamakake J, Kudo h, Wakasa Y, Hatsuyama Y, Igarashi M, Kasai Atsushi, Li T, Harada T. 2009. Null mutation of the MdACS3 gene, coding for a ripening-specific 1-aminocyclopropane-1-carboxylate synthase, leads to long shelf life in apple fruit. Plant Physiology 151, 391-399.

Wang Z, Kenigsbuch D, Sun L, Harel E, Ong M.S, Tobin E. M, 1997. A Myb-related transcription factor is involved in the phytochrome regulation of an Arabidopsis Lhcb gene. The Plant Cell 9, 491-507.

Wiersma PA, Zhang H, Lu C, Quail A, Toivonen PMA. 2007. Survey of the expression of genes for ethylene synthesis and perception during maturation and ripening of

‘Sunrise’ and ‘Golden Delicious’ apple fruit. Postharvest Biology and Technology. 44, 204–211.

Wroblewski T, Tomczak A, Michelmore R (2005) Optimization of Agrobacterium-mediated transient assays of gene expression in lettuce, tomato and Arabidopsis. Plant Biotechnol J 3: 259–273.

Yamagata H, Yonesu K, Hirata A, Aizono Y. 2002. TGTCACA motif is a novel cis-regulatory enhancer element involved in fruit-specific expression of the cucumisin gene. The journal of biological chemistry, vol. 277, No. 13, pp. 11582-11590.

Yin T, Wu H, Zhang S, Liu j, Lu H, Zhang L, Xu Y, Chen D. 2009. Two negative cis-regulatory regions involved in fruit-specific promoter activity from watermelon (Citrullus vulgaris S.). Journal of Experimental Botany, vol. 60, No. 1, pp. 169-185.

Zhou D. 1999. Regulatory mechanism of plant gene transcription by GT-elements and GT-factors. Treands in plant science 4, 6 210-213.

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

Zhu Y, Zheng P, Varanasi V, Shin S, Main D, Curry E, Mattheis JP. 2012. Multiple plant hormones and cell wall metabolism regulate apple fruit maturation patterns and texture attributes. Tree Genetics & Genomes 8:1389–1406.

APPENDIX

Appendix A

Media

LB medium

NaCl	10 g/L
Yeast extract	5 g/L
Tryptone	10 g/L
pH 7.0	

2XYT

Tryptone	16 g/L
Yeast extract	10 g/L
NaCl	5 g/L

YEP

NaCl	5 g/L
Yeast extract	10 g/L
Tryptone	10 g/L
pH 7.2 or 5,6	

SOC medium

Tryptone	20 g/L
Yeast extract	5 g/L
NaCl	10 mM
KCl	2,5mM
MgCl2	10 mM
MgSO4	10 mM
Glucose	20 mM

Infiltration medium

MS salts	4.3 g/L

MES*	10 mM
Sucrose	20 g/L
Acetosyringone	200 μM
Proline	1mM
pH 5.6	

*2-(N-morpholino)ethanesulfonic acid

Appendix B

Alignment of BAC96N17 and BAC22F23 sequences with *MdACS1-1* (accession no. U89156) and *MdACS1-2 1* (accession no. AB10102) Golden Delicious gene bank sequences.

| Single base change between MdACS1-2 alleles and MdACS1-1 allels

. Single base change between the two BAC clones

```
BAC96N17        ---------------------------------------------TTTACACATGCCGG 14
MDACS1-2        AAATCTCTGCATTCTTTGTCATACTTGTGAAGAACCACCAAGCACCTTTACACATGCCGG 240
BAC22F23        ------------------------------------------------------------
MDACS1-1        AAATCTCTGCGTTCTTTGTCATACTTGTGAAGAACCACCAAGCACCTTTACACATGCCGG 240

BAC96N17        TTCCTCCATCGCCATTAGCCAAAACCCTGAGTCGTTTGTTTATTCGAGATCAAGTCATCA 74
MDACS1-2        TTCCTCCATCGCCATTAGCCAAAACCCTGAGTCGTTTGTTTATTCGAGATCAAGTCATCA 300
BAC22F23        ------------------------------------------------------------
MDACS1-1        TTCCTCCATCGCCATTAGCCAAAACCCTGAGGCATTTGTTTATTCGAGATCAAGTCATCA 300

BAC96N17        CGATTTTCGGATCAACAACACACACTTTTTTTTCACCCAGAAGATCGAATCAGAGGATTA 134
MDACS1-2        CGATTTTCGGATCAACAACACACACTTTTTTTTCACCCAGAAGATCGAATCAGAGGATTA 360
BAC22F23        -------CGGATCAACAACACACACTTTTTTT-CACCCAGAAGATCGAATCAGAGGATTA 52
MDACS1-1        CGATTTTCGGATCAACAACACACACTTTTTTT-CACCCAGAAGATCGAATCAGAGGATTA 359
                       ************************ ***************************

BAC96N17        AAAATTGTAGCAGAGATTGTAACCCTAAATTCATTAATACCAATTATTACTTTGTATACG 194
MDACS1-2        AAAATTGTAGCAGAGATTGTAACCCTAAATTCATTAATACCAATTATTACTTTGTATACG 420
BAC22F23        AAAATTGTAGCAGAGATTGTAACCCTAAATTCATTAATACCAATTATTACTTTGTATACG 112
MDACS1-1        AAAATTGTAGCAGAGATTGTAACCCTAAATTCATTAATACCAATTATTACTTTGTATACG 419
                ************************************************************

BAC96N17        TATTCTTGGGTTATTTATTGCAAGAATTTCGTGTTTACAACTCTTTTTCTAGCACTTCCA 254
MDACS1-2        TATTCTTGGGTTATTTATTGCAAGATTTTCGTGTTTACAACTCTTTTTCTAGCACTTCCA 480
BAC22F23        TATTCTTGGGTTATTTATTGCAAGAATTTCGTGTTTACAACTCTTTTTCTAGCACTTCCA 172
MDACS1-1        TATTCTTGGGTTATTTATTGCAAGAATTTCGTGTTTACAACTCTTTTTCTAGCACTTCCA 479
                ************************ *********************************

BAC96N17        TCGACTTATAAGTAATTTAGGCTATTCTTATATTACCAATTAATTTTTAGTGGAATCTCA 314
MDACS1-2        TCGACTTATAAGTAATTTAGGCTATTCTTATATTACCAATTAATTTTTAGTGGAATCTCA 540
BAC22F23        TCGACTTATAAGTAATTTAGGCTATTCTTATATTACCAATTAATTTTTAGTGGAATCTCA 232
MDACS1-1        TCGACTTATAAGTAATTTAGGCTATTCTTATATTACCAATTAATTTTTAGTGGAATCTCA 539
                ************************************************************

BAC96N17        ACTTTTTTAAAATTATTTATCTCATGGAAAATCCAAATTCTCCTCTAAATGAACGGTTAA 374
MDACS1-2        ACTTTTTTAAAATTATTTATCTCATGGAAAATCCAAATTCTCCTCTAAATGAACGGTTAA 600
BAC22F23        ACTTTTTTAAAATTATTTATCTCATGGAAAATCCAAATTCTCCTCTAAATGAACGGTTAA 292
MDACS1-1        ACTTTTTTAAAATTATTTATCTCATGGAAAATCCAAATTCTCCTCTAAATGAACGGTTAA 599
                ************************************************************

                Tandem repeat
BAC96N17        CAAAAAGGGTTAACAAAAAGGAAACTTTAACGCAAAACTCTTGGTACTGTTCACTTTAAT 434
MDACS1-2        CAAAAAGGGTTAACAAAAAGGAAACTTTAACGCAAAACTCTTGGTACTGTTCACTTTAAT 660
BAC22F23        CAAAAAG------------TAAACTTTAACGCAAAACTCTCGGTACTGTTCACTTTAAT 339
MDACS1-1        CAAAAAG------------TAAACTTTAACGCAAAACTCTCGGTACTGTTCACTTTAAT 646
                *******             ********************.******************

BAC96N17        GAAAAATCATATTTTTACACTAAAAAGTCAATCTTGGTACTATTCACTTTACCATTTATT 494
MDACS1-2        GAAAAATCATATTTTTACACTAAAAAGTCAATCTTGGTACTATTCACTTTACCATTTATT 720
BAC22F23        GAAAAATCATATTTTTACATTAAAAAGTCAATCTTGTTACTATTCACTTTACCCTTTATT 399
MDACS1-1        GAAAAATCATATTTTTACATTAAAAAGTCAATCTTGTTACTATTCACTTTACCCTTTATT 706
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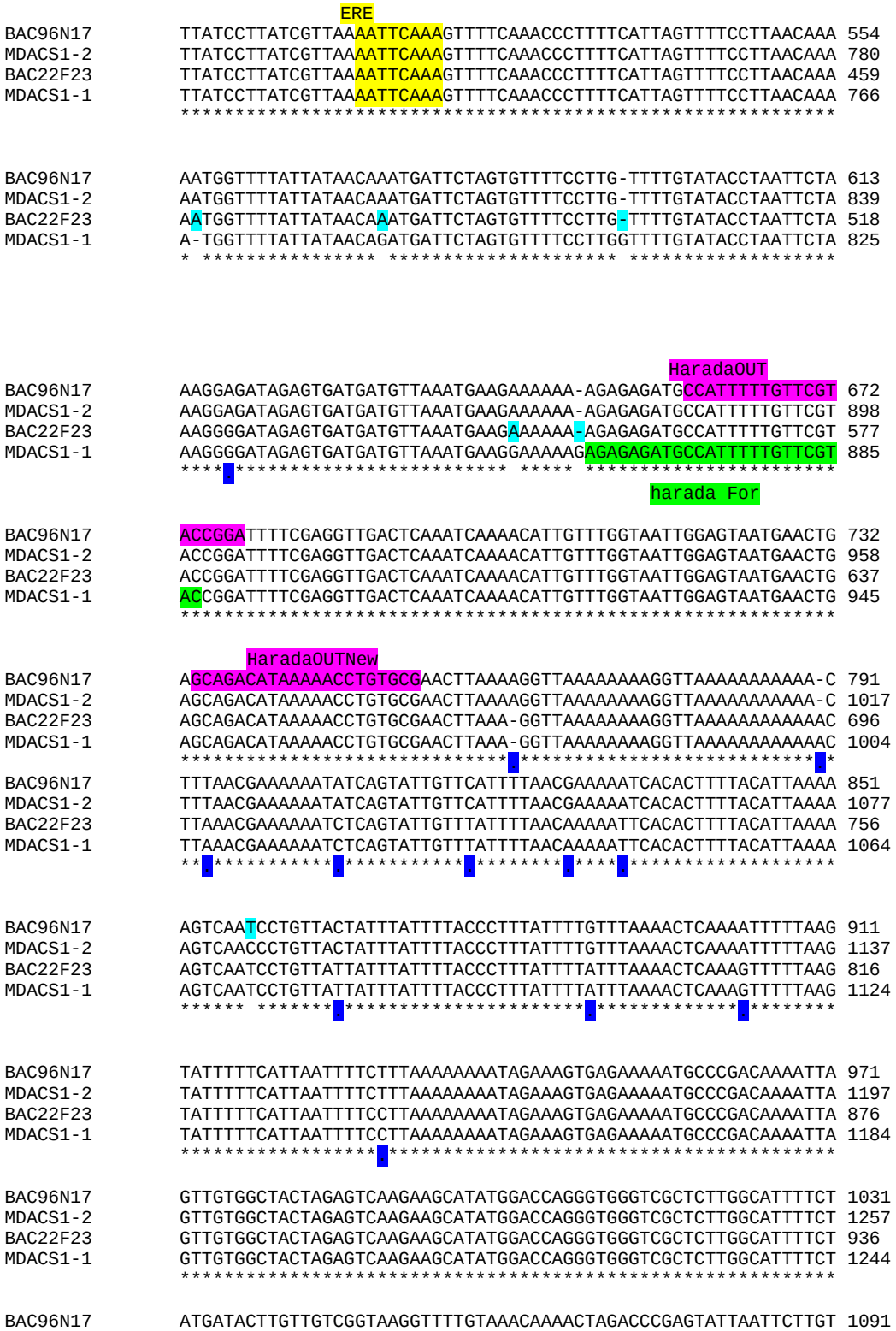
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BAC96N17 TTATCCTTATCGTTAAAATTCAAAGTTTTCAAACCCTTTTCATTAGTTTTCCTTAACAAA 554
MDACS1-2 TTATCCTTATCGTTAAAATTCAAAGTTTTCAAACCCTTTTCATTAGTTTTCCTTAACAAA 780
BAC22F23 TTATCCTTATCGTTAAAATTCAAAGTTTTCAAACCCTTTTCATTAGTTTTCCTTAACAAA 459
MDACS1-1 TTATCCTTATCGTTAAAATTCAAAGTTTTCAAACCCTTTTCATTAGTTTTCCTTAACAAA 766
BAC96N17 AATGGTTTTATTATAACAAATGATTCTAGTGTTTTCCTTG-TTTTGTATACCTAATTCTA 613
MDACS1-2 AATGGTTTTATTATAACAAATGATTCTAGTGTTTTCCTTG-TTTTGTATACCTAATTCTA 839
BAC22F23 AATGGTTTTATTATAACAAATGATTCTAGTGTTTTCCTTG-TTTTGTATACCTAATTCTA 518
MDACS1-1 A-TGGTTTTATTATAACAGATGATTCTAGTGTTTTCCTTGGTTTTGTATACCTAATTCTA 825
HaradaOUT
BAC96N17 AAGGAGATAGAGTGATGATGTTAAATGAAGAAAAAA-AGAGAGATGCCATTTTTGTTCGT 672
MDACS1-2 AAGGAGATAGAGTGATGATGTTAAATGAAGAAAAAA-AGAGAGATGCCATTTTTGTTCGT 898
BAC22F23 AAGGGGATAGAGTGATGATGTTAAATGAAGAAAAAA-AGAGAGATGCCATTTTTGTTCGT 577
MDACS1-1 AAGGGGATAGAGTGATGATGTTAAATGAAGGAAAAAGAGAGAGATGCCATTTTTGTTCGT 885
harada For
BAC96N17 ACCGGATTTTCGAGGTTGACTCAAATCAAAACATTGTTTGGTAATTGGAGTAATGAACTG 732
MDACS1-2 ACCGGATTTTCGAGGTTGACTCAAATCAAAACATTGTTTGGTAATTGGAGTAATGAACTG 958
BAC22F23 ACCGGATTTTCGAGGTTGACTCAAATCAAAACATTGTTTGGTAATTGGAGTAATGAACTG 637
MDACS1-1 ACCGGATTTTCGAGGTTGACTCAAATCAAAACATTGTTTGGTAATTGGAGTAATGAACTG 945
HaradaOUTNew
BAC96N17 AGCAGACATAAAAACCTGTGCGAACTTAAAAGGTTAAAAAAAAGGTTAAAAAAAAAAA-C 791
MDACS1-2 AGCAGACATAAAAACCTGTGCGAACTTAAAAGGTTAAAAAAAAGGTTAAAAAAAAAAA-C 1017
BAC22F23 AGCAGACATAAAAACCTGTGCGAACTTAAA-GGTTAAAAAAAAGGTTAAAAAAAAAAAAC 696
MDACS1-1 AGCAGACATAAAAACCTGTGCGAACTTAAA-GGTTAAAAAAAAGGTTAAAAAAAAAAAAC 1004
BAC96N17 TTTAACGAAAAAATATCAGTATTGTTCATTTTAACGAAAAATCACACTTTTACATTAAAA 851
MDACS1-2 TTTAACGAAAAAATATCAGTATTGTTCATTTTAACGAAAAATCACACTTTTACATTAAAA 1077
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BAC96N17 TATTTTTCATTAATTTTCTTTAAAAAAAATAGAAAGTGAGAAAAATGCCCGACAAAATTA 971
MDACS1-2 TATTTTTCATTAATTTTCTTTAAAAAAAATAGAAAGTGAGAAAAATGCCCGACAAAATTA 1197
BAC22F23 TATTTTTCATTAATTTTCCTTAAAAAAAATAGAAAGTGAGAAAAATGCCCGACAAAATTA 876
MDACS1-1 TATTTTTCATTAATTTTCCTTAAAAAAAATAGAAAGTGAGAAAAATGCCCGACAAAATTA 1184
BAC96N17 GTTGTGGCTACTAGAGTCAAGAAGCATATGGACCAGGGTGGGTCGCTCTTGGCATTTTCT 1031
MDACS1-2 GTTGTGGCTACTAGAGTCAAGAAGCATATGGACCAGGGTGGGTCGCTCTTGGCATTTTCT 1257
BAC22F23 GTTGTGGCTACTAGAGTCAAGAAGCATATGGACCAGGGTGGGTCGCTCTTGGCATTTTCT 936
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BAC22F23        ATGATACTTGTTGTCGGTAAGGTTTTGTAAACAAAACTAGACCCGAGTATTAATTCTTGT 996
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                ************************************************************

BAC96N17        TTCACTAGTACATAACAGCATATTTACCACATCAATTCCATCACGGGCATCAACAGCTCG 1151
MDACS1-2        TTCACTAGTACTTAACAGCATATTTACCACATCAATTCCATCACGGGCATCAACAGCTCG 1377
BAC22F23        TTCTTTGTT----------TTTTT---------------------------------- 1010
MDACS1-1        TTCTTTGTT----------TTTTT---------------------------------- 1318
                ***  *  *           * ***

                                    SINE INSERTION
BAC96N17        TGGTAGAAACTATTCAATTACCACGGTTACATGTAGATCGTGGTAGATAACATTAATTCA 1211
MDACS1-2        TGGTAGAAACTATTCAATTACCACGGTTACATGTAGATCGTGGTAGATAACATTAATTCA 1437
BAC22F23        ------------TTCAATTAC---------------------------------------- 1019
MDACS1-1        ------------TTCAATTAC---------------------------------------- 1327
                          *********

BAC96N17        CCACGGTCAAATTCTGGCCCGTTGTTAAAAGTAAATAATTCACCACGATTAATGCTTCTA 1271
MDACS1-2        CCACGGTCAAATTCTGGCCCGTTGTTAAAAGTAAATAATTCACCACGATTAATGCTTCTA 1497
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                                    HaradaIN
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                ************************************************************
                        HaradaREV

BAC96N17        CCCACTTCCCAGTCCAAGCAAATAGTGAGAAAATAAATTAATGGATGATACTAGGAAAAT 1391
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                ************************************************..*******

BAC96N17        TAAATTTGGAGATAAAATTTGCAAATTATATAATATGTCACCTATACGACTTAACACATT 1451
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BAC22F23        TAAATTTGGAGATAAAATTTGCAAATTATATAATATGTCACCTATACGAATTAACACATT 1218
MDACS1-1        TAAATTTGGAGATAAAATTTGCAAATTATATAATATGTCACCTATACGAATTAACACATT 1526
                ************************************************.**********

BAC96N17        TATCAATATTTAAATAATAAATCAATCATCAACTACCATATAATTTAGTTTCCAAAATTT 1511
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MDACS1-1        TATCAATATTTAAATAATAAATCAATCATCAACTACCATATAATTTAGTTTCCAAAATTT 1586
                ************************************************************

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                ********************************.***************************
                                                          Harada 1521
BAC96N17        CACTAAAACTTCATTGCTTTGGGATTTGGGAGTGTCTGAAGGTCCTTCATGATCAAAGTC 1631
MDACS1-2        CACTAAAACTTCATTGCTTTGGGATTTGGGAGTGTCTGAAGGTCCTTCATGATCAAAGTC 1857
BAC22F23        CACTAAAACTTCATTGCTTTGGGATTTGGGAGTGTCTGAAGGTCCTTCATGATCAATGTC 1398
MDACS1-1        CACTAAAACTTCATTGCTTTGGGATTTGGGAGTGTCTGAAGGTCCTTCATGATCAATGTC 1706
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BAC96N17        TTTAGATGGTGGAGCAAAAGCGCGTACAATTAATTATCATGTTGTTTTTGGATTTTTATT 1691
MDACS1-2        TTTAGATGGTGGAGCAAAAGCGCGTACAATTAATTATCATGTTGTTTTTGGATTTTTATT 1917
BAC22F23        TTTAGATGGTGGAGCAAAAGCGCGTACAATTAATTATCATGTTGTTTTTGGATTTTTATT 1458
MDACS1-1        TTTAGATGGTGGAGCAAAAGCGCGTACAATTAATTATCATGTTGTTTTTGGATTTTTATT 1766
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BAC96N17        GAATCAAAATACTTGGATCATAATGTTAAGAAAAAGAACCAGAGAAATCTAAAGAGACTT 1751
MDACS1-2        GAATCAAAATACTTGGATCATAATGTTAAGAAAAAGAACCAGAGAAATCTAAAGAGACTT 1977
BAC22F23        GAATCAAAATACTTGGATCATAATGTTAAGAAAAAGAACCAGAGAAATCTAAAGAGACTT 1518
MDACS1-1        GAATCAAAATACTTGGATCATAATGTTAAGAAAAAGAACCAGAGAAATCTAAAGAGACTT 1826
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BAC96N17        TCTTAAAAGTGAGATTCTTCATAATTTATTTATCATGTTTTTGGTACAATATTTATAATA 1811
MDACS1-2        TCTTAAAAGTGAGATTCTTCATAATTTATTTATCATGTTTTTGGTACAATATTTATAATA 2037
BAC22F23        TCTTAAAAATGAGATTCTTCATAATTTATTTATCATGTTTTTGGTACAATATTTATAATA 1578
MDACS1-1        TCTTAAAAATGAGATTCTTCATAATTTATTTATCATGTTTTTGGTACAATATTTATAATA 1886
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BAC96N17        TCGGCGCAAAAATTAATGTTAAAATGTAAGATAACAGAGAATTCATAGAAAGCACAATTT 1871
MDACS1-2        TCGGCGCAAAAATTAATGTTAAAATGTAAGATAACAGAGAATTCATAGAAAGCACAATTT 2097
BAC22F23        TCGGGGCAAAAATTAATGTTAAAATGTAAGATAACAGAGAATTCATAGAAAGCACAATTT 1638
MDACS1-1        TCGGGGCAAAAATTAATGTTAAAATGTAAGATAACAGAGAATTCATAGAAAGCACAATTT 1946
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                                                                Harada-1814
BAC96N17        TAAGATAATCTCCTTAACATTTATAAAAAATATGACTACTCAGTGTGACGTGTCATTCCT 1931
MDACS1-2        TAAGATAATCTCCTTAACATTTATAAAAAATATGACTACTCAGTGTGACGTGTCATTCCT 2157
BAC22F23        TAAGATAATCTCCTTAACATTTATAAAAAATATGACTACTCAGTGTGACGTGTCATTCCT 1698
MDACS1-1        TAAGATAATCTCCTTAACATTTATAAAAAATATGACTACTCAGTGTGACGTGTCATTCCT 2006
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BAC96N17        TTGTTAGACAAATAATTTCTATATATTTAAATTTATATTATTACTTTTTTGCTATATATA 1991
MDACS1-2        TTGTTAGACAAATAATTTCTATATATTTAAATTTATATTATTACTTTTTTGCTATATATA 2217
BAC22F23        TTGTTAGACAAATAATTTCTATATATTTAAATTTATATTATTACTTTTTTGCTATATATA 1758
MDACS1-1        TTGTTAGACAAATAATTTCTATATATTTAAATTTATATTATTACTTTTTTGCTATATATA 2066
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BAC96N17        GACCCCTCCAGTCCAACAACATCCAATATCCCACTTCAAACTTGTAATCCAAAACCAAAA 2051
MDACS1-2        GACCCCTCCAGTCCAACAACATCCAATATCCCACTTCAAACTTGTAATCCAAAACCAAAA 2277
BAC22F23        GACCCCTCCAGTCCAACAACATCCAATATCCCACTTCAAACTTGTAATCCAAAACCAAAA 1818
MDACS1-1        GACCCCTCCAGTCCAACAACATCCAATATCCCACTTCAAACTTGTAATCCAAAACCAAAA 2126
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BAC96N17        CCTCAAACTCTCTCTCTATTGCTTTCTCTTCCTTTCCACACTTCTTTCTTACAGCTTGTA 2111
MDACS1-2        CCTCAAACTCTCTCTCTATTGCTTTCTCTTCCTTTCCACACTTCTTTCTTACAGCTTGTA 2337
BAC22F23        CCTCAAACTCTCTCTCTATTGCTTTCTCTTCCTTTCCACACTTCTTTCTTACAGCTTGTA 1878
MDACS1-1        CCTCAAACTCTCTCTCTATTGCTTTCTCTTCCTTTCCACACTTCTTTCTTACAGCTTGTA 2186
                ************************************************************


BAC96N17        TCCATACAGTAGAAAATTAACCAAAATGCGCATGTTATCCAGAAACGCTACGTTCAACTC 2171
MDACS1-2        TCCATACAGTAGAAAATTAACCAAAATGCGCATGTTATCCAGAAACGCTACGTTCAACTC 2397
BAC22F23        TCCATACACAAGAAAATTAACCAAAATGCGCATGTTATCCAGAAACGCTACGTTCAACTC 1938
MDACS1-1        TCCATACAGTAGAAAATTAACCAAAATGCGCATGTTATCCAGAAACGCTACGTTCAACTC 2246
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BAC96N17        TCACGGCCAAGACTCCTCCTACTTCTTAGGTTGGCAAGAGTATGAGAAGAACCCCTACCA 2231
MDACS1-2        TCACGGCCAAGACTCCTCCTACTTCTTAGGTTGGCAAGAGTATGAGAAGAACCCCTACCA 2457
BAC22F23        TCACGGCCAAGACTCCTCCTACTTCTTAGGTTGGCAAGAGTATGAGAAGAACCCCTACCA 1998
MDACS1-1        TCACGGCCAAGACTCCTCCTACTTCTTAGGTTGGCAAGAGTATGAGAAGAACCCCTACCA 2306
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BAC96N17        TGAGGTCCACAACACAAACGGGATTATTCAGATGGGTCTAGCAGAAAATCAGGTAATTAA 2291
MDACS1-2        TGAGGTCCACAACACAAACGGGATTATTCAGATGGGTCTAGCAGAAAATCAGGTAATTAA 2517
BAC22F23        TGAGGTCCACAACACAAACGGGATTATTCAGATGGGTCTAGCAGAAAATCAGGTAATTAA 2058
MDACS1-1        TGAGGTCCACAACACAAACGGGATTATTCAGATGGGTCTAGCAGAAAATCAGGTAATTAA 2366
                ************************************************************
                                                        Harada 2237
BAC96N17        TTATTATAATTTACGAGCTTAATTTTTTATTACTACCATGCATATATGTTACCATATGTA 2351
MDACS1-2        TTATTATAATTTACGAGCTTAATTTTTTATTACTACCATGCATATATGTTACCATATGTA 2577
BAC22F23        TTATTATAATTTACGAGCTTAATTTTTTATTACTACCATGCATATATGTTACCATATGTA 2118
MDACS1-1        TTATTATAATTTACGAGCTTAATTTTTTATTACTACCATGCATATATGTTACCATATGTA 2426
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BAC96N17        GTTATATTTAGTATATAAACTTTGTGCCCGTTTCAATATTTTTTTTTTCTAGCTCTGTTT 2411
MDACS1-2        GTTATATTTAGTATATAAACTTTGTGCCCGTTTCAATATTTTTTTTTTCTAGCTCTGTTT 2637
BAC22F23        GTTATATTTAGTATATAAACTTTGTGCGCGTTTCAATATTTTTTTTTTCTAGCTCTGTTT 2178
MDACS1-1        GTTATATTTAGTATATAAACTTTGTGCGCGTTTCAATATTTTTTTTTTCTAGCTCTGTTT 2486
                ****************************.*************************************


BAC96N17        TGATCTTCTCGAGTCATGGCTGGCTAAGAATCCAGAAGCAGCTGCATTTAAAAAAAATGG 2471
MDACS1-2        TGATCTTCTCGAGTCATGGCTGGCTAAGAATCCAGAAGCAGCTGCATTTAAAAAAAATGG 2697
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BAC22F23        TGATCTTCTCGAGTCATGGCTGGCTAAGAATCCAGAAGCAGCTGCATTTAAAAAAAATGG 2238
MDACS1-1        TGATCTTCTCGAGTCATGGCTGGCTAAGAATCCAGAAGCAGCTGCATTTAAAAAAAATGG 2546
                ************************************************************

BAC96N17        AGAATCCATATTTGCAGAGCTTGCTCTCTTCCAAGATTATCATGGCCTTCCCGCGTTCAA 2531
MDACS1-2        AGAATCCATATTTGCAGAGCTTGCTCTCTTCCAAGATTATCATGGCCTTCCCGCGTTCAA 2757
BAC22F23        AGAATCCATATTTGCAGAGCTTGCTCTCTTCCAAGATTATCATGGCCTTCCCGCGTTCAA 2298
MDACS1-1        AGAATCCATATTTGCAGAGCTTGCTCTCTTCCAAGATTATCATGGCCTTCCCGCGTTCAA 2606
                ************************************************************

BAC96N17        AAAGGTAAACTTATAAATTATAATAATTTATAGTATAAGCTCACTTTTCGTTAATGCAAT 2591
MDACS1-2        AAAGGTAAACTTATAAATTATAATAATTTATAGTATAAGCTCACTTTTCGTTAATGCAAT 2817
BAC22F23        AAAGGTAAACTTATAAATTATAATAATTTATAGTATAAGCTCACTTTTCGTTAATGCAAT 2358
MDACS1-1        AAAGGTAAACTTATAAATTATAATAATTTATAGTATAAGCTCACTTTTCGTTAATGCAAT 2666
                ************************************************************

BAC96N17        TAAAAGCTACTACTAGAACAAGTCTTCTAGCCAGTTGCATGTCTAACTCAGCTTTTGATT 2651
MDACS1-2        TAAAAGCTACTACTAGAACAAGTCTTCTAGCCAGTTGCATGTCTAACTCAGCTTTTGATT 2877
BAC22F23        TAAAAGCTACTACTAGAACAAGTCTTCTAGCCGGTTGCATGTCTAACTCAGCTTTTGATT 2418
MDACS1-1        TAAAAGCTACTACTAGAACAAGTCTTCTAGCCGGTTGCATGTCTAACTCAGCTTTTGATT 2726
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BAC96N17        ATTTTTTTCTTACAGGCAATGGTAGATTTCATGGCGGAAATCCGAGGGAACAAAGTGACC 2711
MDACS1-2        ATTTTTTTCTTACAGGCAATGGTAGATTTCATGGCGGAAATCCGAGGGAACAAAGTGACC 2937
BAC22F23        ATTTTTTTCTTACAGGCAATGGTAGATTTCATGGCGGAAATCCGAGGGAACAAAGTGACC 2478
MDACS1-1        ATTTTTTTCTTACAGGCAATGGTAGATTTCATGGCGGAAATCCGAGGGAACAAAGTGACC 2786
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                          Harada 2618
BAC96N17        TTTGATCCCAACCACTTAGTGCTCACCGCCGGTGCAACTTCAGCGAATGAGACCTTTATC 2771
MDACS1-2        TTTGATCCCAACCACTTAGTGCTCACCGCCGGTGCAACTTCAGCGAATGAGACCTTTATC 2997
BAC22F23        TTTGATCCCAACCACTTAGTGCTCACCGCCGGTGCAACTTCAGCGAATGAGACCTTTATT 2538
MDACS1-1        TTTGATCCCAACCACTTAGTGCTCACCGCCGGTGCAACTTCAGCGAATGAGACCTTTATT 2846
                ************************************************************.

BAC96N17        TTCTGCCTTGCTGACCCCGGCGAAGCCGTTCTTATTCCTACCCCATACTACCCAGGGTAC 2831
MDACS1-2        TTCTGCCTTGCTGACCCCGGCGAAGCCGTTCTTATTCCTACCCCATACTACCCAGGGTAC 3057
BAC22F23        TTCTGCCTTGCTGACCCCGGCGAAGCCGTTCTTATTCCTACCCCATACTACCCAGGGTAC 2598
MDACS1-1        TTCTGCCTTGCTGACCCCGGCGAAGCCGTTCTTATTCCTACCCCATACTACCCAGGGTAC 2906
                ************************************************************

BAC96N17        GTATATTAACCTTCACTTCATTTTTTTTTCTTTGTTTTTAACAAACAATTTGAACTGTAA 2891
MDACS1-2        GTATATTAACCTTCACTTCATTTTTTTTTCTTTGTTTTTAACAAACAATTTGAACTGTAA 3117
BAC22F23        GTATATTAACCTTCACTTCATTTTTTTTTCTTTGTTTTTAACAAACAATTTGAACTGTAA 2658
MDACS1-1        GTATATTAACCTTCACTTCATTTTTTTTTCTTTGTTTTTAACAAACAATTTGAACTGTAA 2966
                ************************************************************

BAC96N17        GACCTACTTTCTTAGTATTCTGACGTGGGGCATGAACTCCATTTCGGGATAAGAAATAAC 2951
MDACS1-2        GACCTACTTTCTTAGTATTCTGACGTGGGGCATGAACTCCATTTCGGGATAAGAAATAAC 3177
BAC22F23        GACCTACTTTCTTAGTATTCTGACGTGGGGCATGAACTCCATTTCGGGATAAGAAATAAC 2718
MDACS1-1        GACCTACTTTCTTAGTATTCTGACGTGGGGCATGAACTCCATTTCGGGATAAGAAATAAC 3026
                ************************************************************

BAC96N17        TAAACACTAATTTCATCCCTAATAGCCGAGTATTTTCTTACGTGTCTTACAGATTTGATA 3011
MDACS1-2        TAAACACTAATTTCATCCCTAATAGCCGAGTATTTTCTTACGTGTCTTACAGATTTGATA 3237
BAC22F23        TAAACACTAATTTCATCCCTAATAGCCGAGTATTTTCTTACGTGTCTTACAGATTTGATA 2778
MDACS1-1        TAAACACTAATTTCATCCCTAATAGCCGAGTATTTTCTTACGTGTCTTACAGATTTGATA 3086
                ************************************************************

BAC96N17        GAGACCTTAAGTGGCGAACTGGAGTCGAGATTGTACCCATTCACTGCACAAGCTCCAATG 3071
MDACS1-2        GAGACCTTAAGTGGCGAACTGGAGTCGAGATTGTACCCATTCACTGCACAAGCTCCAATG 3297
BAC22F23        GAGACCTTAAGTGGCGAACTGGAGTCGAGATTGTACCCATTCACTGCACAAGCTCCAATG 2838
MDACS1-1        GAGACCTTAAGTGGCGAACTGGAGTCGAGATTGTACCCATTCACTGCACAAGCTCCAATG 3146
                ************************************************************

BAC96N17        GCTTCCAAATTACTGAAACCGCTCTGGAAGAAGCCTACCAAGAAGCCGAAAAACGCAATC 3131
MDACS1-2        GCTTCCAAATTACTGAAACCGCTCTGGAAGAAGCCTACCAAGAAGCCGAAAAACGCAATC 3357
BAC22F23        GCTTCCAAATTACTGAAACCGCTCTGGAAGAAGCCTACCAAGAAGCCGAAAAACGCAATC 2898
MDACS1-1        GCTTCCAAATTACTGAAACCGCTCTGGAAGAAGCCTACCAAGAAGCCGAAAAACGCAATC 3206
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BAC96N17        TCAGAGTCAAAGGAGTCTTGGTCACGAACCCATCAAACCCATTGGGCACCACAATGACCA 3191
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MDACS1-2        TCAGAGTCAAAGGAGTCTTGGTCACGAACCCATCAAACCCATTGGGCACCACAATGACCA 3417
BAC22F23        TCAGAGTCAAAGGAGTCTTGGTCACGAACCCATCAAACCCATTGGGCACCACAATGACCA 2958
MDACS1-1        TCAGAGTCAAAGGAGTCTTGGTCACGAACCCATCAAACCCATTGGGCACCACAATGACCA 3266
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BAC96N17        GAAACGAACTCTACCTCCTCCTTTCCTTCGTTGAAGACAAGGGCATCCACCTCATTAGCG 3251
MDACS1-2        GAAACGAACTCTACCTCCTCCTTTCCTTCGTTGAAGACAAGGGCATCCACCTCATTAGCG 3477
BAC22F23        GAAACGAACTCTACCTCCTCCTTTCCTTCGTTGAAGACAAGGGCATCCACCTCATTAGCG 3018
MDACS1-1        GAAACGAACTCTACCTCCTCCTTTCCTTCGTTGAAGACAAGGGCATCCACCTCATTAGCG 3326
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                          Harada 3168
BAC96N17        ATGAAATTTACTCCGGCACAGCTTTTAGCTCCCCATCCTTTATAAGCGTCATGGAAGTTC 3311
MDACS1-2        ATGAAATTTACTCCGGCACAGCTTTTAGCTCCCCATCCTTTATAAGCGTCATGGAAGTTC 3537
BAC22F23        ATGAAATTTACTCCGGCACAGCTTTTAGCTCCCCATCCTTTATAAGCGTCATGGAAGTTC 3078
MDACS1-1        ATGAAATTTACTCCGGCACAGCTTTTAGCTCCCCATCCTTTATAAGCGTCATGGAAGTTC 3386
                ************************************************************

BAC96N17        TCAAAGATAGAAACTGTGATGAGAATTCCGAAGTTTGGCAGCGAGTTCACGTTGTCTATA 3371
MDACS1-2        TCAAAGATAGAAACTGTGATGAGAATTCCGAAGTTTGGCAGCGAGTTCACGTTGTCTATA 3597
BAC22F23        TCAAAGATAGGAACTGTGATGAGAATTCCGAAGTTTGGCAGCGAGTTCACGTTGTCTATA 3138
MDACS1-1        TCAAAGATAGGAACTGTGATGAGAATTCCGAAGTTTGGCAGCGAGTTCACGTTGTCTATA 3446
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BAC96N17        GCCTCTCTAAGGATCTTGGCCTTCCGGGTTTTCGAGTTGGCGCCATTTACTCCAACGACG 3431
MDACS1-2        GCCTCTCTAAGGATCTTGGCCTTCCGGGTTTTCGAGTTGGCGCCATTTACTCCAACGACG 3657
BAC22F23        GCCTCTCTAAGGATCTTGGCCTTCCGGGTTTTCGAGTTGGCGCCATCTACTCCAACGACG 3198
MDACS1-1        GCCTCTCTAAGGATCTTGGCCTTCCGGGTTTTCGAGTTGGCGCCATCTACTCCAACGACG 3506
                **********************************************-*************

BAC96N17        ACATGGTTGTGGCCGCCGCTACAAAAATGTCAAGCTTTGGTCTTGTTTCTTCTCAAACTC 3491
MDACS1-2        ACATGGTTGTGGCCGCCGCTACAAAAATGTCAAGCTTTGGTCTTGTTTCTTCTCAAACTC 3717
BAC22F23        ACATGGTTGTGGCCGCCGCTACAAAAATGTCAAGCTTTGGTCTTGTTTCTTCTCAAACTC 3258
MDACS1-1        ACATGGTTGTGGCCGCCGCTACAAAAATGTCAAGCTTTGGTCTTGTTTCTTCTCAAACTC 3566
                ************************************************************

BAC96N17        AGCACCTTCTCTCCGCCATGCTATCCGACAAAAAACTCACTAAGAACTACATAGCCGAGA 3551
MDACS1-2        AGCACCTTCTCTCCGCCATGCTATCCGACAAAAAACTCACTAAGAACTACATAGCCGAGA 3777
BAC22F23        AGCACCTTCTCTCCGCCATGCTATCCGACAAAAAACTCACTAAGAACTACATAGCCGAGA 3318
MDACS1-1        AGCACCTTCTCTCCGCCATGCTATCCGACAAAAAACTCACTAAGAACTACATAGCCGAGA 3626
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BAC96N17        ACCACAAAAGACTCAAACAACGTCAGAAAAAGCTCGTCTCCGGCCTTCAGAAATCTGGCA 3611
MDACS1-2        ACCACAAAAGACTCAAACAACGTCAGAAAAAGCTCGTCTCCGGCCTTCAGAAATCTGGCA 3837
BAC22F23        ACCACAAAAGACTCAAACAACGTCAGAAAAAGCTCGTCTCCGGCCTTCAGAAATCTGGCA 3378
MDACS1-1        ACCACAAAAGACTCAAACAACGTCAGAAAAAGCTCGTCTCCGGCCTTCAGAAATCTGGCA 3686
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BAC96N17        TTAGCTGCCTCAACGGCAATGCTGGCTTGTTCTGTTGGGTGGATATGAGGCACTTACTTA 3671
MDACS1-2        TTAGCTGCCTCAACGGCAATGCTGGCTTGTTCTGTTGGGTGGATATGAGGCACTTACTTA 3897
BAC22F23        TTAGCTGCCTCAACGGCAATGCTGGCTTGTTCTGTTGGGTGGATATGAGGCACTTGCTTA 3438
MDACS1-1        TTAGCTGCCTCAACGGCAATGCTGGCTTGTTCTGTTGGGTGGATATGAGGCACTTGCTTA 3746
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BAC96N17        GGTCCAACACCTTTGAAGCCGAAATGGAGCTCTGGAAAAAGATTGTATACGAAGTTCACC 3731
MDACS1-2        GGTCCAACACCTTTGAAGCCGAAATGGAGCTCTGGAAAAAGATTGTATACGAAGTTCACC 3957
BAC22F23        GGTCCAACACCTTTGAAGCCGAAATGGAGCTCTGGAAAAAGATTGTATACGAAGTTCACC 3498
MDACS1-1        GGTCCAACACCTTTGAAGCCGAAATGGAGCTCTGGAAAAAGATTGTATACGAAGTTCACC 3806
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BAC96N17        TCAATATATCTCCTGGATCGTCTTGTCATTGCACGGAACCTGGTTGGTTCCGTGTCTGCT 3791
MDACS1-2        TCAATATATCTCCTGGATCGTCTTGTCATTGCACGGAACCTGGTTGGTTCCGTGTCTGCT 4017
BAC22F23        TCAATATATCTCCTGGATCGTCTTGTCATTGCACGGAACCTGGTTGGTTCCGTGTCTGCT 3558
MDACS1-1        TCAATATATCTCCTGGATCGTCTTGTCATTGCACGGAACCTGGTTGGTTCCGTGTCTGCT 3866
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BAC96N17        TTGCCAACTTGCCCGAGAGAACTCTGGACTTGGCAATGCAGAGACTGAAGGCATTTGTGG 3851
MDACS1-2        TTGCCAACTTGCCCGAGAGAACTCTGGACTTGGCAATGCAGAGACTGAAGGCATTTGTGG 4077
BAC22F23        TTGCCAACTTGCCCGAGAGAACTCTGGACTTGGCAATGCAGAGACTGAAGGCATTTGTGG 3618
MDACS1-1        TTGCCAACTTGCCCGAGAGAACTCTGGACTTGGCAATGCAGAGACTGAAGGCATTTGTGG 3926
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BAC96N17        GGGAGTATTACAACGTCCCTGAGGTCAATGGCGGCAGCCAAAGCAGCCATTTAAGCCACT 3911
MDACS1-2        GGGAGTATTACAACGTCCCTGAGGTCAATGGCGGCAGCCAAAGCAGCCATTTAAGCCACT 4137
BAC22F23        GGGAGTATTACAACGTCCCTGAGGTCAATGGCGGCAGCCAAAGCAGCCATTTAAGCCACT 3678
MDACS1-1        GGGAGTATTACAACGTCCCTGAGGTCAATGGCGGCAGCCAAAGCAGCCATTTAAGCCACT 3986
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BAC96N17        CGAGAAGACAGTCGCTCACGAAGTGGGTTTCCCGGCTATCCTTCGATGACCGCAGTCCTA 3971
MDACS1-2        CGAGAAGACAGTCGCTCACGAAGTGGGTTTCCCGGCTATCCTTCGATGACCGCAGTCCTA 4197
BAC22F23        CAAGAAGACAGTCGCTCACAAAGTGGGTTTCCCGGCTATCCTTCGATGACCGCGGTCCTA 3738
MDACS1-1        CAAGAAGACAGTCGCTCACAAAGTGGGTTTCCCGGCTATCCTTCGATGACCGCGGTCCTA 4046
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BAC96N17        TTCCCGGTAGATGA---------------------------------------------- 3985
MDACS1-2        TTCCCGGTAGATGAAAGGTAGCCTGGTCTGAGTACAAGAAACCGCTAAGGAAAATTACAT 4257
BAC22F23        TTCCCGGTAGATGA---------------------------------------------- 3752
MDACS1-1        TTCCCGGTAGATGAAAGGTAGCCTGGTCTGAGTACAAGAAACCGCTAAGGAAAATTACAT 4106
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