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SORBITOL DEHYDROGENASE EXPRESSION IN APPLE FRUIT

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ABSTRACT OF DISSERTATION

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The Graduate School

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2006

SORBITOL DEHYDROGENASE EXPRESSION IN APPLE FRUIT

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Agriculture
at the University of Kentucky

By
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Lexington, Kentucky

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Lexington, Kentucky

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Sorbitol, the primary photosynthate and translocated carbohydrate in apple (*Malus x domestica* Borkh.), is converted to fructose by SORBITOL DEHYDROGENASE (SDH; EC 1.1.1.14) which is active in apple fruit throughout fruit development. Apple fruit set and early development is very sensitive to carbohydrate availability, but details on carbohydrate metabolism during this phase are limited. The first objective of this work was to determine if SORBITOL DEHYDROGENASE, the primary enzyme responsible for metabolism of the major phloem-transported carbohydrate sorbitol, is present and active during apple fruit set and early development. The second objective of this work was to determine if *SDH* genes are differentially expressed and how their patterns of expression may relate to SDH activity in apple seed and cortex during early fruit development. Nine different genes encoding SDH were determined from analysis of a cDNA library and genomic-clones. Northern, Western and ELISA analyses showed that *SDH* transcripts and SDH protein were present in the fruit during the first 5 weeks after bloom and comprised 7 to 8 % of the total extractable protein. Whole fruit SDH activity was highest at 2 to 3 weeks after bloom in each of three cultivars, Lodi, Redchief Delicious and Fuji. Seed SDH activity was found to be much higher than cortex SDH activity per mg and g FW, and seed SDH activity contributed significantly to whole fruit SDH activity during the first five weeks of development after bloom. Five of the nine *SDH* genes present in apple genome were expressed in apple fruit (*SDH1, SDH2, SDH3, SDH6, SDH9*). Expression of *SDH6* and *SDH9* was seed-specific and expression of *SDH2* was cortex-specific. Using 2D SDS-PAGE and Western analyses, SDH isomers with pI values 4.2, 4.8, 5.5 and 6.3 were found in seeds, and SDH isomers with pI values 5.5, 6.3, 7.3 and 8.3 were found in cortex. The present work is the first to show that *SDH* is differentially expressed and highly active in seed and cortex during early development. Thus, SDH during apple fruit set and early development may play a primary role in defining fruit sink activity.

KEYWORDS: *Malus x domestica* Borkh, sorbitol, sorbitol dehydrogenase, SDH isoforms, apple seed and cortex.

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TABLE OF CONTENTS

ACKNOWLEDGMENTS iii

TABLE OF CONTENTS iv

LIST OF TABLES vi

LIST OF FIGURES vii

LIST OF FILES viii

Chapter 1 Literature Review 1

Importance of sorbitol to apple yield and quality 1

Physiological aspects of apple fruit development 1

Sorbitol production and translocation in apple 2

SORBITOL DEHYDROGENASE as a main sorbitol metabolizing enzyme in apple fruit 3

Sorbitol as a signaling molecule 6

Objectives 8

Chapter 2 Sorbitol Dehydrogenase Expression and Activity During Apple Fruit Set and Early Development 9

Introduction 9

Materials and methods 10

Materials 10
RNA extraction 10
cDNA library screening 10
Northern analysis 11
Genomic clone isolation 12
Sequence determination and alignment 12
SDH enzyme activity measurement 12
ELISA and Western analyses 13

Results 13

cDNA library screening and genomic clones analysis 13
Northern analysis 14
Fruit growth and SDH activity 14
Western and ELISA analyses of SDH 14

Discussion 15

Chapter 3 Tissue specific SDH expression in seed and cortex of apple fruit during early development. 25

Introduction 25

Materials and Methods 27

Fruit collection 27
SDH activity measurement 27
Northern analysis 28
Western analysis 28
2D PAGE 28
RT-PCR analysis 28

Results 29
SDH activity 29
Western and 2D PAGE analysis 30
Northern analysis 31
RT-PCR analyses 31
Discussion 33
Appendix A ***51***
Appendix B ***54***
Appendix C ***56***
REFERENCES ***60***
VITA ***64***

LIST OF TABLES

Table 2.1 Homology of *SDH* genes. ……. 18
Table 3.1 RT-PCR gene specific primers. …… 37
Table 3.2 SDH isomers found in seed and cortex. …… 38
Table 3.3 *SDH* genes expressed in seed and cortex. ……. 39

LIST OF FIGURES

Figure 2.1	Organization of *SORBITOL DEHYDROGENASE* genes in apple. ...	20
Figure 2.2	Northern blot of *SDH* transcripts in whole fruit. ……..	21
Figure 2.3	Fresh weight accumulation, RGR, and SDH activity in whole fruit.	22
Figure 2.4	Western blot of whole fruit. …….	23
Figure 2.5	Total protein per gram fresh weight, and SDH amount per mg protein in whole fruit. ……..	24
Figure 3.1	SDH activity per mg protein in seed, cortex and whole fruit. …….	40
Figure 3.2	Extractable protein content and SDH activity per gram FW in seed and cortex. …….	41
Figure 3.3	Western blot of seed and cortex protein. ……..	45
Figure 3.4	Seed SDH isomers separated by 2D electrophoresis. ……..	46
Figure 3.5	Cortex SDH isomers separated by 2D electrophoresis. ……	47
Figure 3.6	Northern blot of seed and cortex *SDH* transcripts. ……...	48
Figure 3.7	RT-PCR analysis of RNA transcripts from seed and cortex. ……	49
Figure 3.8	Determination of the specificity of RT-PCR primers. ……..	50

LIST OF FILES

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Chapter 1

Literature Review

Importance of sorbitol to apple yield and quality

Apple is the most important temperate zone tree fruit grown in the United States and around the world, and maintaining the consistency of, or increasing the yield and quality of, the apple fruit crop is essential for good economic returns to growers. All aspects of fruit development and quality are dependent on carbohydrate import and its utilization by the fruit. Apple fruit quality consists of fruit size, color, sugar and acid content, and flavor. With over 90% of the final dry weight of the fruit from carbohydrate, fruit yield and quality depend heavily on imported carbohydrate (Westwood, 1993).Limitations in carbohydrate availability to fruit, and/or inefficient utilization by fruit, can adversely affect yield and quality.

Sorbitol is the major sugar exported from apple leaves, and it is primarily converted to fructose in the fruit by sorbitol dehydrogenase. Apple fruit are considered weaker sinks than shoots and roots, and suffer in competition for carbohydrate resources. Rates of SDH activity, and thus sorbitol utilization by fruit, may be reduced as sorbitol availability declines, though it is not known how this occurs. Understanding the mechanisms regulating *SORBITOL DEHYDROGENASE* expression and activity could lead to new horticultural and/or biochemical or genetic strategies to optimize the competitive position and resulting quality and yield of apple fruit and have a desirable economic impact.

Physiological aspects of apple fruit development

After pollination and fertilization, apple fruit develops via cell division for 4-6 weeks followed by an extended period, 50 to 180 days depending on cultivar, of cell expansion until harvest (Bain and Robertson, 1951). Fruit in the cell division phase exhibits exponential growth that is followed by a linear phase during cell expansion (Lakso et al., 1995). Final size is a function of cell number produced during the first 4 to 6 weeks (Goffinet et al., 1995) and is also a function of the number of cells recruited from the L3 layer of the primordium and the degree of final cell expansion. Fruit relative growth rate, or sink activity, is higher during the early time period than at any other time

during growth (Lakso et al., 1998, Byers et al., 1990). In developing fruit, most of the accumulated soluble carbohydrate is present as fructose (45-60%) with smaller quantities of glucose (20%), sucrose (10%) and sorbitol (3-8%) (Beruter, 1983). However, from the start of cell expansion until ripening a significant amount of carbohydrate is stored in the starch pool. When ripening commences, the starch is ultimately converted to glucose and fructose, increasing their levels in ripe fruit.

Apple fruit set and early development is very sensitive to carbohydrate availability, as shown in partial shading studies (Bepete and Lakso, 1998; Byers et al., 1991). This availability is affected by competition among the many reproductive and vegetative sinks. Though the initial number of flowers that transition to become growing fruit may vary due to environmental conditions and insect pollinator activity during bloom, the initial levels of fruit set are high enough that some fruit must be removed, or thinned, within the first 4 weeks to ensure sufficient fruit size and quality at harvest. During this early period, some fruit will be shed naturally, commonly called 'June drop', and some will be removed by chemical application, or chemical thinning. The latter practice is essential to achieving high yield and quality, and it must be done within the first 4 weeks or so after bloom to maximize effects on fruit cell division so that high cell numbers per fruit are created, leading to good fruit size. Because fruit are weaker sinks than growing shoots (Corelli Grappadelli et al., 1994; Lakso et al., 1998), the inability of many fruit to persist and grow, and/or the low growth rate of some fruit that do persist and that results in poor size and quality at harvest, may be due to less efficient utilization of uploaded carbohydrates compared to shoots and other vegetative sinks. Were fruit more competitive for carbohydrate resources at this time, more fruit could be allowed to develop as size and quality would not be compromised. Zhang et al. (2005) have recently shown that fruit growth in pear (*Pyrus pyrifolia*), which like apple uses sorbitol as the major photoassimilate, is limited by sink strength of the fruit rather than the capacity of the transport pathway. This strongly suggests that the capacity for utilization of carbohydrate is critical to achieving sufficient yield and quality.

Sorbitol production and translocation in apple

In apple, sorbitol is the primary translocated carbohydrate, estimated to comprise about 80% of total translocated sugars while sucrose comprises the rest (20%) (Bieleski,

1969; Webb and Burley, 1962). The high sorbitol concentration in the phloem is correlated with the high rate of sorbitol synthesis in source leaves via activity of NADP-SORBITOL-6-PHOSPHATE DEHYDROGENASE (S6PDH, also named ALDOSE-6-PHOSPHATE REDUCTASE) and inhibition of sucrose synthesis by SUCROSE PHOSPHATE SYNTHASE (Zhou et al., 2001). S6PDH catalyses the reversible reduction of glucose-6-phosphate to sorbitol-6-phosphate, and it is inhibited by Pi which may be one of the mechanisms controlling S6PDH activity and correlating S6PDH activity with photosynthetic rate in apple leaves (Zhou et al., 2001). Sorbitol concentration in apple leaves increases gradually after dawn, reaches its highest level in late afternoon, and then declines to its lowest level at the end of the dark period.

Upon entering the fruit, sorbitol is unloaded from the phloem although the precise pathway from phloem to parenchyma storage cells has not been determined. Sorbitol and sucrose unloading in apple fruit seems to be apoplastic due to symplastic isolation of the fruit cortex cells from the sieve element-companion cell complex. Unloading probably occurs across the plasma membrane of the sieve elements, not companion cells; this process may depend on both H^+-ATPase and monosaccharide transporters (Zhang et al., 2004). Post-phloem transport may occur both apoplastically as well as symplastically due to the many symplastic connections between parenchyma cells in the apple cortex. Two sorbitol transporter genes, *MdSOT1* and *MdSOT2,* have been identified and encode integral membrane proteins with their highest expression in all sink tissues of apple including fruit (Gao et al., 2005). These transporters are energy–dependent H^+ sorbitol symporters driven by the proton motive force across the plasma membrane and are similar to tart cherry transporters (Gao et al., 2003), plantain sorbitol transporters (Ramsperger-Gleixner et al., 2004), and the celery mannitol transporter (Noiraud et al., 2001). Low expression of *MdSOT1* and *MdSOT2* in watercore-affected apple fruit is correlated with sorbitol accumulation in the intercellular apoplastic space of the fruit cortex (Gao et al., 2005).

SORBITOL DEHYDROGENASE as a main sorbitol metabolizing enzyme in apple fruit

Although sorbitol concentration in phloem sap is high, the sorbitol levels of the fruit are very low. This, and the fact that fructose accumulates and is the most abundant

soluble carbohydrate in the fruit, strongly suggest that the sorbitol-to-fructose conversion may be the key rate-limiting step in sorbitol utilization by apple fruit. SORBITOL DEHYDROGENASE (SDH, E.C.1.1.1.14) has been identified as the primary enzyme that metabolizes sorbitol in apple fruit (Beruter, 1985; Yamaki and Ishiwaka, 1986; Yamaguchi et al, 1996). Sorbitol is converted to fructose by SDH and uses NAD^+ as a cofactor (Beruter, 1985; Yamaki, 1980; Yamaki and Ishiwaka, 1986). Sorbitol can also be metabolized by SORBITOL OXIDASE (SOX) to yield glucose, but this enzyme has a 10-fold lower activity than SDH throughout fruit development (Yamaki and Ishiwaka, 1986).

Four highly homologous *SDH* genes (*SDH1, SDH2, SDH3, SDH4*) have been found to be expressed in apple fruit (Park et al., 2002). The presence of multiple *SDH* genes in apple genome suggests tissue-specific expression and in that work expression of three of these genes was restricted to sink tissues only (immature leaves, stems, roots, maturing fruit), while a fourth *SDH* gene was expressed in both immature and mature leaves. Similar results were obtained from the developing buds of Japanese pear (*Pyrus serotina,* another Rosaceae family species) where partial fragments of five *SDH* genes were isolated (Ito et al., 2005). The derived amino acid sequence of one of the pear isomers (PpySDH5) has 94-98% homology with apple SDH1 and is distinct from the other four isomers of pear SDH (71% homology). The other four isomers are similar to each other with 88% to 95% homology. Due to the observed expression pattern in buds, the authors suggested that the pear SDH isomers could be categorized into two groups: one expressed in the bud for growth and development (i.e., PpySDH1) and the others of unknown tissue specificity (like PpySDH5).

The expression patterns of the known SDH isomers during apple fruit set and development have not been determined, although the presence of two isomers (SDH1 and SDH2) was detected in fruit starting at 90 days after bloom and two other isomers (SDH3 and SDH4) started at 120 days after bloom (Park et al., 2002). None of these four SDH transcripts were detected at 30 days after bloom.

SDH, a member of the medium chain dehydrogenase/reductase protein family has been intensively studied in mammalian systems since SDH is the second enzyme of the polyol pathway of glucose metabolism. Human SDH is a homotetramer (155 kDa) with a

catalytic Zn atom bound in the active site of each subunit (37 kDa) (Darmanin et al., 2003). Studies have demonstrated that the human SDH enzyme follows the ordered Theorell-Chance kinetic mechanism where the coenzyme initially binds with SDH, then substrate binding occurs followed by the reaction, after which the product disassociates first and the coenzyme last. The rate-determining step of the enzymatic reaction is the dissociation of the SDH-NADH complex. Reported Kcat and Km for human SDH are 506 min^{-1} and 1.5 mM, respectively (El-Kabbani et al., 2004). SDH purified from Japanese pear fruit is also a tetramer (160 kDa) with a subunit size of 40 kDa (Oura et al., 2000). There have not been consistent results specifying SDH catalytic activity in apple (Yamada et al., 1998; Park et al., 2002). To date, apple SDH3 and pear SDH have been reported to have similar Km values, 83mM and 96.4 mM, respectively (Park et al., 2002; Oura et al., 2000).

Apparent changes in SDH activity may be due to limitations in sorbitol availability to the fruit. With defoliation of fruit-bearing spurs in mid-season and girdling the phloem to stop sorbitol import into the fruit, fruit growth stopped and SDH activity was negligible within 14 days (Archbold, 1999; Beruter and Feusi, 1997). More importantly, incubation of fruit cortex tissue obtained from control or girdled and defoliated stems with or without sorbitol showed that sorbitol was essential to maintaining SDH activity in sections that had activity when collected, and induced increased activity in those from defoliated and girdled spurs (Archbold, 1999). It is unknown if SDH protein was degraded or was deactivated by modification in fruit tissue that lost activity *in situ* and during incubation, or if existing SDH proteins were activated or SDH gene expression resumed when sorbitol became available. Pear fruit SDH, during a similar induction study, exhibited upregulation on a transcriptional level, but neither SDH protein amount in the tissue nor its activity were well-correlated with transcript level (Iida et al., 2004).

As discussed previously, a natural fruit drop called June drop occurs within 4 weeks of bloom/pollination. This fruit drop is believed to be due to the competition for resources between fruit and vegetative tissues. Recent work in our lab suggests that there is a substantial decline in sap exudate sorbitol content coincident with this natural thinning, perhaps evidence of this competition (Archbold, unpublished). This is a period

in which new foliage is developing, so new photoassimilate is gradually being added to the available carbohydrate pool, though stored carbohydrate remains a major part of that available (Corelli Grappadelli et al., 1994). Whether the decline in sap sorbitol content causes the decline in fruit SDH activity, and if this makes the young fruit weaker sinks at a critical time in development, remains to be explored.

Protein phosphorylation may be important for targeting proteins to specific sites and controlling protein turnover. Such a mechanism has been observed with sucrose synthase which has a nonspecific affinity for membranes, and phosphorylation status regulates this association (Koch, 2004). In addition, phosphorylation status regulates SUCROSE SYNTHASE degradation. First, the phosphorylation that activates the enzyme at the S15 site makes this enzyme available for a second phosphorylation at a second site, S170. This second phosphorylation leads to ubiquitin-mediated degradation (Hardin et al., 2003). Apple SDH has been considered a cytosolic protein but the possibility of association with membranes has not been investigated.

Sorbitol as a signaling molecule

The majority of plant species use sucrose as the main phloem-transported carbohydrate, and sucrose cleavage initiates hexose-based sugar signals in importing tissues (Koch, 2004). The gene families encoding either invertases or sucrose synthases that cleave sucrose respond at transcriptional and posttranscriptional levels to internal and external environmental signals and can dramatically alter plant development. An important aspect of sucrose function as a signal molecule is the physical path of sucrose import and sites of sucrose cleavage. Sucrose can move from phloem into the cytoplasm of sink cells with or without crossing the plasma membrane or the cell wall space. Sucrose and hexose sensing occur when sucrose crosses the membrane. A sucrose transporter is sucrose-responsive at a transcriptional level making this transporter an important factor in sucrose sensing and sucrose distribution (Vaughn et al., 2002). If sucrose moves via plasmodesmatal connections, SUCROSE SYNTHASE action producing UDP-glucose and fructose from sucrose and vacuolar invertases can initiate hexose-based signals (Koch, 2004). SUCROSE NONFERMANTING FACTOR-RELATED KINASE (SnRK), a SNF1 ortholog, is required for upregulation of

SUCROSE SYNTHASE during sugar induction and is linked to the first phosphorylation event of sucrose synthase (Halford et al., 2003).

Sorbitol is the main translocated sugar in apple, but the role of sorbitol in sugar signaling and sensing has not been studied. A sorbitol induction pathway has been determined for only one species, *Bacillus subtilis*. *B. subtilis* has a unique pathway for sorbitol (glucitol) metabolism. In the presence of sorbitol in the culture medium, sorbitol utilization (known as gut for *g*lucitol *ut*ilization) operon is induced (Poon et al., 2001). Sorbitol utilization operon encodes two genes: a SORBITOL PERMEASE (GutA, GLUCITOL PERMEASE) which transports sorbitol into the cell, and a SORBITOL DEHYDROGENASE (GutB, GLUCITOL DEHYDROGENASE). The sorbitol operon induction by a transcription activator, GutR occurs in the presence of sorbitol. In the presence of sorbitol, GutR (95-kDa protein) binds tightly to its binding site located upstream of the sorbitol operon promoter. Sorbitol induces GutR to change its conformation and triggers GutR to bind ATP efficiently. After sequential binding of sorbitol and ATP to GutR, GutR adopts a new conformation by forming a compact structure that is resistant to trypsin digestion. In this condition, the ATP·sorbitol·GutR complex can dissociate slowly from the GutR-binding site. Therefore, one of the functions of sorbitol as an inducer is to induce GutR to tightly bind to its target site and ATP so that subsequent reactions can occur and lead to the activation of the sorbitol operon.

Since sorbitol is the main soluble sugar in apple, it may play a role similar to sucrose in other species. As sucrose has been implicated in sugar signaling, it is possible that sorbitol may play a similar role in sugar sensing in apple. Sugar signaling has been found to be a very important aspect of plant development. In addition to sucrose sensing systems, plants also use other systems involving hexoses (Smeekens, 2000). Hexoses may act through hexokinase–dependent and hexokinase–independent pathways, where the dependent pathway involves phosphorylation of hexoses while the independent does not (Smeekens, 2000). Glucose can be phosphorylated to glucose-6-phosphate by hexokinase to signal a response (Rolland et al., 2002), but sorbitol has not been studied in this regard.

Objectives

The first objective of this work was to determine if *SDH* transcript is detectable, if SDH protein accumulates, and if SDH activity is evident during apple fruit set and early development. The present work used three apple cultivars to determine if contrasting patterns of SDH transcript accumulation and enzyme activity may occur among apple cultivars.

The second objective of this study was to determine if *SDH* is expressed and is active in apple seed and cortex, and how analyses of whole fruit *SDH* expression and activity patterns might be related to those in seed and cortex separately.

The third objective of this study was to determine if differences in activity in seed and cortex are related to tissue-specific expression of SDH isomers.

Chapter 2

Sorbitol Dehydrogenase Expression and Activity During Apple Fruit Set and Early Development

Introduction

Apple fruit set and early development are critically important in the production of the annual crop. Following pollination and fertilization, fruit develops via cell division for 4 to 6 weeks followed by an extended period of cell expansion until harvest (Bollard, 1970; Pratt, 1988). Fruit relative growth rate, or sink activity, is higher during this period than at any other time during growth (Lakso et al, 1995; Schecter et al., 1993), but fruit are weaker sinks than growing shoots and are sensitive to carbohydrate availability (Bepete and Lakso, 1998; Byers et al., 1990). Sorbitol comprises 80% or more of the carbohydrate translocated in the phloem of apple (Bieleski, 1969; Loescher et al., 1982; Webb and Burley, 1962), and is thus the main carbohydrate resource imported by vegetative and fruit sinks. Sucrose comprises the bulk of the remaining carbohydrate in the phloem. The ability of a fruit to persist and grow while competing with other reproductive and vegetative sinks may be due in significant part to the ability to utilize these carbohydrates rapidly and efficiently (Ho, 1988).

SORBITOL DEHYDROGENASE (SDH, EC 1.1.1.14) has been identified as the primary enzyme that metabolizes sorbitol in apple fruit (Beruter, 1985; Yamaki and Ishiwaka, 1986; Yamaguchi et al., 1996), so it may play a critical role in defining sink activity. Patterns of SDH transcript accumulation and enzyme activity have been described for periods starting 4 to 6 weeks after bloom and ending at harvest (Park et al., 2002; Yamaguchi et al., 1996; Yamada et al., 1998, 1999), but that during the early fruit development period has not been reported. As this is the most critical period for defining apple yield, it is imperative that the factors determining apple fruit set and early growth be elucidated.

The objective of this work was to determine if SDH transcript is detectable, if SDH protein accumulates, and if SDH activity is evident during apple fruit set and early

development. The present work used three apple cultivars to determine if contrasting patterns of SDH transcript accumulation and enzyme activity may occur in apple. Preliminary studies indicated that both SDH enzyme activity and protein, the latter determined using an antibody we developed, were present in 'Gala' apple during early fruit development (Archbold et al., 2004).

Results from this work have been published (Nosarzewski et al., 2004 Physiologia Plantarum 121:391-398,).

Materials and methods

Materials

The apple cultivars Lodi, Redchief Delicious, and Red Fuji were harvested from the University of Kentucky orchard, Lexington, KY, once a week for 5 weeks in 2002 starting immediately after full bloom (AFB) when receptacle growth of fruit was first evident. Harvested fruits (taken from trees between the hours of 10AM and 12AM) were immediately frozen in liquid N_2 for transport to -80 °C storage until further use. In addition, a sample of fresh fruits was placed on ice for subsequent determination of individual fruit fresh weight (FW). Fruit relative growth rate (RGR) was estimated from weekly FW measurements. The RGR was determined as the difference between the natural logarithms of the FWs on the two dates divided by the days between measurements, or [(Ln (FW 2) – Ln (FW 1)]/days between measurements (Hunt, 1982).

RNA extraction

Apple fruit total RNA was extracted using a hot borate protocol (Wan and Wilkins, 1994).

cDNA library screening

Drs. Ian Wilson and David Dilley kindly provided a cDNA library made from combined RNA extractions from various ripening stages of Mutsu apple fruit. Five hundred thousand recombinants from the cDNA library were screened. A probe was made using the 812 bp SDH cDNA purified from *Eco*R I-digested plasmid and the released fragment was labeled using the ECL direct nucleic acid labeling and detection system protocol (Amersham, Piscataway, NJ, USA). Using this method, 125 positive clones were identified of which 40 recombinants were purified to homogeneity in subsequent screens. cDNA in plasmid form was recovered by *in vivo* excision using the

lambda ZAP–vector system (Stratagene Cloning Systems, La Jolla, CA). The clones were sequenced at the Macromolecular Structure Analysis Laboratory (University of Kentucky, Lexington, KY, USA). This facility used an ABI Prism 377 DNA Sequencer (ABI; Perkin-Elmer, Foster City, CA, USA) and dye termination chemistry with AmpliTaq DNA polymerase, FS (Taq; FS; Perkin-Elmer/Applied Biosystems Division [PE/ABI], Foster City, CA, USA) to read cycle-sequencing reactions employing a combination of universal and gene–specific primers (Genset Corporation, Operon Technologies, Alameda, CA, USA).

Northern analysis

Total RNA (10 μg $lane^{-1}$) was first size fractionated through 1.3% w/v agarose denaturating formaldehyde gels and then vertically transferred using capillary action onto positively charged nylon membranes (Amersham Life Science Inc., Arlington Heights, IL, USA) in 10 x SSC (1 x SSC is 150 mM NaCl, 15 mM Na citrate, pH 7) overnight and UV cross–linked at 120,000 μJoules/cm^2 on a FB-UVXL-1000 Stratalinker (Fisher Scientific, Santa Clara, CA, USA). After rinsing the membranes for 5 min in 2 x SSC, they were placed in pre-hybridization solution (100 μg/ml sheared salmon sperm DNA, DIG Easy-Hyb buffer) (Roche, Mannheim, Germany) for 4-6 h at 68ºC.

In the process of generating antisense RNA probes, a SDH fragment was amplified using a cDNA clone in a plasmid as template, Advantage Taq polymerase (ClonTech, Palo Alto, CA, USA), a degenerate forward primer (5' - RTT CAC YAC YTC ARG AMC ATG) and a degenerate reverse primer containing a 5' non-homologous extension encoding the T7 RNA polymerase promoter sequence (5' - GTA ATA CGA CTC ACT ATA GGG TCC BAC AAG GCA RAC TTT KCC) at an annealing temperature of 50ºC for 36 cycles. The amplicon was size fractionated on 1% agarose gel and purified using a QIA quick gel purification kit (Qiagen, Valencia, CA, USA) and used in the transcription reaction at 37ºC for 3 h with T7 RNA polymerase (Roche) in the presence of dioxigenin-UTP (Roche).

The probe was added to the pre-hybridization solution described above and the membrane probed for 12 h at 68ºC. The primary wash was done twice in 2 x SSC with 0.1% SDS at room temperature for 15 min. The two final high stringency washes were at 0.2 x SSC, 0.1% SDS, and 68ºC for 15 min each.

Following the final high stringency wash, the membrane was briefly rinsed in washing buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5, 0.3% v/v Tween 20) and incubated for 30 min in blocking solution (1% w/v blocking reagent (Roche), 0.1 M maleic acid, 0.15 M NaCl, pH 7.5), followed by incubation for 30 min in antibody (75 mg/mL of anti-dioxigenin–AP in blocking solution). After two subsequent 15 min washes in washing buffer, the membrane was equilibrated for 2-5 min in detection buffer (0.1 M Tris-HCl, 0.1M NaCl, pH 9.5) and placed between leaves of a transparent sheet protector with a 1:100 dilution of CSPD (0.25 mM final concentration) in detection buffer. The membrane was exposed to X-ray film for 15-25 min and then developed.

Genomic clone isolation

Genomic DNA was extracted from expanding leaf tissue collected from Mutsu apple trees. Using the Extract–N-Amp Plant PCR kit (Sigma, St. Louis, MO, USA), a 900 bp fragment of SDH was amplified using primers to conserved SDH sequence (forward 5' – AGA TYC WAC CTT WCA AGC TYC and reverse 5' - GCT TCT TCC ACC TCC TTC) at a 53ºC annealing temp for 36 cycles. The amplicon was ligated by T4 DNA ligase at 16ºC overnight into a homemade T/A cloning vector (Xu and Downie, unpublished). Sequencing of the partial-length, genomic DNA amplicons was performed at the Macromolecular Structure Analysis Laboratory (University of Kentucky, Lexington, KY, USA).

Sequence determination and alignment

From the sequence data derived as above, Sequencher software (Gene Codes Corp., Ann Arbor, MI, USA) was used for gene and cDNA consensus sequence alignments and base calling. Multiple nucleic acid sequence alignments were performed using the Clustal W algorithm (Thompson et. al., 1994).

SDH enzyme activity measurement

SDH enzyme was extracted from whole apple fruit, cortex and seeds, and assayed as in Archbold (1999) with the exception that 0.6 M Tris buffer (pH 7) was used to extract the tissues in place of K-phosphate buffer. The protein content of the Sephadex-purified extracts was determined spectrophotometrically at 595 nm using the Bradford Assay (Bradford, 1976). There were 3 extractions of composite samples of apple fruit per

weekly sampling date. Enzyme activity is averaged by weekly sampling date and is reported as nmol NAD^+ reduced • min^{-1} • mg $protein^{-1}$.

ELISA and Western analyses

ELISA assays and Western blots were performed using the ImmunoPure ABC Phosphatase Staining Kit (Pierce) at room temperature. For the ELISA procedure, 100 µL of 10 mg protein/mL apple extract in coating buffer (0.1 M sodium bicarbonate, pH 9.2) was placed into each well of a microtiter plate and incubated for 1 h. After three 200 µL rinses of each well with wash buffer (0.05% w/v Tween 20, 0.1% w/v BSA in TBS), wells were incubated with 200 µL blocking buffer (ImmunoPure ABC Phosphatase Staining Kit, Pierce) for 1 h and later incubated with primary immunopurified SDH antibody (Nosarzewski et al., 2004) for 30 min. Wells were washed in the same manner and exposed to 100 µL of secondary antibody (biotinylated affinity purified goat anti-rabbit IgG 1.5mg/mL, Pierce) for 30 min. After washing, wells were incubated with 100 µL ABC solution (Avidin, biotinylated alkaline phosphatase, Pierce) for 30 minutes and finally developed with 100 µL para-nitro-phenyl phosphate (PNPP) solution (1 mg in 1 ml diethanolamine buffer, pH 9.5). Absorbance was measured at 405 nm using a Uniskan I ELISA plate reader (Labsystems Inc., Morton Grove, IL, USA). The quantity of SDH was calculated using a standard curve derived using purified sheep liver SDH (Sigma).

Western blots were obtained after transferring protein from 12.5% SDS-PAGE gels, run with 9 µg of apple protein per lane, to nitrocellulose membranes and treating them in the same manner as the ELISA above. The signal was developed using a NBT/BCIP solution (Pierce). Purified antibody interacted with the recombinant apple SDH recovered from *E. coli* and sheep liver SDH equally well, so the sheep liver SDH was used as a visual standard as its molecular mass was close to apple SDH.

Results

cDNA library screening and genomic clones analysis

Three isoforms of NAD-SDH (accession numbers AY244806, AY244807, and AY244810 for SDH1, SDH2, and SDH9, respectively; Figure 2.1) were determined from analysis of complete cDNAs obtained from an apple cDNA library. The three full length cDNAs consisted of 1359, 1497, 1432 bp and contained open reading frames of 1107, 1116, 1107 bp, encoding proteins of 369, 372, 369 amino acids, respectively. Using

ProtParam (Appel et al., 1994), the protein sizes were estimated to be 39 - 40 kDa. In addition to the mRNA sequence of one of the isoforms of NAD-SDH (AY244807, or SDH2), the partial genomic sequence of the gene encoding SDH2 was also determined (AY244808). Five other partial genomic sequences of NAD-SDH were distinguished by differences in intron sequences (AY244809, AY244811, AY244814, AY244813, and AY244812 for SDH3, SDH5, SDH6, SDH7, and SDH8, respectively; Table 2.1, Figure 2.1, Appendix A). All apple SDH isoforms except SDH1 were highly homologous at the protein (Table 2.1 B, Appendix B) and nucleic acid (Table 2.1 A, Appendix C) levels.

Northern analysis

Generally, RNA blot results indicated the presence of approximately the same amount of NAD-SDH transcript throughout the first five weeks after bloom in all three apple varieties (Figure 2.2). However, transcript abundance tended to be greatest during the first week after full bloom and, at least for Lodi and Red Fuji, the least 5 weeks after full bloom (Figure 2.2).

Fruit growth and SDH activity

Lodi apple fresh weight increased most rapidly during the 5-week period AFB, while Redchief Delicious and Red Fuji exhibited nearly identical FW through week 5 (Figure 2.3 A). Fruit RGRs were similar among the three cultivars through 5 weeks AFB, declining from week 1 to week 2, increasing at week 3, and declining in the remaining weeks (Figure 2.3 B). SDH activity in all 3 cultivars increased from the first to the second week AFB. Lodi and Red Fuji exhibited peak activity at 2 weeks AFB, while Redchief Delicious reached a peak at 3 weeks AFB. Lodi SDH activity was very low at 4 and 5 weeks AFB, while Red Fuji exhibited another peak at 5 weeks AFB (Figure 2.3 C).

Western and ELISA analyses of SDH

Apple SDH was detected by immunoblotting with a band at approximately 37 kDa, while sheep liver SDH was detected at approximately 40 KDa (Figure 2.4). The apple SDH band was most intense in the first 2 – 3 weeks AFB. The extractable protein per g FW showed some variation but no dominant trend, as Lodi and Red Fuji showed a decline from week 1 to week 2, and an increase over the remaining weeks while Red Delicious exhibited a decline over the 5 weeks (Figure 2.5 A). ELISA results indicated

that 6 to 8% of the total extractable protein was SDH across the 5-week period AFB (Figure 2.5 B). The cultivars were similar with only Redchief Delicious lower at week 3.

Discussion

Nine genes for SDH were revealed in this study by unique genomic and mRNA sequences. SDH4 provided the initial sequence upon which the antibody was developed, although the sequence is shared by all isoforms and with the sheep liver SDH used as a standard. Park et al. (2002) reported 4 full-length sequences for Fuji apple, but found 24 positive clones in a screen. Sequences of SDH1, SDH2, and SDH9 in our work show greater than 97% identity with three isoforms, MdSDH1, MdSDH2, and MdSDH4, from Fuji apple (Park et al., 2002).

The full length of the cDNA sequences in our work fall within the range of those previously reported (Park et al., 2002; Yamada et al., 1998), 1359-1521 bp, encoding SDH proteins of 367 to 379 amino acids with a molecular mass of 39 to 40 kDa. However, our Western analyses do not correlate with the calculated protein sizes as our signal, identified as the most intense band and closest to the estimated molecular mass, occurred closer to 37 kDa (Figure 2.4). Other reports are inconsistent in their description of apple SDH, with one suggesting a 62 kDa protein (Yamaguchi et al., 1996), and another indicating a 42 kDa protein (Yamada et al., 1998), both of which are larger than the molecular mass observed in the present work as well as that estimated from the sequence length. Although the entire SDH sequence length suggests protein larger than 37 kDa, the lower molecular mass may be a result of a different initiation site for translation of SDH or a posttranslational modification of SDH protein length. Interestingly, a second initiation codon (AUG, methionine) occurs at the 23rd amino acid into the open reading frame. The first methionine may be bypassed, leading to initiation with the second (Mathews, 2002). This would produce a protein of 37 kDa, the same as indicated by the Western blots.

Northern analysis indicated that SDH transcript was present during each week of this study. The probes used in this work did not distinguish among isoforms. Park et al. (2002) reported that 4 isoforms were expressed in sink tissues including fruit as they approached ripening while one isoform was expressed in source leaves as well. Thus it is

possible that not all of the isoforms reported in our work were expressed each week or even at all during early fruit development.

The amount of SDH protein did not vary appreciably as did SDH activity, evidence that there may be posttranslational modification of an inactive SDH pool that affects activity as others have suggested (Yamaguchi et al., 1996). A second alternative is that different SDH isoforms are produced during the first weeks after full bloom and that these isoforms differ in their kinetics. Support for either contention comes from the observation that SDH activity for Redchief Delicious peaks when the least amount of SDH protein is detected (3 weeks after full bloom, compare Figure 2.3 C and Figure 2.5 B). It is possible that a highly active isoform predominates at this time. This contrasts with late season SDH expression when relative protein quantity and activity appeared to change proportionally (Park et al. 2002; Yamada et al., 1999). However, neither of the prior reports quantified SDH protein as was done using ELISA in our work, but rather assessed quantity from band intensity in Western analyses. Although SDH activity may be affected by sorbitol availability (Archbold, 1999), it is not clear from the present data why the activity varied.

The pattern of change in SDH activity may be related to fruit sink activity. Even though the maturation dates varied over 90 days for the three cultivars in this work, fruit RGR, an expression of sink activity, exhibited high values at weeks 1 and 3 AFB for all three, confirming prior studies that fruit RGR is highest in the period immediately after bloom and is similar among cultivars with differing maturation dates (Lakso et al., 1995; Schecter et al., 1993). SDH activity was highest at week 2 for Lodi and Red Fuji and at week 3 for Redchief Delicious. Since the patterns of fruit RGR and SDH activity did not match, other enzymes of sorbitol and sucrose metabolism, including sorbitol oxidase and invertase (Yamaki and Ishiwaka, 1986), may also play important roles in defining sink activity and fruit RGR. It should be noted that this work did not distinguish between seed/embryo SDH and that in the flesh. As both are rapidly-growing sinks for sorbitol, SDH expression and activity may have occurred in both tissues.

In conclusion, the present work is the first to show SDH is expressed and exhibits significant activity in apple fruit immediately after fruit growth starts. One or more of the multiple isoforms reported here are expressed shortly after fruit growth commences. It is

likely that SDH plays a critical role in establishing young apple fruit as sinks and in their persistence during the critical fruit set period.

Table 2.1. A) Sequence identity at the nucleic acid level for the Apple *SORBITOL DEHYDROGENASE* cDNAs and; B) sequence identity (lower triangle) and similarity (upper triangle) among the amino acid sequences deduced from the cDNAs retrieved during the course of this investigation. Genbank accession numbers for each clone are provided at the bottom of the tables.

Table 2.1. A)

Identity of *Nucleic Acids*

SDH	1	2	3	4	5	6	7	8	9
1		75	74	75	74	75	75	76	76
2	75		91	91	91	89	96	89	89
3	74	91		89	89	88	91	89	89
4	75	91	89		93	89	89	92	96
5	74	91	89	93		89	90	91	92
6	75	89	88	89	89		92	92	88
7	75	96	91	89	90	92		88	88
8	76	89	89	92	91	92	88		94
9	76	89	89	96	92	88	88	94	
	AY244806	AY244807	AY244809	AY053504	AY244811	AY244814	AY244813	AY244812	AY244810

Identity of Nucleic Acids

Table 2.1. B)

Similarity of Amino Acids

SDH	1	2	3	4	5	6	7	8	9
1		93	92	93	93	93	93	94	94
2	72		100	99	100	99	99	99	99
3	71	92		99	99	98	99	99	99
4	72	96	90		100	99	99	99	100
5	72	95	92	94		99	99	99	100
6	71	95	93	93	95		98	99	99
7	71	96	94	93	94	97		99	99
8	73	95	92	94	96	96	95		99
9	73	94	91	96	96	95	94	96	

Identity of Amino Acids

AY244806 AY244807 AY244809 AY053504 AY244811 AY244814 AY244813 AY244812 AY244810

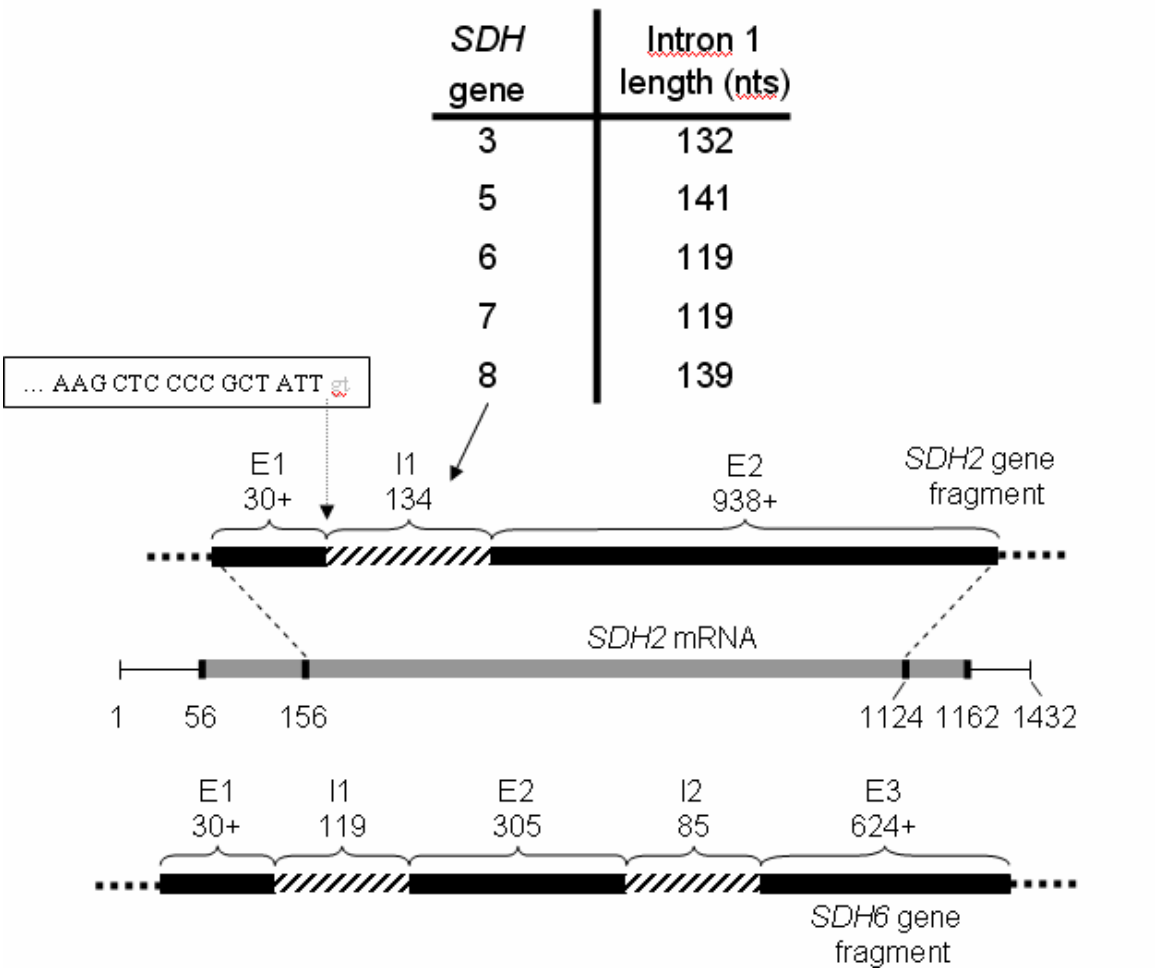

Figure 2.1. A depiction of the organization of *SORBITOL DEHYDROGENASE* genes in apple. The number of introns/exons present in each gene fragment, their lengths (in nucleotides), and placement in the genes, are provided. Genbank accession numbers for SDH3, SDH5, SDH6, SDH7, and SDH8 are AY244809, AY244811, AY244814, AY244813, and AY244812, respectively.

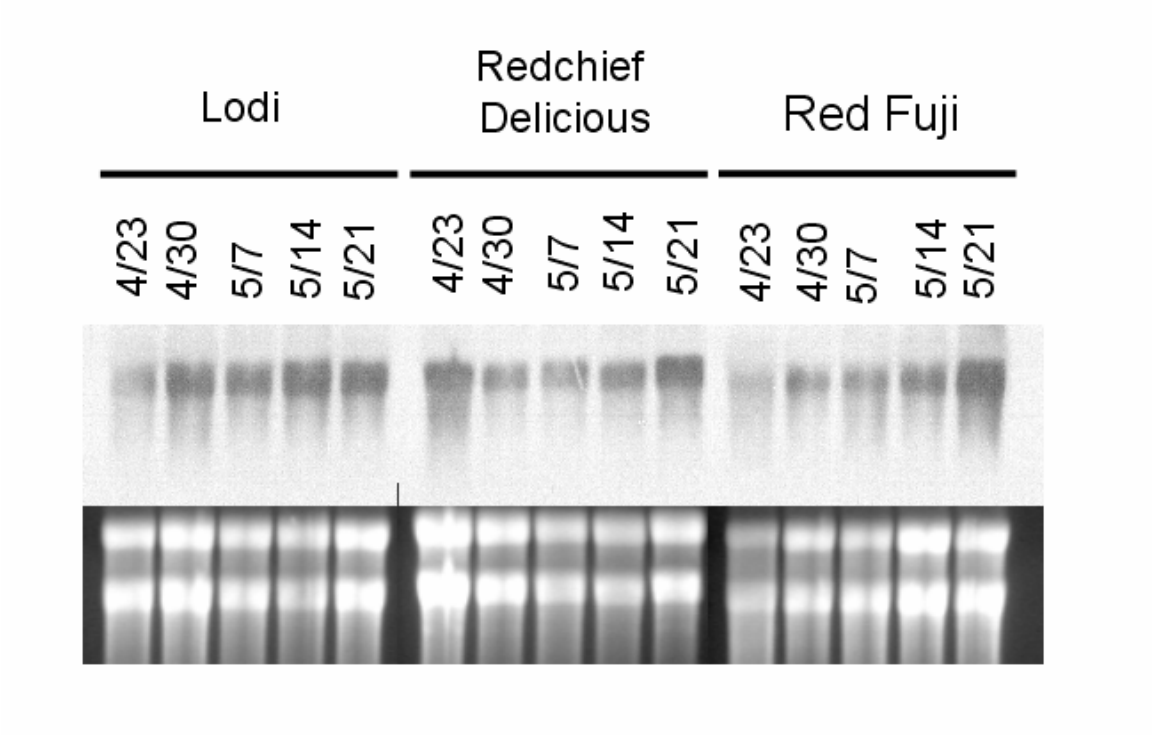

Figure 2.2. Northern blot of an amalgam of *SORBITOL DEHYDROGENASE* transcripts from all genes being transcribed in apple fruit from three cultivars during early fruit development. The upper panel is the signal obtained from a non-discriminating, DIG-labeled *SDH* probe used to challenge a blot of apple fruit total RNA (10 μg lane^{-1}). The lower panel is a photograph of the ethidium bromide-stained, denaturing RNA gel prior to RNA transfer to assess the equality of the RNA loading.

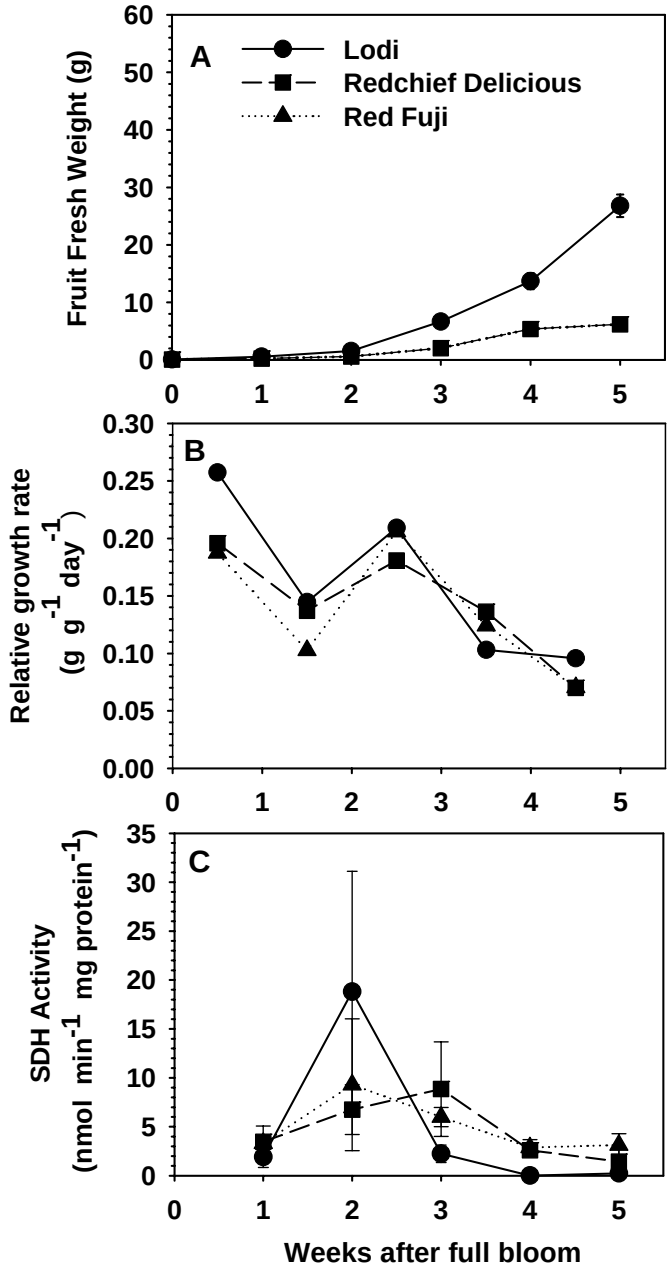

Figure 2.3. SORBITOL DEHYDROGENASE activity is detectable in fruit as early as 1 week after full bloom. A) Fruit fresh weight accumulation during the first five weeks after full bloom in three cultivars, Lodi, Redchief Delicious, and Red Fuji. B) The relative growth rate of fruits from three different apple cultivars during the first 5 weeks after full bloom. C) Sorbitol dehydrogenase activity in fruits harvested weekly during the first five weeks after full bloom.

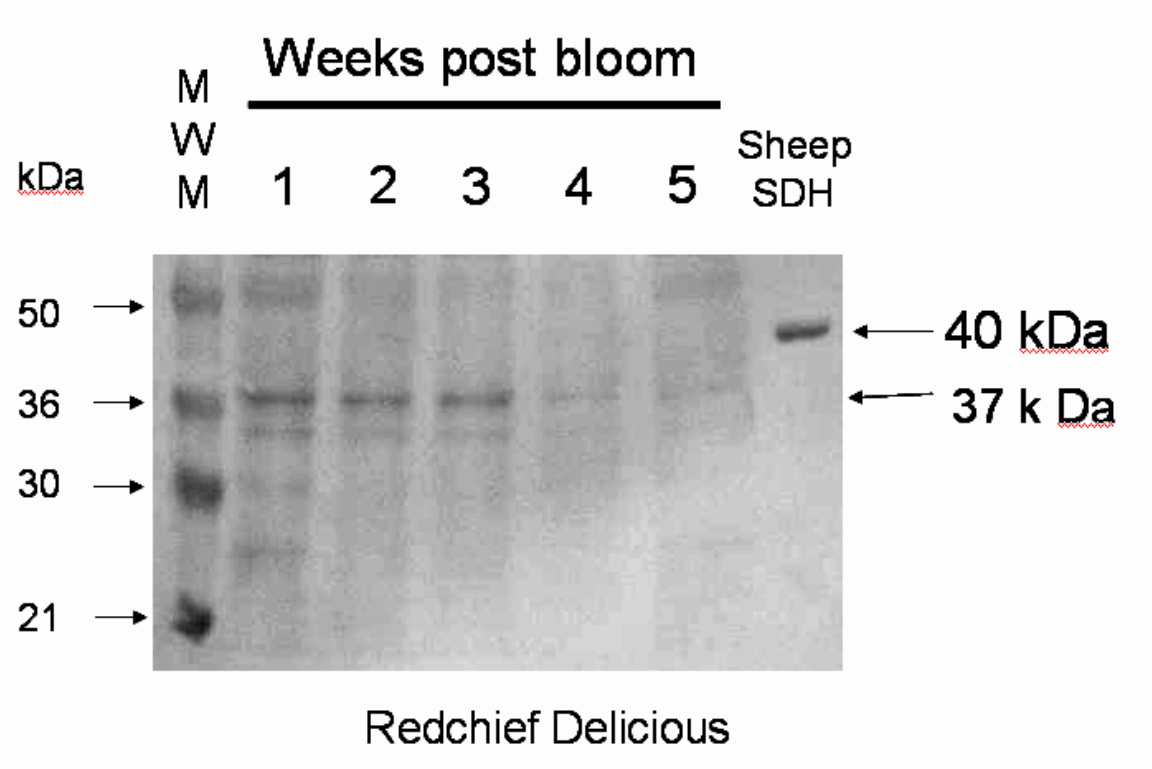

Figure 2.4. Western blot of apple fruit protein (20 μg·lane^{-1}; cv. Redchief Delicious) during the first 5 weeks after full bloom. SORBITOL DEHYDROGENASE protein is present in the fertilized ovary from the first week and remains detectable through 5 weeks, though it is faint at weeks 4 and 5. Purified sheep SDH (40 kDa) was loaded (9 ng · lane^{-1}) as an internal control. MWM: molecular weight marker.

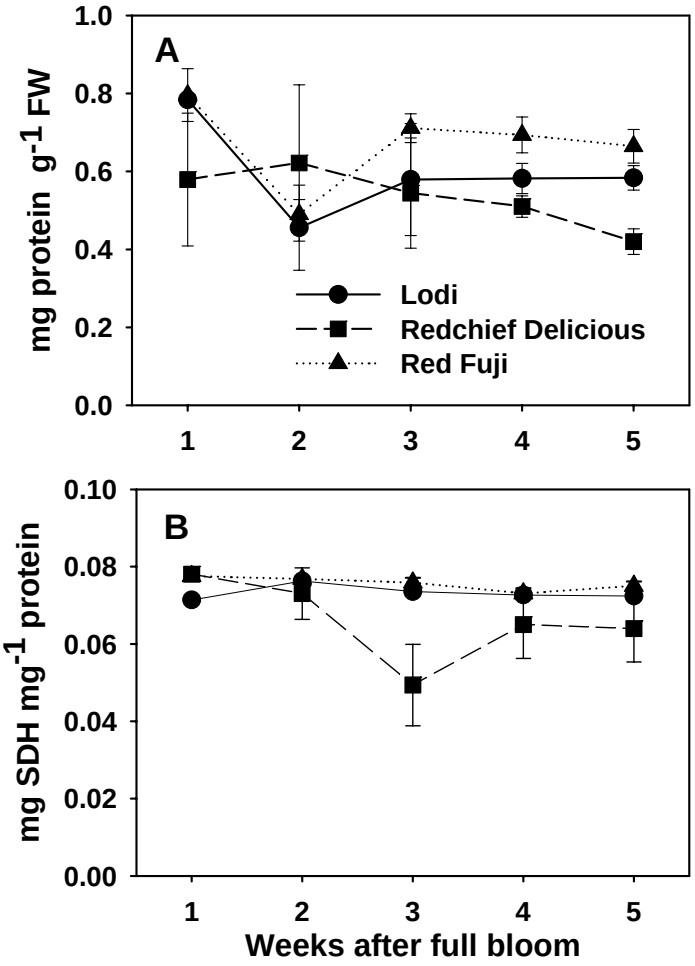

Figure 2.5. A) The protein content per gram fresh weight of fruit, and B) the SDH amount per mg protein during the first five weeks after full bloom in three cultivars of apple: Lodi, Redchief Delicious, and Red Fuji.

Chapter 3

Tissue specific *SDH* expression in seed and cortex of apple fruit during early development.

Introduction

Apple (*Malus X domestica* Borkh.) fruit set and development depend on carbohydrate import and metabolism. The main translocated carbohydrate in apple is sorbitol, and oxidation of sorbitol to fructose by SORBITOL DEHYDROGENASE (SDH, E.C.1.1.1.14), using NAD^+ as a co-factor, is the first step of sorbitol utilization. SDH has been identified as the primary enzyme that metabolizes sorbitol in apple fruit (Beruter, 1985; Yamaki and Ishiwaka, 1986; Yamaguchi, 1996). A significant level of SDH activity per unit fresh weight (FW) and per mg protein has been found immediately after fruit growth starts, one week after bloom, and during the ensuing weeks (Nosarzewski et al., 2004). In addition, SDH exhibits high levels of activity at the transition from cell division to cell expansion and during ripening (Yamaguchi, 1996; Yamada et al, 1999; Park et al, 2002). Analysis of SDH protein levels during the first five weeks of apple fruit development, often termed fruit set, showed that the amount of protein present in the tissue did not change significantly, and that SDH activity per mg protein fluctuated depending on the cultivar during that time period (Nosarzewski et al., 2004).

Variation in SDH activity may be due to limitations in sorbitol availability to the fruit. Apple fruit set and early development is very sensitive to carbohydrate availability, as shown by partial shading and treatments reducing photosynthetic activity (Bepete and Lakso, 1998; Byers et al., 1990). This availability is affected by competition among the many reproductive and vegetative sinks. Because fruit are weaker sinks than growing shoots (Corelli Grappadelli et al., 1994; Lakso et al., 1998), the inability of many fruit to persist and grow, and/or the low growth rate of some fruit that do persist and that results in poor size and quality at harvest, may be due to less efficient utilization of uploaded carbohydrates compared to shoots and other vegetative sinks. Were fruit more competitive for carbohydrate resources during early development, more fruit could

develop without compromising size and quality. Zhang et al. (2005) have recently shown that fruit growth in pear (*Pyrus pyrifolia*) is limited by sink strength of the fruit rather than the capacity of the transport pathway; sorbitol is the major photoassimilate in pear as it is in apple. This strongly suggests that the capacity for utilization of carbohydrate is critical for achieving sufficient yield and quality. It is likely that SDH plays a critical role in establishing young apple fruit as sinks during the fruit set phase.

Nine SDH isoforms were isolated and characterized in apple, each with an approximate molecular weight (deduced from mRNA sequences) of 39-40 kDa (Nosarzewski et al., 2004). The presence of such an abundance of *SDH* genes in apple suggests tissue-specific regulation of *SDH* expression as indicated by Park et al. (2002). In that work, expression of three isomers was restricted to sink tissues like young leaves, stems, roots, and maturing fruit, while a fourth isomer was expressed in both immature and mature leaves. Similar results were obtained from the developing buds of Japanese pear (*Pyrus serotina,* another Rosaceae family species) where partial fragments of five *SDH* genes were isolated (Ito et al., 2005). The derived amino acid sequence of one of the pear isomers (PpySDH5) has 94-98% homology with apple SDH1 and is distinct from the other four isomers of pear SDH (71% homology). The other four isomers are similar to each other with 88% to 95% homology. Due to the observed expression pattern in buds, the authors suggested that the pear SDH isomers could be categorized into two groups: one expressed in the bud for growth and development (i.e., *PpySDH1*) and the others of unknown tissue specificity (like *PpySDH5*). The expression patterns of the known *SDH* genes during apple fruit set and development have not been determined, although the presence of two isomers (SDH1 and SDH2) was detected in apple fruit starting at 90 days after bloom and two other isomers (SDH3 and SDH4) were present at 120 days after bloom (Park et al., 2002). Though none of these four *SDH* transcripts were detected at 30 days after bloom, we found *SDH* transcript without distinguishing between *SDH* genes during the first five weeks after bloom (Nosarzewski et al., 2004).

Prior to the present work, studies of fruit SDH activity have focused on either whole fruit or cortex activity only. There has been no data for apple that compares SDH activity in seeds to that of cortex tissue. As the early fruit development period after fertilization is critical to eventual yield, an analysis of both tissues can provide a greater

understanding of the dynamics of sorbitol metabolism during this important phase. One of the objectives of this study was to determine if *SDH* is expressed and is active in apple seed and cortex, and how analyses of whole fruit *SDH* expression and activity patterns might be related to those in seed and cortex separately. Another objective of this study was to determine if differences in activity in seed and cortex are related to *SDH* tissue-specific expression. For these purposes, seed and cortex tissues from apple collected during early fruit development were subjected to analysis of SDH activity, detection of SDH isomeric protein by 2D-PAGE and Western analyses, and identification of individual transcripts using RT-PCR and Northern analysis.

Materials and Methods

Fruit collection

The apple cultivars 'Redchief Delicious' and 'Mutsu' were harvested from the University of Kentucky South Farm orchard, Lexington, KY, once a week for 4 weeks in 2003, 2004, and 2005 ('Redchief Delicious') and 2005 only ('Mutsu') starting immediately after full bloom (AFB). Week 1 was defined as the time when fruit growth was first evident. Fruit was removed from the trees between the hours of 9 AM and 11 AM. When separation of seed from cortex was first possible at week 2 AFB, fruit were placed on ice and transported to the laboratory, where seeds were separated from cortex, and both were frozen in liquid N_2 and stored at -80 ^{o}C until further use. The apple cultivar 'Redchief Delicious' was used for all assays except RT-PCR analyses. The cultivar 'Mutsu' was used for SDH activity assays and RT-PCR analyses.

SDH activity measurement

Cortex and seed tissue samples of apple were weighed, and SDH enzyme was extracted and assayed from these samples as in Nosarzewski et al. (2004) except for exclusion of DTT from the extraction solution. The protein content of extracts was determined spectrophotometrically at 595 nm using the Bradford assay (Bradford, 1976). There were 5 extractions (replicates) of composite samples of seed and cortex tissues per weekly sampling date across three years. Enzyme activity was averaged by weekly sampling date and is reported as nmol NAD^+ reduced • mg protein^{-1} • min^{-1}. SDH activity per mg protein and protein amount per g FW tissue and tissue mass were used to determine SDH

activity per mg protein, per g FW and values per whole fruit. To calculate SDH activity per fruit, the SDH activity per g FW of the cortex was multiplied by the mass of the cortex, the SDH activity per g FW of the seeds was multiplied by the mass of the seeds (7 per fruit) and the products added.

Northern analysis

Total RNA was extracted from a composite sample comprised of weekly samples of seed or cortex tissue of 'Redchief Delicious' using a hot borate protocol (Wan and Wilkins, 1994). Northern analyses were run as described in Nosarzewski et al. (2004).

Western analysis

Western blots were performed on weekly composite samples using the ImmunoPure ABC Phosphatase Staining Kit (Pierce) at room temperature as described in Nosarzewski et al. (2004).

2D PAGE

Extracted proteins, as described above, from weekly composite samples were precipitated with 4X volume of cold acetone overnight in -20 ºC. After centrifugation for 10 min at 10000g and 4 ºC, the pellet was air-dried and dissolved in sample solubilization solution (8 M urea, 2 mM tributyl phosphine (TBP), 4% 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid (CHAPS), 0.2% carrier ampholyte, 0.0002% bromophenol blue). Each Immobilized Protein Gradient (IPG) strip was passively rehydrated with 125 µl of prepared sample containing 200-500 µg protein for 24 h. First dimension focusing was done using a Protean IsoElectric Focusing (IEF) Cell (BioRad) and using rapid ramp mode up to 4000 V and 20000 Vhours. After a 10 min equilibration of the IPG strip in equilibration solution (6 M urea, 20% w/v SDS, 1.5 M Tris (pH 8.8), 50% glycerol) containing 2% w/v DTT and another 10 min equilibration of the IPG strip in equilibration solution (6 M urea, 20% w/v SDS, 1.5 M Tris (pH 8.8), 50% v/v glycerol) containing 2.5% w/v iodoacetamide, the second dimension resolution was performed using SDS containing 12.5% w/v acrylamide gels. The resulting gels were subjected to Western analysis as described in Nosarzewski et al. (2004).

RT-PCR analysis

Total RNA was isolated from seeds and cortex of 'Mutsu' apple (composite across weekly samples) using a hot borate technique (Wan and Wilkins, 1994). Total

RNA isolated from the above tissues was pretreated with DNase I using a kit (DNA-*free*, Ambion, Austin, TX) according to the manufacturers protocol. The first strand cDNA for RT-PCR analysis was synthesized with oligo $(dT)_{18}$ primers using 1 µg of total RNA and SUPERSCRIPT III (Invitrogen) at 50°C for 1 h. The reaction was terminated (75°C, 15 min) and treated with RNase cocktail (Ambion; 37°C, 20 min). PCR was performed on 2 μL of first strand cDNA, using gene-specific primers (Table 3.1) for all 9 *SDH* mRNA species at 40 PCR cycles (annealing temperatures in Table 3.1). Amplicons were isolated on 1% (w/v) agarose gels and subjected to ethidium bromide staining.

Every gene-specific primer was tested by PCR to ensure its gene specificity. DNA templates representing each of the nine *SDH* genes were obtained from available 'Mutsu' *SDH* cDNAs (*SDH1, SDH2, SDH9*) and genomic clones (*SDH3, SDH4, SDH5, SDH6, SDH7, SDH8*). PCR cycles were performed on ~5 pg DNA template. PCR using each of the nine pairs of gene specific primers was performed for each *SDH* cDNA (annealing temperatures in Table 3.1).

Results

SDH activity

Seed SDH activity per mg extractable protein during weeks 2-5 AFB of 'Redchief Delicious' was up to 8-fold greater than cortex and whole fruit SDH activity (Figure 3.1). Seed SDH activity per mg protein increased 3-fold from week 2 to week 3, remained at that level through week 4, and rose again at week 5. In contrast, cortex and whole fruit SDH activity per mg protein were similar with slightly higher activity levels for whole fruit (Figure 3.1). Both cortex and whole fruit activity per mg protein appeared to decline slightly from weeks 3 to 5.

Average seed and cortex SDH activity for 'Redchief Delicious' during four weeks of development (starting at the second week AFB) was 56.6±5.2 and 14.8±5.9 nmols NADH/min/mg protein, respectively. This is consistent (within one standard deviation) with the values found during a similar period for 'Mutsu' apple (53.4 ±3.8 and 13.3±3.4 nmol NADH/min/mg protein for seed and cortex, respectively). The patterns of change of seed and cortex SDH activity of 'Mutsu' over weekly sampling dates were consistent with 'Red Delicious' as well (data not shown).

Seed had much greater protein content per g FW than cortex or whole fruit (Figure 3.2 A). Due to changes in seed extractable protein content per g FW (Figure 3.2 A) and in seed SDH activity per mg protein (Figure 3.1), seed SDH activity pattern per g FW (Figure 3.2 B) varied. At the 3rd week AFB, seed SDH activity reached its peak (a four-fold increase over the 2nd week) due to a three-fold increase in SDH activity per mg protein and an increase in protein content per g FW. The seed SDH activity per mg protein did not change much by the 4th week but, due to a significant decrease in protein content to a level similar to that at the 2nd week, seed SDH activity per g FW dropped to half its activity at the 3rd week. Another increase in seed SDH activity per g FW at the 5th week was related to elevated seed SDH activity per mg protein, not an increase of seed extractable protein. Since the cortex and whole fruit protein content and SDH activity per mg protein changed only slightly over the 4 weeks, cortex and whole fruit SDH activity per g FW was fairly constant and considerably lower than seed SDH activity (Figure 3.1, Figure 2.1 A, B).

Seed fresh weight accumulation and extractable protein content per fruit increased gradually during weeks 2 to 5 AFB (Figure 3.3 A-B, Figure 3.4 A-B). Cortex fresh weight accumulation and extractable protein content increased gradually from the 2nd to 4th week AFB, then increased rapidly up to 5 fold by the 5th week AFB. Total SDH activity per fruit fluctuated (Figure 3.5 A). After a 4-fold increase from week 2 to week 3 and a small decline from week 3 to week 4, SDH activity per fruit nearly doubled from week 4 to week 5, reaching values nearly 8-fold greater than week 2 activity. This pattern was similar to the cortex SDH activity per fruit, and may be explained by the high contribution of cortex biomass to whole fruit biomass (Figure 3.3, Figure 3.4). In contrast to the relatively small contribution of seed to whole fruit biomass and protein content (Figure 3.3 C, Figure 3.4 C), total seed SDH activity contributed significantly to total SDH activity per fruit (Figure 3.4 A-B), reaching its highest level of contribution (30%) to total SDH activity per fruit at weeks 4 and 5.

Western and 2D PAGE analysis

The presence of SDH during early development of apple seed and cortex tissues was confirmed by immunoblotting (Figure 3.3). A 37 kDa band representing SDH protein was clearly visible in both tissues every week. Furthermore, after 2D SDS-PAGE followed by

Western blot detection of SDH protein, six SDH isomers with different pI values were detected in whole fruit tissue (Table 3.2). Four of the SDH isomers were found in seeds at pIs of 4.2, 4.8, 5.5, and 6.3 (Figure 3.4) and four SDH isomers were also found in cortex tissue at pIs of 5.5, 6.3, 7.3, and 8.3 (Figure 3.5). Two of the SDH isomers (with pI 5.5 and 6.3) were shared by the seed and cortex tissues, while the other isomers were tissue specific.

Northern analysis

RNA blot results indicated the presence of *SDH* transcript in seed and cortex tissues of apple fruit (Figure 3.6). Despite the slightly lower level of seed RNA loaded on the gel (lower panel Figure 3.6), the amounts of *SDH* transcript per μg total RNA appeared to be much greater in seed then in cortex (upper panel Figure 3.6).

RT-PCR analyses

RT-PCR analyses (Figure 3.7) were performed using total RNA extracted from seed and cortex tissues of 'Mutsu' apple collected during weeks 2-5 of fruit development. Two *SDH* genes, *SDH1* and *SDH3,* were expressed in both seed and cortex tissues. *SDH2* expression was limited to cortex, while *SDH6* and *SDH9* expression were found in seed tissue only. Of the nine *SDH* genes present in apple, five were found in apple fruit during this period (Table 3.3).

The gene specificity of the primers generating amplicons in apple fruit was confirmed by PCR analyses on *SDH* cDNAs and genomic DNA previously obtained from 'Mutsu' apple (Nosarzewski et al., 2004) (Figure 3.8). The *SDH1*-specific primers recognized *SDH1* cDNA only from nine *SDH* templates by amplifying a 281 bp amplicon (40 PCR cycles at 50^0C annealing temperature) (Figure 3.8 A). The *SDH2*-specific primers recognized *SDH2* cDNA from nine *SDH* templates by amplifying a 650 bp amplicon (40 PCR cycles at 55^0C annealing temperature) (Figure 3.8 B). The *SDH3*-specific primers recognized *SDH3* and *SDH5* templates (40 PCR cycles at 55^0C annealing temperature) (Figure 3.8 C). Forty PCR cycles at 55 ^{0}C were performed on *SDH5* and *SDH3* templates using *SDH5*-specific primers (Figure 3.8 D). *SDH5*-specific primers were able to recognize *SDH5* template (not *SDH3*) and amplify the expected 611 bp fragment. Since *SDH5*-specific primers were not capable of recognizing *SDH3* template, and since *SDH5* expression was not evident in seed or cortex tissue, *SDH3*-

specific primers are valid gene-specific primers under the present conditions. The *SDH6*-specific primers should recognize *SDH6* cDNA during 40 PCR cycles (at a 65^0C annealing temperature) and a 283 bp fragment should be amplified but when *SDH6* templates is obtained from genomic DNA then a 367 bp fragment should be amplified. The difference in amplicon sizes is a consequence of the presence of a second intron in the *SDH6* gene. *SDH6* is the only apple SDH gene which posses a second intron. The *SDH6*-specific primers recognized *SDH6* and *SDH5* templates (40 PCR cycles at a 55^0C annealing temperature) amplifying the expected 367 bp and a close-to-expected 283 bp fragment, respectively (Figure 3.8 E). The *SDH6*-specific primers in the presence of *SDH2* and *SDH7* templates also amplified a ~367bp amplicon (higher than the expected size) in seed, only. The *SDH6*-specific primers are a valid gene-specific primer combination based on the following experimental conditions:

1. *SDH5* expression was not found in seed and the *SDH5*-specific primers was not capable of recognizing *SDH6* template but the expected 611 bp fragment was amplified only in the presence of *SDH5* template (Figure 3.8 D).
2. *SDH7* expression was not found in the seed and the *SDH7* primers failed to amplify a band in the presence of *SDH6* template despite being capable of strong amplification when *SDH7* template was present (Figure 3.8 H).
3. *SDH2* expression was not found in seed and the *SDH2*-specific primers did not recognized *SDH6* template despite being capable of a strong amplification when *SDH2* template is present (Figure 3.11 B).

The *SDH9*-specific primers recognized *SDH9* and *SDH5* templates (40 PCR cycles at a 60^0C annealing temperature) by amplification of the expected 235 bp fragment (Figure 3.8 F). Forty PCR cycles at 55 ^{0}C were performed on *SDH5* and *SDH9* templates using *SDH5*-specific primers (Figure 3.8 D). *SDH5*-specific primers recognized *SDH5* template only (not *SDH9* template). Since *SDH5*-specific primers were not capable of recognizing *SDH9* template, and since *SDH5* expression was not found in seed or cortex

tissue, the *SDH9*-specific primer pair is a valid gene-specific primer under these conditions.

The other four primers, designed to recognize *SDH4, SDH5, SDH7* and *SDH8* templates, were capable of recognizing the targeted *SDH* templates (Figure 3.8 G, D, H, I). Since *SDH4*, *SDH5*, *SDH7* and *SDH8* were not expressed in the apple fruit tissues, any possible interactions between those primers and *SDH* templates other than the targeted ones are inconsequential. The *SDH4*-specific primers recognized the *SDH4* template during 40 PCR cycles (at a 60^0C annealing temperature), and the expected 375 bp fragment was amplified (Figure 3.8 H). The *SDH5*-specific primers recognized *SDH5* template by amplifying 611 bp amplicon during 40PCR cycles (at a 55 ^{0}C annealing temperature) (Figure 3.8 D). The *SDH7*-specific primer recognized *SDH7* template during 40 PCR cycles (at a 65^0C annealing temperature), and the expected 283 bp fragment was amplified (Figure 3.8 H). The *SDH8*-specific primer recognized *SDH8* template during 40 PCR cycles (at a 65^0C annealing temperature), and the expected 323 bp fragment was amplified (Figure 3.8 I).

Discussion

SDH expression and activity in the seed and cortex of apple fruit were revealed in this study. Development of apple fruit during the first five weeks after fertilization is correlated with increasing SDH activity per whole fruit in all tissues (Figure 3.5) and can be explained by the constant increase in the biomass of all tissues of apple fruit (Figure 3.3 A-B). The small decline in SDH activity per fruit around the fourth week could be explained by the slight decrease in extractable protein content per fruit (Figure 3.4 A-C) coincident with slower FW accumulation (Figure 3.3 A-C). SDH activity in the whole fruit represents a composite of SDH activities extracted from both seed and cortex tissues. Despite the low total quantity of seed extractable protein (Figure 3.4 A-C) and seed fresh weight (Figure 3.3 A-C) relative to total fruit mass, seed SDH activity contributed significantly (up to 30%), to total SDH activity per fruit, peaking during the 4^{th} and 5^{th} weeks (Figure 3.5 A-B).

Northern blot (Figure 3.6) results indicated the presence of greater amounts of *SDH* transcript in seed than in cortex per µg total RNA, which is correlated to the finding that seed SDH activity per mg protein was several-fold greater than in the cortex. This

high level of SDH transcript in seed might be due to more *SDH* genes being expressed (Figure 3.7) in seed than in cortex (4 versus 3, respectively). There is also a possibility that high seed SDH activity could be related to catalytic differences between SDH isomers since two of them (SDH6, SDH9) are seed-specific (Table 3.2). Posttranslational modification of seed SDH protein may also be considered as a possible factor affecting high seed SDH activity. Western blot detected SDH protein in seed to a greater extent (greater signal strength) than in the the cortex, possibly signifying that the SDH transcripts in seeds produce more total or longer-lasting protein amounts.

Though seed extractable protein contributed only about 5% to whole fruit protein content (Figure 3.4 A-C), seed SDH activity contributed 30% of total fruit SDH activity (Figure 3.5 A-B). Since our Northern analysis (Figure 3.6) and RT-PCR data (Figure 3.7) did not distinguish between weekly samples but were a composite of all four weeks together, we cannot associate this high level of seed SDH activity per mg protein can not be directly associated with the high expression level of specific *SDH* genes at any particular time.

The much lower intensity of the signal for *SDH* transcript in cortex than seed observed in the Northern blot (Figure 3.6) suggests that levels of *SDH* mRNA were lower in cortex This could be related to a low transcriptional rate, low stability of *SDH* transcript, or fewer *SDH* genes being expressed in cortex then in seed, all of which would be consistent with the significantly lower SDH activity level per mg protein (Figure 3.1) in cortex than in seed during early fruit development.

The patterns of cortex SDH and whole fruit SDH activity per mg protein (Figure 3.1) are similar with slightly higher values for whole fruit. This similarity can be explained by the overwhelmingly high cortex biomass contribution to the total whole fruit biomass (around 90%) during the first five weeks of fruit development (Figure 3.3 A-C). The variation of cortex and whole fruit SDH activity per mg protein over time were small (Figure 3.1). The pattern of whole fruit SDH activity per mg protein agrees with the previously-reported whole fruit SDH activity pattern for ‘Red Delicious’ (Nosarzewski et al., 2004), though we have since determined that the previous data on SDH activity was generally lower due to the use of DTT in the extraction buffer. DTT is known to be an SDH inhibitor (El-Kabbani et al., 2004).

The overall lower than seed and constant level of cortex SDH activity per mg protein (Figure 3.1) during the first weeks of development is curious given the relatively high need for carbohydrate during this period. Perhaps this low level of cortex SDH activity per g FW (Figure 3.2B) is sufficient for providing substrate compounds during cortex cell division and early cell expansion process during these first weeks of fruit development since, even without considering seed SDH activity, total cortex SDH activity per fruit increased. It is also possible that at this time the other sorbitol metabolizing enzyme, sorbitol oxidase, which has not been studied in this early period for apple, assumes an important role. As well, sucrose or other sugars may also be important for sustaining fruit growth in this period. Acid invertase utilizes sucrose as a substrate, and it was active during all developmental stages in apple fruit starting from 10 days after full bloom (Zhang et al., 2001). In peach fruit, another Rosaceae species with sorbitol as a main translocated carbohydrate, acid invertase was found around the third week after bloom, reached a peak in the fourth week, rapidly dropped to negligible levels, and was again detected in the ninth week after bloom (Lo Bianco et al, 1999)

Different *SDH* transcripts found by RT-PCR (Figure 3.7) may be associated with SDH isomers detected by 2D-PAGE analysis. *SDH1* and *SDH3* were expressed in both seed and cortex tissues. Similarly, two of the SDH isomers, one with a pI value of 5.5 and another with a pI value of 6.3 were also found by 2D-PAGE analysis in both seed and cortex tissues (Table 3.1). It is possible that those two proteins could be a product of the *SDH1* and *SDH3* genes, as the predicted pI value by Protein Modification Screening Tool (http://proteomics.mcw.edu/promost) for SDH1 is 6.2. Since the *SDH3* sequence is only partially known, we can only speculate that if that isomer with a pI of 6.3 is in fact a product of *SDH1* then the isomer with a pI value of 5.5 would be a product of *SDH3*.

The two other SDH isomers found in the cortex have higher pI values (7.3 and 8.3) than the two remaining isomers found in the seed (pI's of 4.2 and 4.8). Possibly, the isomer with a pI of 7.3 found only in the cortex could be a product of *SDH2* since the predicted pI value according to Protein Modification Screening Tool for SDH2 is 7.0. The two SDH isomers (pI values 4.2 and 4.8) found only in the seed could be a products of SDH6 or SDH9 though the correspondence between the individual isomer and individual gene is undetermined at this time. More SDH isomers than expressed SDH

genes, 4 versus 3, respectively, were found in the cortex. This may be the result of posttranslational modifications altering the predicted pIs, an artifact of 2D analysis, or an indication that not all *SDH* genes have yet been discovered.

Our Northern analysis, RT-PCR and 2D gel results appear contradict the findings by Park et al. (2001), since in their work none of their four *SDH* genes gound in our work (*SDH1, SDH2, SDH3, SDH4*) were expressed in apple fruit tissue during early development from 4 to 8 weeks AFB. However, Park et al. found expression of four *SDH* genes during the later period of fruit development (from ~12 to ~20 weeks after bloom) where the presence of *SDH1, SDH2, SDH3* and *SDH4* transcripts were detected in apple fruit. Additionally, they found that *SDH2, SDH3* and *SDH4* transcripts were limited to young leaves while *SDH1* transcript was found in both young and old leaves.

In conclusion, the present work is the first to show that *SDH* was expressed and was active in both seed and cortex tissues of apple fruit during weeks 2-5 of fruit development and that *SDH* genes were expressed in a tissue-specific manner during the fruit set period. Five of nine *SDH* genes were expressed in apple fruit, four in seed and three in cortex. The significantly higher level of SDH activity, combined with greater *SDH* transcript levels and more *SDH* genes being expressed, in seeds than in cortex tissues of apple suggest that SDH plays an important role in both apple seed and cortex development. It is probable that SDH is a critical enzyme responsible for carbohydrate metabolism during seed and cortex development in apple during early fruit development.

Table 3.1. Gene specific primers, annealing temperatures and expected amplicon sizes.

Gene-specific primer	Forward (F) and reverse (R) primers	Annealing temperature	Expected amplicon size bp
SDH1	F 5'-CTCCAAATAATGGTTGTC-3' R 5'-CAACTAACGTTTCTCAGAA-3'	50^0C	281
SDH2	F 5'-GCATCAGCTGCGCACATT-3' R 5'-CAAAAACTCAAGGCAAAGC-3'	55^0C	650
SDH3	F 5'-ACGTGAAGCATCTGGTTT-3' R 5'-TCCCAGAGACTTGGCCAC-3'	55^0C	407
SDH4	F 5'-TCGGCGAGCCAATGTTGA-3' R 5'-GCAGCGGGAGTCAGAGGC-3'	60^0C	375
SDH5	F 5'-AGGCCGCTACAATCTCTGT-3' R 5'-CGAGGAGTTCCAAGCTT-3'	55^0C	611
SDH6	F 5'-TCAGCAGTGCAAAGGAGGGCAG-3' R 5'-CCCGAAAGCAAGAGCGGCCAAA-3	65^0C	283 (or 367with second intron)
SDH7	F 5'-CGTCAGCAGTGCAAAGGAGGGCAG-3' R 5'-TCCGAAAGCACGAGCAGCCAGC-3'	65^0C	283
SDH8	F 5'-TTGCTTTAAATTGCCAA-3' R 5'-TTTCCAGTGTAGGTGG-3'	50^0C	323
SDH9	F 5'-AAGTTTTCGCCACCCCC-3' R 5'-CCCGAAAGCACGAGCGGT-3'	60^0C	235

Table 3.2. SDH isomers in seed and cortex of 'Red Delicious' apple during fruit development in weeks two to five AFB.

Estimated pI	Tissue where isomer was found	
	seed	cortex
4.2	X	-
4.8	X	-
5.5	X	X
6.3	X	X
7.3	-	X
8.3	-	X

Table 3.3. *SDH* genes expressed in seed and cortex of 'Redchief Delicious' apple during fruit development in weeks two to five AFB.

Gene	Tissue where gene was expressed.	
	Seed	Cortex
SDH1	**X**	**X**
SDH2	**-**	**X**
SDH3	**X**	**X**
SDH6	**X**	**-**
SDH9	**X**	**-**

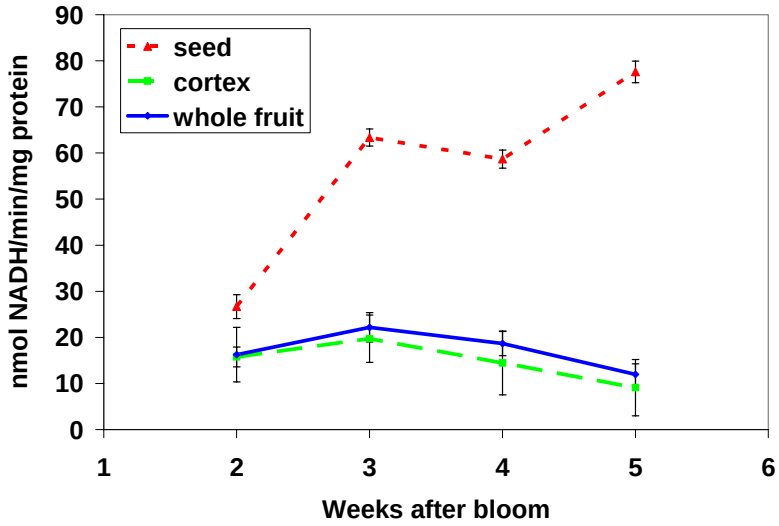

Figure 3.1. SDH activity per mg protein in seed, cortex and whole fruit extracts during fruit development in weeks two to five after bloom of 'Redchief Delicious' apple. Values are the mean ± SE of 5 samples per week across 3 years.

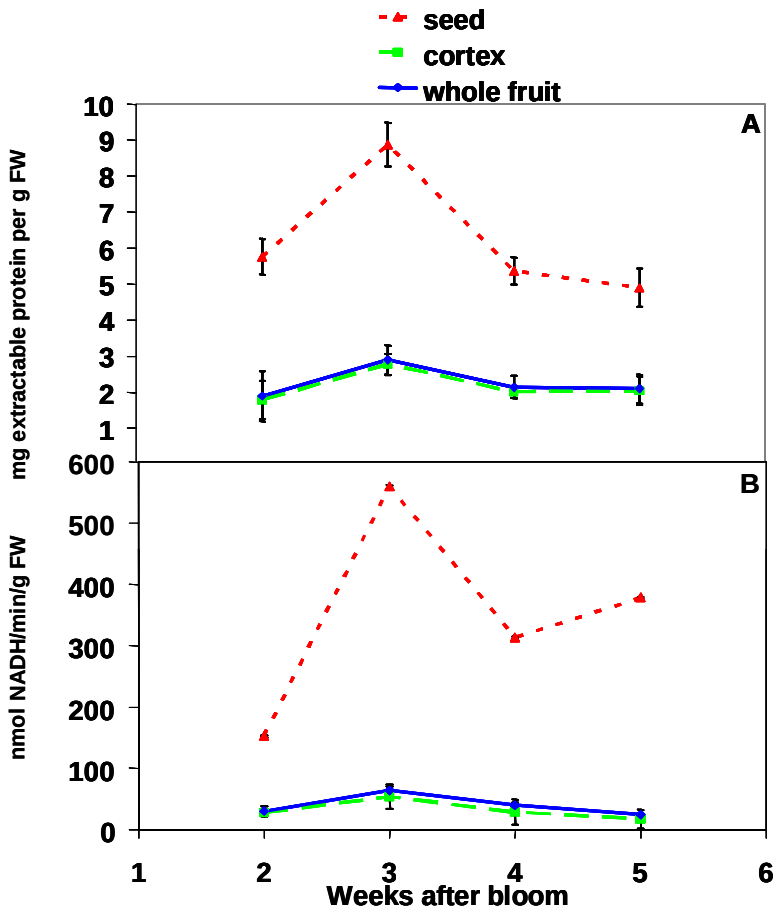

Figure 3.2. A) Extractable protein content per g FW for seed, cortex and whole fruit during fruit development in weeks two to five AFB of 'Redchief Delicious' apple. B) SDH activity per g FW for seed, cortex and whole fruit. Values are the mean ± SE of 5 samples per week across 3 years.

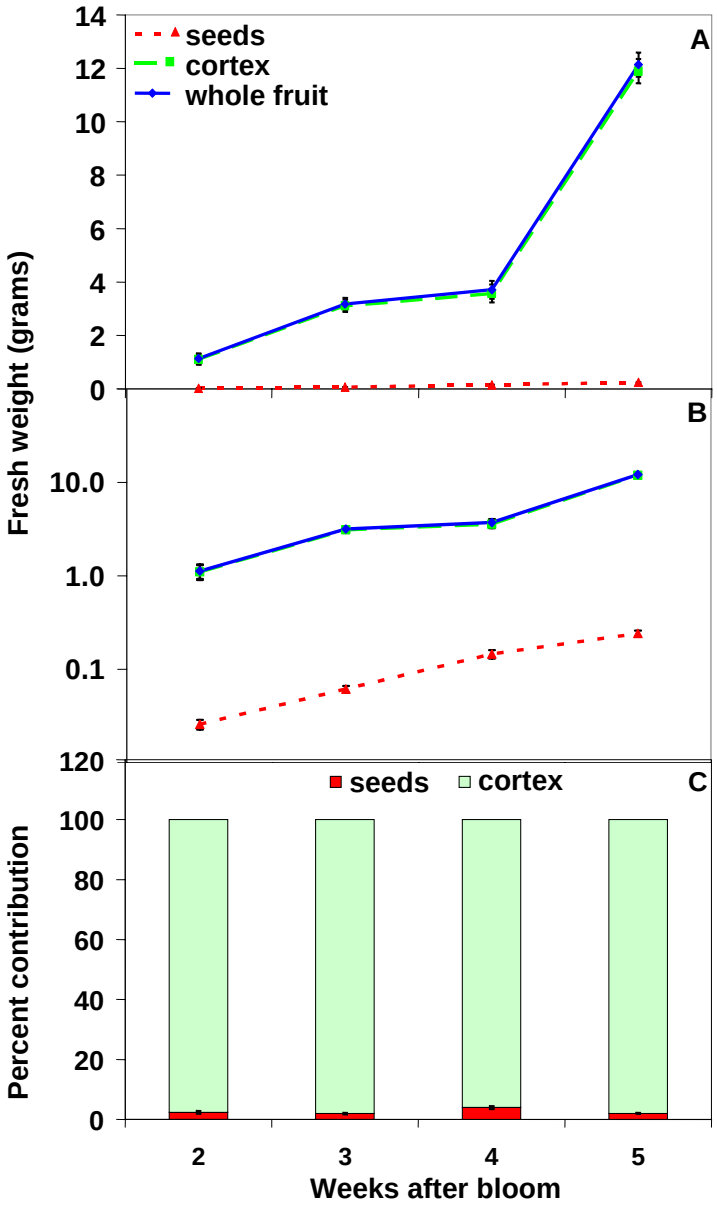

Figure 3.3 Fresh weight accumulation (per fruit) of seeds, cortex or whole fruit of 'Redchief Delicious' apple during fruit development in weeks two to five AFB on a linear scale (A) and a logarithmic (B) scale. C) Seed and cortex contribution (%) to whole fruit fresh weight. Values are the mean ± SE of 5 samples per week across 3 years.

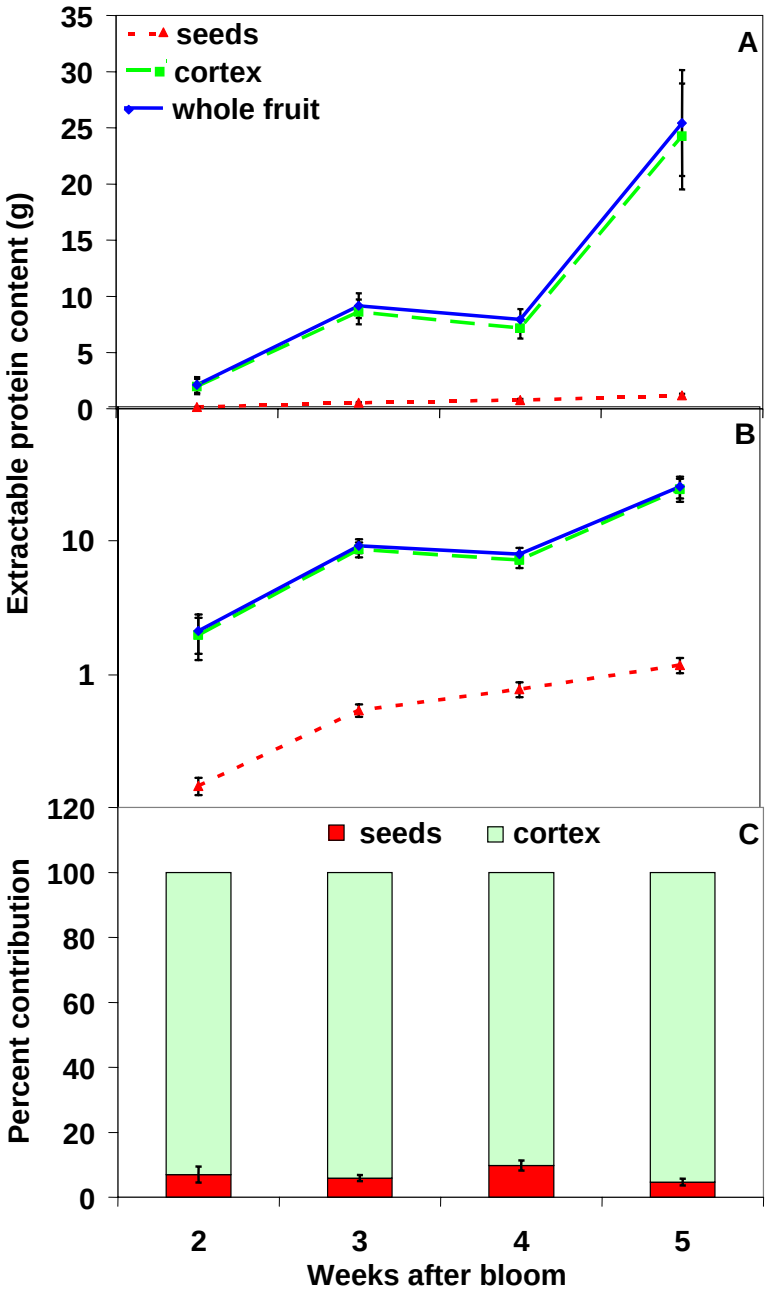

Figure 3.4. Extractable protein content per fruit of seeds, cortex or whole fruit of 'Redchief Delicious' apple during fruit development in weeks two to five AFB on a linear scale (A) and a logarithmic (B) scale. C) Seed and cortex contributions to whole fruit extractable protein content.

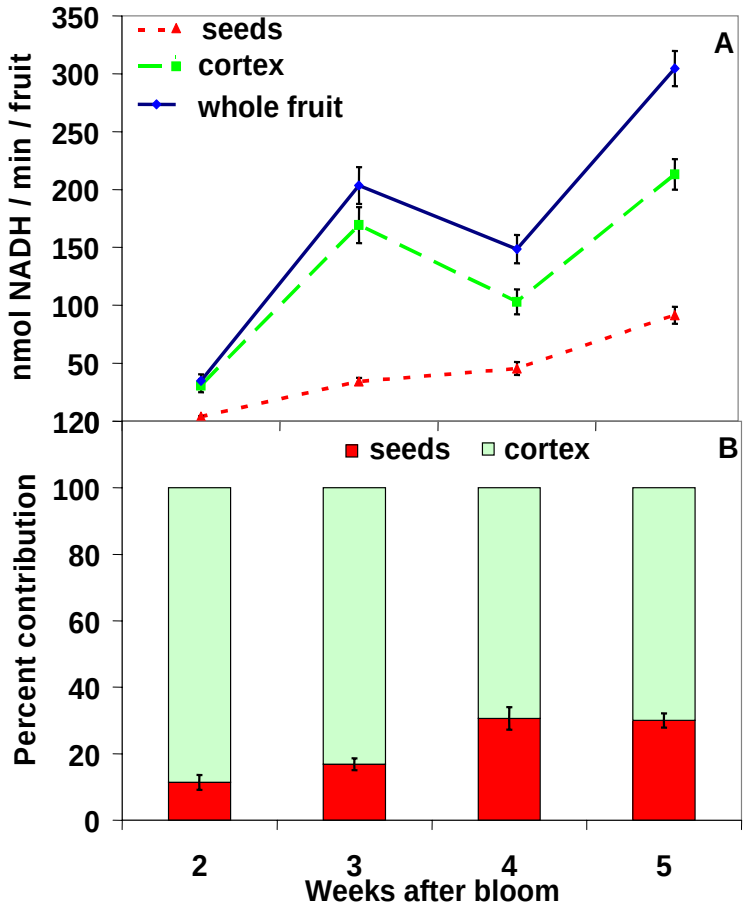

Figure 3.5. A) Whole fruit, cortex and total seed SDH activity per fruit during fruit development in weeks two to five AFB of 'Redchief Delicious' apple. B) Seed and cortex contribution (%) to total SDH activity per fruit.

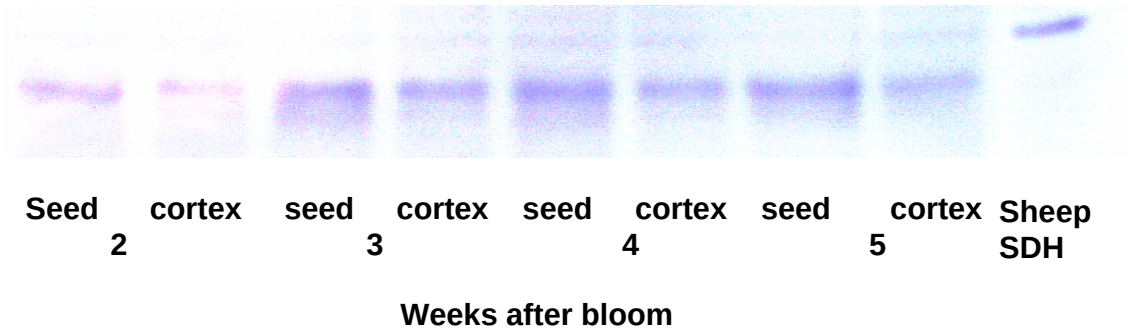

Figure 3.3. Western blot of seed and cortex protein (10 μg per lane) from 'Redchief Delicious' during fruit development in weeks two to five AFB using antiSDH primary antibody. A 37 kDa band (arrow) was consistently present from both seed and cortex tissue over the four weeks examined. Purified sheep SDH (40 kDa) was loaded (9 μg per lane) as a control.

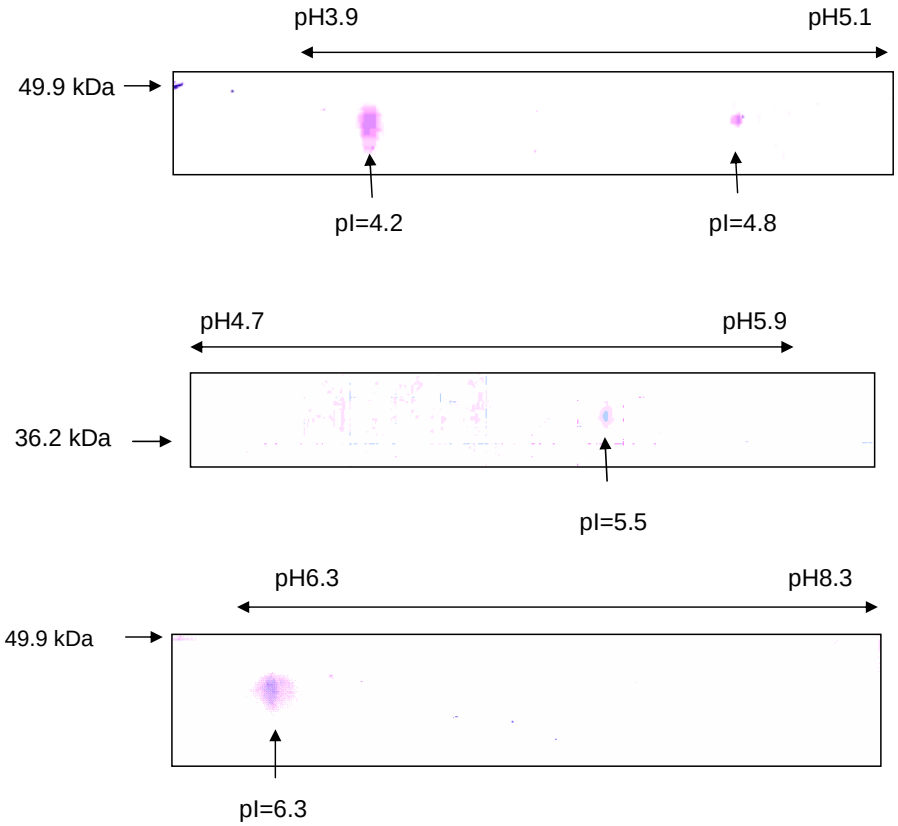

Figure 3.4. Seed SDH isomers of 'Redchief Delicious' apple separated by 2D PAGE and detected by Western blotting.

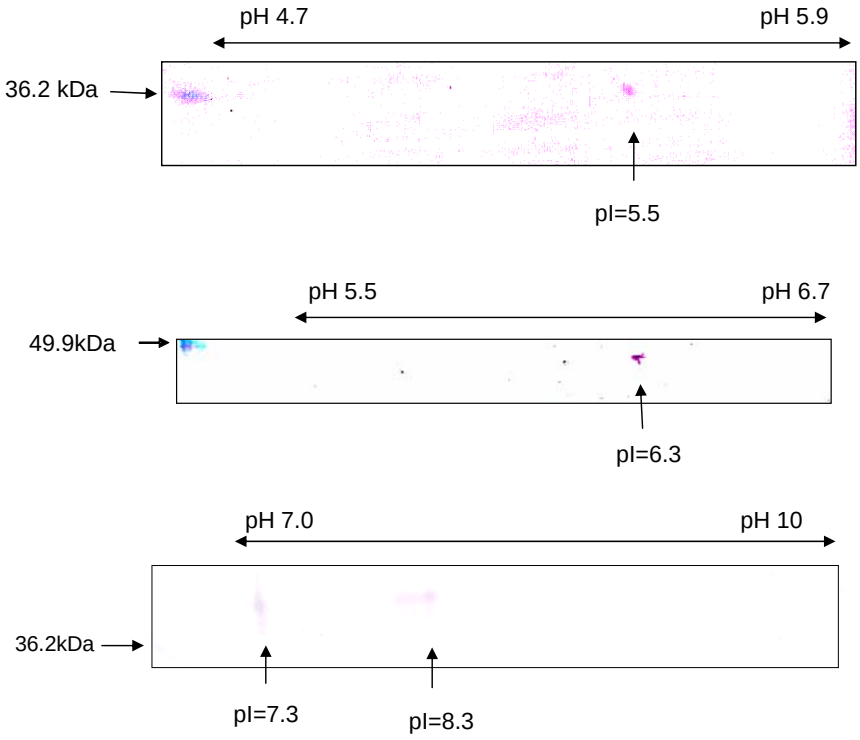

Figure 3.5. Cortex SDH isomers of 'Redchief Delicious' apple separated by 2D PAGE and detected by immunoblotting.

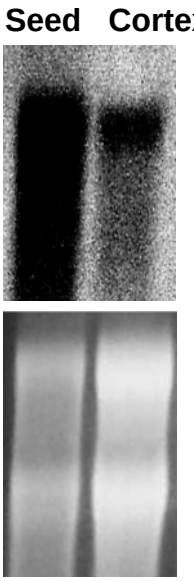

Figure 3.6. Northern blot of an amalgam of *SDH* transcripts from all genes transcribed in seed or cortex of 'Redchief Delicious' apple during fruit development in weeks two to five AFB. The upper panel is the signal obtained from a non-discriminating, DIG-labeled *SDH* probe used to challenge a blot of apple fruit total RNA (10 μg lane^{-1}). The lower panel is a photograph of the ethidium bromide-stained, denaturing RNA gel prior to RNA transfer to assess the equality of the RNA loading.

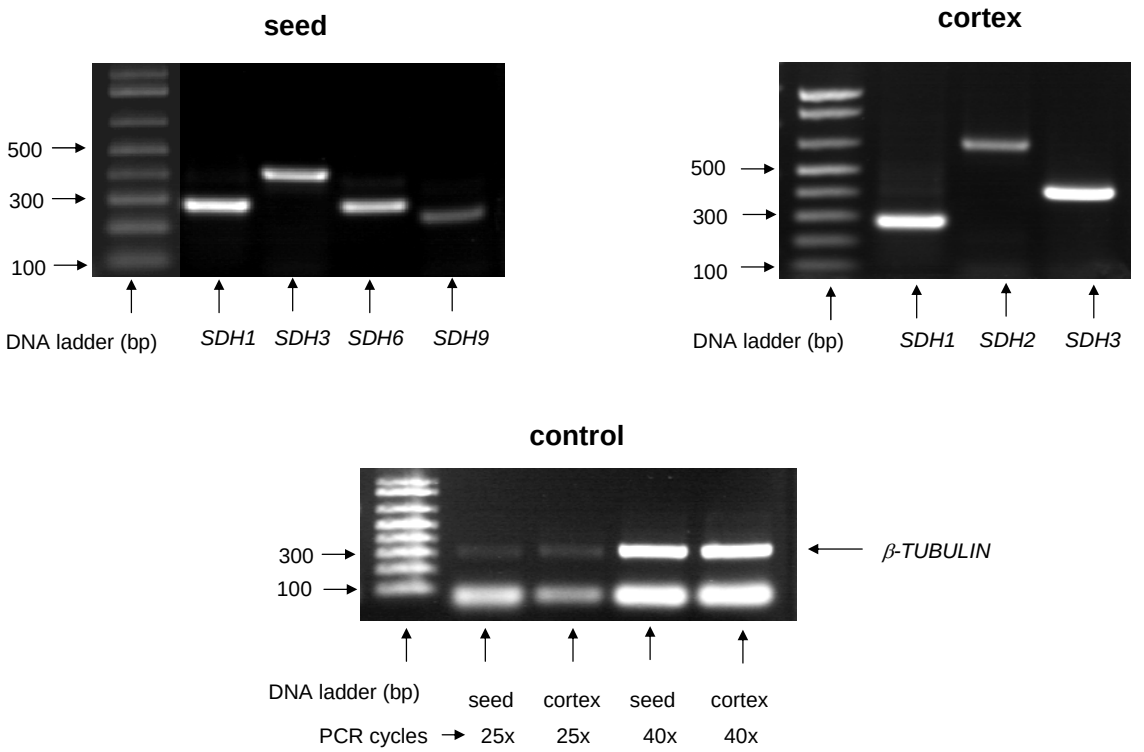

Figure 3.7. RT-PCR analysis of RNA transcripts extracted from seed and cortex tissue of 'Mutsu' apple during early fruit development. The RT-PCR products were obtained at 40 reaction cycles using gene-specific primers for *SDH* cDNAs. -*TUBULIN* transcripts were amplified at 25 and 40 reaction cycles to show similar cDNA abundance in seed and cortex RT-PCR reactions used for *SDH* cDNA detection.

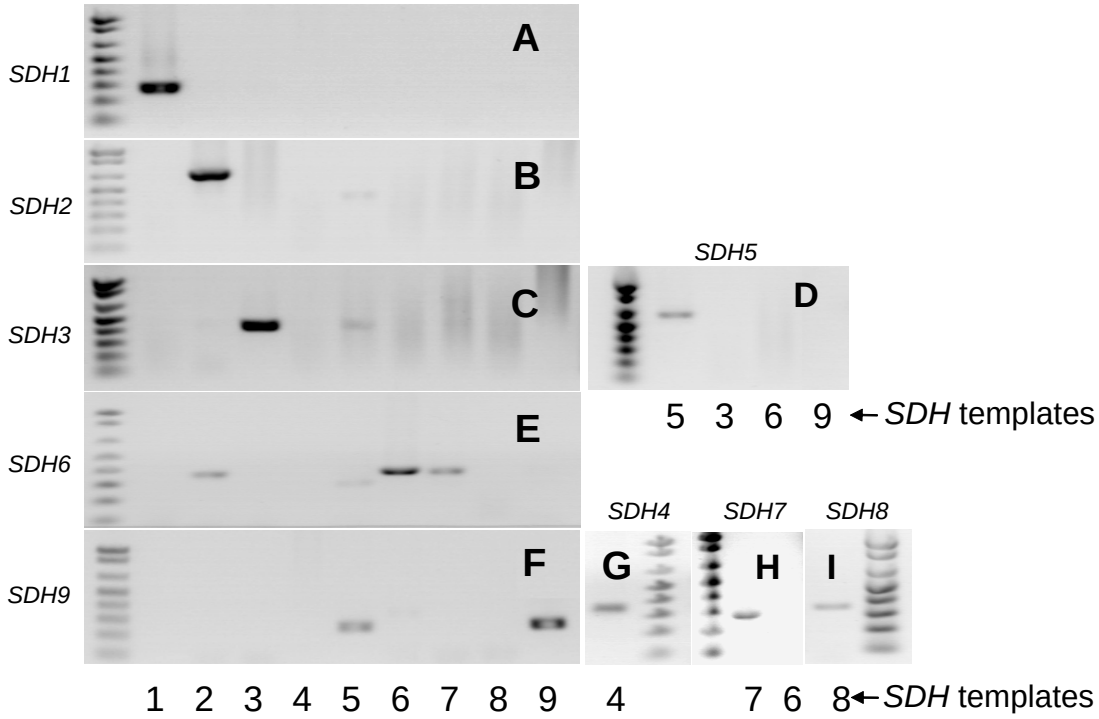

Figure 3.8. Determination of the specificity of RT-PCR primers. Five pg of each of nine different *SDH* templates for every 40 PCR cycle was used in the presence of the following: A) *SDH1*-specific primers, B) *SDH2*-specific primers, C) *SDH3*-specific primers, D) *SDH5*-specific primers, E) *SDH6*-specific primers, F) *SDH9*-specific primers G) *SDH4*-specific primers, H) *SDH7*-specific primers, I) *SDH8*-specific primers. See Table 3.1 for annealing temperatures used for each reaction. 1 Kb DNA plus ladder (GibcoBRL) was used as MWM (bands represent sizes of: 100bp, 200bp, 300bp, 400bp, 500bp, 650bp, 850bp, 1000bp).

Appendix A

Nucleic acid CLUSTAL W alignment for *SDH2, SDH3, SDH5, SDH6, SDH7* and *SDH8* listed as 2, 3, 5, 6, 7 and 8 respectively in the left hand column (introns in red):

```
2               AGATCCTACCTTTCAAGCTCCCCGCTATTGGTATGT-ACTTATCTCCCTACTTTT-ATTT 58
7               AGATCCTACCTTTCAAGCTCCCCGCTATTGGTATTT-ACTTCTCTCCCTTCTTTT-AAAT 58
3               AGATCCTACCTTTCAAGCTCCCCGCTATTGGTATTTTACTTATCTCCCTACTTTTTAATT 60
6               AGATCCTACCTTTCAAGCTCCCCGCTATTGGTATTT-ACTTCTCTCCCTTCTTTT-AAAT 58
5               AGATCCTACCTTTCAAGCTCCCCGCTATTGGTAATA-ATCTCTCTCCTT---TTT-AATT 55
8               AGATCCTACCTTTCAAGCTCCCCGCTATTGGTATGT-ATCTCTCTCCTTGTTCTT-AATT 58
                ********************************    *  * ***** *    ** *  *

2               TCATCGCTAGTTTTTTT--TTTTCGGTTTTTGATTATCAGTTTTCAAGTTTCGTTGAAGT 116
7               TCATCGTTA----TTTT--TTTTCGGTTTTTGGTTTTCA----------CCGTTGTAGT 101
3               TCATCGCTAA---TTTT--TTTTTCCTTTTTATTTTTTTATTTTCAGTTTTCATGGATGT 115
6               TCATCGTTA----TTTT--TTTTCCGTTTTTGGTTTTCA----------CCGTTGTAGT 101
5               TCGTTGTTGTTCTGTTCGGTTTTCAGTTCCA-GTTTGCAGTTCTATATTTTTTTGGTTTT 114
8               CCGTCCTTCTTTCCTTCGGTTTTCAATTTTTTGTTTTTAATTCTCGGTTTTCTTAGTTGT 118
                 * *   *      **   ****   **     **                  * *   *

2               ATTGGC--------TAACATCTTGCTCTCCTGTT-TATTGGGATTTCTTAATTTTAGGAC 167
7               GATG-C--------TAACATGCTGCTCCCCTGTTCTCTTGGGATTTCTTAATTTTAGGAC 152
3               ATTGAC--------TAACATGTTGCTC-CCTGTTTTGTTTGGATTTCTTAATTT-AGGAC 165
6               GATG-C--------TAACATGCTGCTCCCCTGTTCTCTTGGGATTTCTTAATTTTAGGAC 152
5               CTTGGATGTATAGCTAACTTGTTTTTCCCCTATTTTGTTGGGATTTCTTTATTTTAGGAC 174
8               ATTGAT------GCTAACGTCTTGCTCCCCTGTTTTGTTGGGATTTCTTAATTTTAGGAC 172
                  **          **** *  *  ** *** ** * ** ********* **** *****

2               CGAATGATGTTCGAATTCGGATTAAGGCTGTTGGCATTTGCGGAAGTGATATTCACTACC 227
7               CCAATGATGTCCGGATTCGGATTAAGGCGGTTGGTATTTGTGGAAGCGATGTTCACTACC 212
3               TCAATGATGTTCAAATTCGGATTAAGGCTGTCGGCATTTGTGGAAGCGATGTTCACTACC 225
6               CCAATGATGTCCGGATTCGGATTAAGGCGGTTGGTATTTGTGGAAGCGATGTTCACTACC 212
5               CGAATGATGTTCAAATTCGAATTAAGGCTGTCGGCATTTGCGGAAGTGATGTTCACTACC 234
8               CCAATGATGTTCGAATTCGGATCAAGGCTGTCGGCATTTGTGGAAGTGATGTTCACTACC 232
                  ******** *  ***** ** ***** ** ** ***** ***** *** *********

2               TCAAGACCATGAAATGTGGGGATTTTCAGGTTAAGGATCCGATGGTGATCGGACATGAGT 287
7               TCAGGACCATGAAATGTGCGGATTTTGAGGTTAAAGAACCGATGGTGATCGGACATGAGT 272
3               TCAAGAACATGAAACTGGCGGATTTTGAGGTGAAAGAACCAATGGTGATCGGACATGAGT 285
6               TCAGGACCATGAAATGTGCGGATTTTGAGGTTAAAGAACCGATGGTGATCGGACATGAGT 272
5               TCAAGACCATGAAATGTGCGGATTTTGAGGTTAAAGAGCCGATGGTGATCGGACATGAGT 294
8               TCAAGACCATGAAATGCGCGGATTTTGAGGTTAAAGAGCCAATGGTAATCGGACATGAGT 292
                *** ** *******   * ******* **** ** ** ** ***** *************

2               GTGCTGGGATCGTAGACAAAGTTGGGAGCGAGGTGAAGCATCTGGTGCCTGGTGACCGTG 347
7               GTGCTGGGATCGTAGACAAAGTTGGGAGCGAGGTGAAGCATCTGGTGCCTGGTGACCGGG 332
3               GTGCTGGGATCGTAGAAAAAGTTGGGAGCGACGTGAAGCATCTGGTTTCTGGTGACCGCG 345
6               GTGCTGGGATCGTAGACAAAGTTGGGAGCGAGGTGAAGCATCTGGTGCCTGGTGACCGGG 332
5               GTGCTGGGATCGTAGACAAAGTTGGGAGCGAGGTGAAGCATCTGGTGCCTGGTGACCGAG 354
8               GTGCCGGGATCGTAGACAAAGTTGGGAGCGAGGTGAAGCATTTGGTGCCTGGTGACCGCG 352
                **** *********** ************** ********* ****  ********** *

2               TGGCTGTTGAGCCCGGCATCAGCTGCGCACATTGCCAGCAGTGCAAGGGCGGCCGCTACA 407
7               TGGCGGTTGAGCCCGGTATCAGTTGCTCACGGCGTCAGCAGTGCAAAGGAGGGCAGTACA 392
3               TGGCTGTAGAGCCCGGCATCAGCTGCTCACGGTGCCAGCAGTGCAAAGGAGGCCGCTACA 405
6               TGGCGGTTGAGCCCGGTATCAGTTGCTCACGGTGTCAGCAGTGCAAAGGAGGGCAGTACA 392
5               TGGCGGTTGAGCCCGGCATCAGCTGCGCACATTGCCAGCAGTGCAAAGGAGGCCGCTACA 414
8               TGGCGGTAGAGCCTGGCATCAGCTGCGCACGGTGCCAGCAGTGCAAAGGAGGCCGCTATA 412
                **** ** ***** ** ***** *** ***   * *********** ** ** *  ** *
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2              ATCTATGCCCTGACATGAAGTTTTTCGCCACCCCACCTGTTCATGGTTCATTGGCGAATC 467
7              ATCTTTGCCCCGACATGAAGTTTTTCGCCACCCCACCGGTTCATGGTTCATTGGCGAATC 452
3              ATCTCTGCCCCGACATGAAGTTTTTCGCCACCCCACCGGTTCATGGTTCCTTGGCTAACC 465
6              ATCTTTGCCCCGACATGAAGTTTTTCGCCACCCCACCGGTTCATGGTTCCTTGGCAAATC 452
5              ATCTCTGTCCCGACATGAAGTTTTTTGCCACCCCACCGGTTCACGGTGCCTTGGCTAATC 474
8              ATCTTTGCCCTGATATGAAGTTTTTCGCCACCCCACCGGTTCATGGTTCCTTGGCTAATC 472
               **** ** ** ** ********** ********** ***** *** * ***** ** *

2              AG---------------------------------------------------------- 469
7              AG---------------------------------------------------------- 454
3              AG---------------------------------------------------------- 467
6              AGGTATAAATTTCGACTTTCTTAACCTTTGTGACATATGAATTCTATGGCATCAGGACCG 512
5              AG---------------------------------------------------------- 476
8              AG---------------------------------------------------------- 474
               **

2              ---------------------------ATTGTGCACCCCGCGGATCTGTGCTTTAAATTG 502
7              ---------------------------ATTGTGCACCCCGCGGATCTGTGCTTTAAATTG 487
3              ---------------------------ATTGTGCACCCTGCAGATCTGTGCTTTAAACTG 500
6              TAAATAATGACCATCTGTTTTTAACAGATTGTGCATCCTGCGGATCTATGCTTCAAGCTG 572
5              ---------------------------ATTGTGCACCCTGCAGATCTGTGCTTTAAGTTG 509
8              ---------------------------ATTGTGCACCCTGCAGATCTTTGCTTTAAATTG 507
                                          ******** ** ** ***** ***** **  **

2              CCGGAAAATGTGAGCTTGGAAGAAGGGGCAATGTGTGAGCCCTTGAGTGTTGGGGTTCAC 562
7              CCGGAAAATGTGAGCTTGGAAGAAGGGGCAATGTGTGAGCCCTTGAGTGTTGGGGTTCAC 547
3              CCCGAAAACGTGAGCTTGGAGGAAGGAGCAATGTGTGAGCCCTTGAGTGTTGGGGTTCAC 560
6              CCAGAGAATGTGAGCTTGGAGGAAGGGGCAATGTGCGAGCCCTTGAGTGTTGGAGTTCAT 632
5              CCGGAAAATGTGAGCTTGGAGGAAGGGGCAATGTGTGAACCCTTGAGTGTTGGGGTTCAC 569
8              CCAAAAAACGTGAGTTTGGAGGAAGGGGCAATGTGTGAGCCCTTGAGTATTGGGGTTCAC 567
               **  * ** ***** ***** ***** ******** ** ********* **** *****

2              GCTTGTCGGCGAGCCAATGTTGGTCCCGAAACAACTGTTCTGATCGTCGGCGCAGGGCCG 622
7              GCTTGTCGGCGAGCCAATGTTGGTCCCGAAACAACTGTTCTGATCGTCGGCGCAGGGCCG 607
3              GCTTGTCGGCGAGCCAATGTTGGTCCCGAAACAACTGTTCTGATCGTCGGTGCAGGGCCG 620
6              GCATGTCGGCGAGCCAATGTTGGTCCCGAAACAACTGTTCTGATCATCGGAGCAGGGCCT 692
5              GCTTGTCGGCGAGCCAATGTTGGTCCTGAAACAACTGTTCTGATCATCGGCGCAGGGCCT 629
8              GCTTGTCGGCGAGCCAATGTTGGTCCCGAAACAACTGTTCTGATCACCGGAGCAGGGCCT 627
               ** ********************** ******************  *** ********

2              ATCGGGCTGGTTTCCGTGCTGGCTGCTCGTGCTTTCGGAGCACCAAGAATTGTCATCGTA 682
7              ATCGGGCTGGTTTCCGTGCTGGCTGCTCGTGCTTTCGGAGCACCAAGAATTGTCATCGTA 667
3              ATTGGGCTGGTTTCCGTGCTCGCTGCTCGTGCTTTCGGAGCACCAAGAATTGTCATCGTA 680
6              ATTGGTCTGGTTTCAGTTTTGGCCGCTCTTGCTTTCGGGGCACCAAGAATTGTCATTGTG 752
5              ATTGGTCTGGTCTCAGTTTTGGCCGCTCGTGCTTTCGGGGCACCAAGAATTGTTATAGTG 689
8              ATTGGTCTCGTTTCAGTTTTAGCCGCTCGTGCTTTCGGGGCACCAAGAATTGTGATTGTG 687
               ** ** ** ** ** **  * ** **** ********* ************** ** **

2              GATATGGATGACAGGCGTTTAGCCATGGCAAAGTCTCTCGGCGCCGATGGCACGGTCAAA 742
7              GATATGGATGACAGGCGTTTAGCCATGGCAAAGTCTCTCGGCGCCGATGGCACGGTCAAA 727
3              GATATGGATGATAAGCGTTTAGCCGTGGCCAAGTCTCTGGGAGCTGATGGAACCGTCAAA 740
6              GATATGGACGACAAGCGTTTAGCCATGGCAAAGTCTCTCGGCGCTGATGGCACCGTCAAA 812
5              GATATGGATGACAAGCGTCTAGCGATGGCAAAGTCTCTCGGGGCTGATGACACCGTCAAA 749
8              GATATGGATGACAAGCGTCTAGCAATGGCAAAGTCTCTCGGTGCTGATGAAACCGTGAAA 747
               ******** ** * **** ****  **** ******** ** ** ****  ** ** ***

2              GTTTCGACAAAAATGGAGGATTTAGATGACGAAGTTGCCAAGATTAAAGAAGCTATGGGA 802
7              GTTTCGATAAAAATGGAGGATTTAGATGACGAAGTTGCCAAGATTAAAGAAGCTATGGGA 787
3              GTTTCAGCAAAAATGGAGGATTTAGATGACGAAGTTGCCAAGATTAAAGAAACCATGGGA 800
6              GTTTCAACAAAAATGGAGGATTTGGATGACGAAGTTGCCAAGATTAAAGAAGCAATGGAA 872
5              GTTTCGACAAAAATGGAGGATTTAGATGATGAAGTTGCCGAAATTAAAAAAGCCATGATC 809
8              GTTTCGACAAAAATGGAGGACTTAGATGATGAAGTTGCTGAAATTAAAAAAGCAATGGAA 807
               *****   ************ ** ***** ********  * ****** ** * ***
```

```
2               TCCGAAGTTGATGTGACCTTCGACTGTGTTGGCTTCAACAAAACCATGTCTACGGGCCTC 862
7               TCCGAAGTTGATGTGACCTTCGACTGTGTTGGCTTCAACAAAACCATGTCTACGGGCCTC 847
3               GCCGAAGTTGATGTGACCTTCGACTGTGTGGGTTTCAACAAAACCATGTCTACGGGCCTC 860
6               TCGGAGGTGGATGTGACCTTTGATTGTGTGGGTTTTAATAAAACCATGTCGACGGGTCTC 932
5               TCCGAAGTGGATGTGACCTTCGATTGTGTGGGTTTCAACAAAACCATGGCGACCGGCCTC 869
8               TCGGAGGTGGATGTGACCTTTGATTGTGTGGGTTTTAATAAAACCATGTCGACGGGTCTC 867
                 * ** ** ********** ** ***** ** ** ** ********* * ** ** ***

2               AATGCCACTCGTCCTGGCGGCAAAGTTTGCCTTGTCGGAATGGGACACGGGGTGATGACA 922
7               AATGCCACTCGTCCTGGCGGCAAAGTCTGCCTTGTCGGAATGGGACACGGGGTGATGACA 907
3               AATGCTACTCGTCCCGGCGGCAAAGTTTGCCTTGTAGGAATGGGGCACAGCATGATGACA 920
6               AACGCCACACGCCCCGGCGGCAAAGTCTGCCTTGTCGGAATGGGACACGGCATGATGACA 992
5               AATGCTACTCGTCCTGGCGGAAAAGTCTGCCTTGTCGGAATGGGACACGGCCTGATGACA 929
8               AACGCCACACGCCCCGGCGGCAAAGTCTGCCTTGTCGGAATGGGACACGGCATGATGACA 927
                ** ** ** ** ** ***** ***** ******** ******** *** *  ********

2               GTCCCTCTCACTCCGGCTGCTGCCAGGGAGGTTGACGTGGTTGGAGTTTTTCGTTACAAG 982
7               GTCCCTCTCACTCCGGCTGCTGCCAGGGAGGTTGACGTGGTTGGAGTTTTTCGTTACAAG 967
3               GTCCCTCTGACACCGGCTGCAGCCAGGGAGGTTGACGTGGTTGGAGTTTTCCGGTATAAG 980
6               GTGCCTCTCACTCCAGCTGCTGCCAGGGAGGTTGATGTGGTTGGTGTTTTCCGGTGCAAG 1052
5               GTGCCTCTCACCCCTGCTGCTGCTAGGGAGGTCGACGTTGTTGGAGTTTTCAGATACAAG 989
8               GTGCCTCTCACTCCAGCTGCTGCCAGGGAGGTTGATGTGGTTGGTGTTTTCCGGTACAAG 987
                ** ***** ** ** ***** ** ******** ** ** ***** *****  * *  ***

2               AACACATGGCCGCTTTGCCTTGAGTTTTTGAGAAGCGGGAAGATCGACGTGAAGCCGCTT 1042
7               AACACATGGCCGCTTTGCCTTGAGTTTTTGAGAAGCGGGAAGATCGACGTGAAGCCGCTT 1027
3               AACACATGGCCTCTTTGCCTTGAGTTTTTGAGAAGCGGGAAGATCGACGTGAAGCCGCTT 1040
6               AACACATGGCCACTTTGCCTCGAGTTTTTGAGAAGTGGGAAGATCGACGTGAAGCCGCTT 1112
5               AACACATG---------------------------------------------------- 997
8               AACACATGGCCACTTTGCCTCGAGTTTTTGAGAAGTGGGAAGATCGACGTGAAACCGCTT 1047
                ********

2               ATTACCCACCGGTTTGGTTTTACCGAGAAGGAGGTGGAAGAAGC---TTTGGAACCAGTG 1099
7               ATTACCCACCGGTTTGGTTTTACCGAGAAGGAGGTGGAAGAAGCAAGCTTGGAATTCT-- 1085
3               ATTACCCACCGGTTTGGATTTACCGAGAAGGAGGTGGAAGAAGCAAGCTTGGAATTCT-- 1098
6               ATTACTCACCGGTTTGGATTTACCGAGAAGGAGGTGGAAGAAGCAAGCTTG--------- 1163
5               ------------------------------------------------------------
8               ATCACGCACCGTTTTGGATTCACGGAGAAGGAGGTGGAAGAAGCAAGCTTGGAATTC--- 1104


2               CTC 1102
7               ---
3               ---
6               ---
5               ---
8               ---
```

Appendix B

Protein CLUSTAL W aligment of apple SDH isomers. Sequences shown on the right are encoded by the *SDH* gene shown in the left hand column (e.g. a 6 in the left hand column indicates that the sequence has been encoded by *SDH6*). Zinc-containing alcohol dehydrogenase signature sequences are highlighted and possible sites susceptible for phosphorylation of serine, threonine, and tyrosine are in different colors.

```
6               ----------------------------------------ILPFKLPAIGPNDVRIRIKA 20
7               ----------------------------------------ILPFKLPAIGPNDVRIRIKA 20
2               MGKGGQSCNGVVRDAK------PVEQENMAAWLVDVNTIKILPFKLPAIGPNDVRIRIKA 54
4               MGKGGQSCNGVVRDAK------PVEQENMAAWLVDVNTIKILPFKLPAIGPNDVRIRIKA 54
9               MGKGGQSCNGMVRQAK------PVEQENMAAWLVDVNTIKILPFKLPSIGPNDVRIRIKA 54
5               ----------------------------------------ILPFKLPAIGPNDVQIRIKA 20
8               ----------------------------------------ILPFKLPAIGPNDVRIRIKA 20
3               ----------------------------------------ILPFKLPAIGLNDVQIRIKA 20
1               MGKGGMSDGDHADRCYGEAINGDVQQENMAAWLLGVKNLKIQPYKLPNLGPHDVRVRLKA 60
                                                        * *:*** :* :**::*:**

6               VGICGSDVHYLRTMKCADFEVKEPMVIGHECAGIVDKVGSEVKHLVPGDRVAVEPGISCS 80
7               VGICGSDVHYLRTMKCADFEVKEPMVIGHECAGIVDKVGSEVKHLVPGDRVAVEPGISCS 80
2               VGICGSDIHYLKTMKCGDFQVKDPMVIGHECAGIVDKVGSEVKHLVPGDRVAVEPGISCA 114
4               VGICGSDIHYLKTMKCGDFQVKDPMVIGHECAGIVDKVGSEVKHLVPGDRVAVEPGISCA 114
9               VGICGSDVHYLKTMKCADFEVKEPMVIGHECAGIVDKVGSEVKHLVPGDRVAVEPGISCA 114
5               VGICGSDVHYLKTMKCADFEVKEPMVIGHECAGIVDKVGSEVKHLVPGDRVAVEPGISCA 80
8               VGICGSDVHYLKTMKCADFEVKEPMVIGHECAGIVDKVGSEVKHLVPGDRVAVEPGISCA 80
3               VGICGSDVHYLKNMKLADFEVKEPMVIGHECAGIVEKVGSDVKHLVSGDRVAVEPGISCS 80
1               VGICGSDVHHFKNMRCVDFIVKEPMVIGHECAGIIEEVGSEVEDLVPGDRVALEPGISCK 120
                *******:*:::.*:  ** **:***********:::***:*:.**.*****:******

6               RCQQCKGGQYNLCPDMKFFATPPVHGSLANQIVHPADLCFKLPENVSLEEGAMCEPLSVG 140
7               RRQQCKGGQYNLCPDMKFFATPPVHGSLANQIVHPADLCFKLPENVSLEEGAMCEPLSVG 140
2               HCQQCKGGRYNLCPDMKFFATPPVHGSLANQIVHPADLCFKLPENVSLEEGAMCEPLSVG 174
4               RCQQCKGGRYNLCPDMKFFATPPVHGSLANQIVHPADLCFKLPENVSLEEGAMCEPLSVG 174
9               RCQQCKGGRYNLCPDMKFFATPPVHGSLANQIVHPADLCFKLPENVSLEEGAMCEPLSVG 174
5               HCQQCKGGRYNLCPDMKFFATPPVHGALANQIVHPADLCFKLPENVSLEEGAMCEPLSVG 140
8               RCQQCKGGRYNLCPDMKFFATPPVHGSLANQIVHPADLCFKLPKNVSLEEGAMCEPLSIG 140
3               RCQQCKGGRYNLCPDMKFFATPPVHGSLANQIVHPADLCFKLPENVSLEEGAMCEPLSVG 140
1               RCNLCKQGRYNLCRKMKFFGSPPNNGCLANQVVHPGDLCFKLPDNVSLEEGAMCEPLSVG 180
                : : ** *:**** .****.:** :*.****:***.*******.**************:*

6               VHACRRANVGPETTVLIIGAGPIGLVSVLAALAFGAPRIVIVDMDDKRLAMAKSLGADGT 200
7               VHACRRANVGPETTVLIVGAGPIGLVSVLAARAFGAPRIVIVDMDDRRLAMAKSLGADGT 200
2               VHACRRANVGPETTVLIVGAGPIGLVSVLAARAFGAPRIVIVDMDDRRLAMAKSLGADGT 234
4               VHACRRANVDPETTVLIIGAGPIGLVSVLAARAFGAPRIVIVDMDDKRLAMAKSLGADEA 234
9               VHACRRANVGPETTVLIIGAGPIGLVSVLTARAFGAPRIVIVDMDDKRLAMAKSLGADEA 234
5               VHACRRANVGPETTVLIIGAGPIGLVSVLAARAFGAPRIVIVDMDDKRLAMAKSLGADDT 200
8               VHACRRANVGPETTVLITGAGPIGLVSVLAARAFGAPRIVIVDMDDKRLAMAKSLGADET 200
3               VHACRRANVGPETTVLIVGAGPIGLVSVLAARAFGAPRIVIVDMDDKRLAVAKSLGADGT 200
1               IHACRRANVCQETNALVVGAGPIGLVTLLAARAFGAPRIVIADVNDERLLIAKSLGADEV 240
                :********  **..*: ********::*:* *********.*::*.** :******* .
```

```
6               VKVSTKMEDLDDEVAKIKEAMESEVDVTFDCVGFNKTMSTGLNATRPGGKVCLVGMGHGM 260
7               VKVSIKMEDLDDEVAKIKEAMGSEVDVTFDCVGFNKTMSTGLNATRPGGKVCLVGMGHGV 260
2               VKVSTKMEDLDDEVAKIKEAMGSEVDVTFDCVGFNKTMSTGLNATRPGGKVCLVGMGHGV 294
4               VKVSTKMEDLDDEVAEIKEAMISEVDVTFDCVGFNKTVSTGLNATRPGGKVCLVGMGHGV 294
9               VKVSTKMEDLDDEVAEIKKAMISEVDVTFDCVGFNKTMSTGLNATRPGGKVCLVGMGHGV 294
5               VKVSTKMEDLDDEVAEIKKAMISEVDVTFDCVGFNKTMATGLNATRPGGKVCLVGMGHGL 260
8               VKVSTKMEDLDDEVAEIKKAMESEVDVTFDCVGFNKTMSTGLNATRPGGKVCLVGMGHGM 260
3               VKVSAKMEDLDDEVAKIKETMGAEVDVTFDCVGFNKTMSTGLNATRPGGKVCLVGMGHSM 260
1               VKVSTNIEDVAEEVAKIQKVLENGVDVTFDCAGFNKTITTALSATRPGGKVCLVGMGQRE 300
                **** ::**: :***:*::.:   *******.*****::*.*.************:

6               MTVPLTPAAAREVDVVGVFRCKNTWPLCLEFLRSGKIDVKPLITHRFGFTEKEVEEASL- 319
7               MTVPLTPAAAREVDVVGVFRYKNTWPLCLEFLRSGKIDVKPLITHRFGFTEKEVEEASLE 320
2               MTVPLTPAAAREVDVVGVFRYKNTWPLCLEFLRSGKIDVKPLITHRFGFTEKEVEEAFAT 354
4               MTVPLTPAAAREVDVVGVFRYQKTWPLCLEFLRSGKIDVKPLITHRFGFTEKEVEEAFAT 354
9               MTVPLTPAAAREVDVVGVFRYQNTWPLCLEFLRSGKIDVKPLITHRFGFTEKEVEEAFAT 354
5               MTVPLTPAAAREVDVVGVFRYKNT---------------------------------- 284
8               MTVPLTPAAAREVDVVGVFRYKNTWPLCLEFLRSGKIDVKPLITHRFGFTEKEVEEASLE 320
3               MTVPLTPAAAREVDVVGVFRYKNTWPLCLEFLRSGKIDVKPLITHRFGFTEKEVEEASLE 320
1               MTLPL---ATREIDVIGIFRYQNTWPLCLEFLRSGKIDVKPLITHRFGFSQKEVEEAFET 357
                **:**   *:**:**:*:** ::*

6               --------------
7               F------------- 321
2               SARGGNAIKVMFTL 368
4               SARGGNAIKVMFKL 368
9               SARGGNAIKVMFKL 368
5               --------------
8               F------------- 321
3               F------------- 321
1               SARGGNAIKVMFNL 371
```

Appendix C

Nucleic acid CLUSTAL W aligment of apple *SDH* genes. Sequences shown on the right belong to the *SDH* gene shown in the left hand column (e.g. a 6 in the left hand column indicates that the sequence belongs to *SDH6*).

```
4               ------------------ATGGGTAAGGGAGGCCAATCCTGCAATGGCGTGGTTAGAGAC 42
9               ------------------ATGGGCAAGGGAGGCCAATCCTGCAATGGCATGGTTAGACAA 42
5               ------------------------------------------------------------
8               ------------------------------------------------------------
6               ------------------------------------------------------------
2               ---------------AAATGGGTAAGGGAGGCCAATCCTGCAATGGCGTGGTTAGAGAC 44
7               ------------------------------------------------------------
3               ------------------------------------------------------------
1               ATGGGAAAGGGAGGCATGTCTGATGGAGATCATGCTGATCGCTGTTATGGGGAAGCAATA 60


4               GCCAAACCTGTTGAGCAGGAAAACATGGCTGCCTGGCTAGTTGATGTTAACACCATCAAG 102
9               GCCAAACCTGTTGAGCAGGAAAACATGGCTGCTTGGCTTGTTGATGTCAACACCATCAAG 102
5               ------------------------------------------------------------AG 2
8               ------------------------------------------------------------AG 2
6               ------------------------------------------------------------AG 2
2               GCCAAACCTGTTGAGCAGGAAAACATGGCTGCCTGGCTAGTTGATGTTAACACCATCAAG 104
7               ------------------------------------------------------------AG 2
3               ------------------------------------------------------------AG 2
1               AATGGTGATGTTCAACAAGAGAACATGGCTGCTTGGCTTCTTGGTGTTAAAAACCTCAAG 120
                                                                          **


4               ATCCTACCTTTCAAGCTCCCCGCTATTGGACCGAATGATGTTCGAATTCGGATTAAGGCT 162
9               ATCCTACCTTTCAAGCTCCCCAGTATCGGACCCAATGATGTTCGAATTCGGATCAAGGCT 162
5               ATCCTACCTTTCAAGCTCCCCGCTATTGGACCGAATGATGTTCAAATTCGAATTAAGGCT 62
8               ATCCTACCTTTCAAGCTCCCCGCTATTGGACCCAATGATGTTCGAATTCGGATCAAGGCT 62
6               ATCCTACCTTTCAAGCTCCCCGCTATTGGACCCAATGATGTCCGGATTCGGATTAAGGCG 62
2               ATCCTACCTTTCAAGCTCCCCGCTATTGGACCGAATGATGTTCGAATTCGGATTAAGGCT 164
7               ATCCTACCTTTCAAGCTCCCCGCTATTGGACCCAATGATGTCCGGATTCGGATTAAGGCG 62
3               ATCCTACCTTTCAAGCTCCCCGCTATTGGACTCAATGATGTTCAAATTCGGATTAAGGCT 62
1               ATTCAACCTTACAAGCTTCCTAATCTTGGACCCCATGATGTTAGAGTCCGGCTGAAGGCT 180
                ** * ***** ****** **   * * ****   *******     * **  * *****


4               GTTGGCATTTGCGGAAGTGATATTCACTACCTCAAGACCATGAAATGTGGGGATTTTCAG 222
9               GTCGGCATTTGTGGAAGTGATGTTCACTACCTCAAGACCATGAAATGCGCGGATTTTGAG 222
5               GTCGGCATTTGCGGAAGTGATGTTCACTACCTCAAGACCATGAAATGTGCGGATTTTGAG 122
8               GTCGGCATTTGTGGAAGTGATGTTCACTACCTCAAGACCATGAAATGCGCGGATTTTGAG 122
6               GTTGGTATTTGTGGAAGCGATGTTCACTACCTCAGGACCATGAAATGTGCGGATTTTGAG 122
2               GTTGGCATTTGCGGAAGTGATATTCACTACCTCAAGACCATGAAATGTGGGGATTTTCAG 224
7               GTTGGTATTTGTGGAAGCGATGTTCACTACCTCAGGACCATGAAATGTGCGGATTTTGAG 122
3               GTCGGCATTTGTGGAAGCGATGTTCACTACCTCAAGAACATGAAACTGGCGGATTTTGAG 122
1               GTTGGCATATGTGGCAGTGATGTTCACCACTTCAAGAACATGAGGTGTGTAGATTTTATA 240
                ** ** ** ** ** ** *** ***** ** *** ** *****     *  ******


4               GTTAAGGATCCGATGGTGATCGGACATGAGTGTGCTGGGATCGTAGACAAAGTTGGGAGC 282
9               GTTAAAGAGCCAATGGTAATCGGACATGAGTGTGCCGGGATCGTAGACAAAGTTGGGAGC 282
5               GTTAAAGAGCCGATGGTGATCGGACATGAGTGTGCTGGGATCGTAGACAAAGTTGGGAGC 182
8               GTTAAAGAGCCAATGGTAATCGGACATGAGTGTGCCGGGATCGTAGACAAAGTTGGGAGC 182
6               GTTAAAGAACCGATGGTGATCGGACATGAGTGTGCTGGGATCGTAGACAAAGTTGGGAGC 182
2               GTTAAGGATCCGATGGTGATCGGACATGAGTGTGCTGGGATCGTAGACAAAGTTGGGAGC 284
7               GTTAAAGAACCGATGGTGATCGGACATGAGTGTGCTGGGATCGTAGACAAAGTTGGGAGC 182
3               GTGAAAGAACCAATGGTGATCGGACATGAGTGTGCTGGGATCGTAGAAAAAGTTGGGAGC 182
1               GTTAAAGAGCCAATGGTTATTGGGCATGAGTGTGCTGGGATCATAGAGGAAGTTGGGAGT 300
                ** ** ** ** ***** ** ** *********** ****** ****  **********
```

```
4               GAGGTGAAGCATCTGGTGCCTGGTGACCGTGTGGCTGTTGAGCCTGGCATCAGCTGCGCA 342
9               GAGGTGAAGCATTTGGTGCCTGGTGACCGCGTGGCGGTAGAGCCTGGCATCAGCTGCGCA 342
5               GAGGTGAAGCATCTGGTGCCTGGTGACCGAGTGGCGGTTGAGCCCGGCATCAGCTGCGCA 242
8               GAGGTGAAGCATTTGGTGCCTGGTGACCGCGTGGCGGTAGAGCCTGGCATCAGCTGCGCA 242
6               GAGGTGAAGCATCTGGTGCCTGGTGACCGGGTGGCGGTTGAGCCCGGTATCAGTTGCTCA 242
2               GAGGTGAAGCATCTGGTGCCTGGTGACCGTGTGGCTGTTGAGCCCGGCATCAGCTGCGCA 344
7               GAGGTGAAGCATCTGGTGCCTGGTGACCGGGTGGCGGTTGAGCCCGGTATCAGTTGCTCA 242
3               GACGTGAAGCATCTGGTTTCTGGTGACCGCGTGGCTGTAGAGCCCGGCATCAGCTGCTCA 242
1               GAGGTCGAGGATTTGGTGCCAGGCGATCGTGTGGCACTAGAGCCTGGTATCAGTTGCAAG 360
                ** **  ** ** ****  * ** ** ** *****  * ***** ** ***** ***

4               CGGTGCCAGCAGTGCAAGGGAGGCCGCTATAATCTTTGCCCTGATATGAAGTTTTTCGCC 402
9               CGGTGCCAGCAGTGCAAGGGAGGCCGCTATAATCTTTGCCCTGATATGAAGTTTTTCGCC 402
5               CATTGCCAGCAGTGCAAAGGAGGCCGCTACAATCTCTGTCCCGACATGAAGTTTTTTGCC 302
8               CGGTGCCAGCAGTGCAAAGGAGGCCGCTATAATCTTTGCCCTGATATGAAGTTTTTCGCC 302
6               CGGTGTCAGCAGTGCAAAGGAGGGCAGTACAATCTTTGCCCCGACATGAAGTTTTTCGCC 302
2               CATTGCCAGCAGTGCAAGGGCGGCCGCTACAATCTATGCCCTGACATGAAGTTTTTCGCC 404
7               CGGCGTCAGCAGTGCAAAGGAGGGCAGTACAATCTTTGCCCCGACATGAAGTTTTTCGCC 302
3               CGGTGCCAGCAGTGCAAAGGAGGCCGCTACAATCTCTGCCCCGACATGAAGTTTTTCGCC 302
1               CGATGCAACCTCTGCAAACAAGGCCGGTACAATCTATGCCGCAAGATGAAGTTTTTTGGC 420
                *   *  * *  *****    ** *  ** ***** ** *   * ********** * *

4               ACCCCACCGGTTCATGGTTCCTTGGCTAATCAGATTGTGCACCCTGCGGATCTGTGCTTT 462
9               ACCCCCCCGGTTCATGGTTCCTTGGCTAATCAGATTGTGCACCCTGCGGATCTGTGCTTT 462
5               ACCCCACCGGTTCACGGTGCCTTGGCTAATCAGATTGTGCACCCTGCAGATCTGTGCTTT 362
8               ACCCCACCGGTTCATGGTTCCTTGGCTAATCAGATTGTGCACCCTGCAGATCTTTGCTTT 362
6               ACCCCACCGGTTCATGGTTCCTTGGCAAATCAGATTGTGCATCCTGCGGATCTATGCTTC 362
2               ACCCCACCTGTTCATGGTTCATTGGCGAATCAGATTGTGCACCCCGCGGATCTGTGCTTT 464
7               ACCCCACCGGTTCATGGTTCATTGGCGAATCAGATTGTGCACCCCGCGGATCTGTGCTTT 362
3               ACCCCACCGGTTCATGGTTCCTTGGCTAACCAGATTGTGCACCCTGCAGATCTGTGCTTT 362
1               TCCCCTCCAAATAATGGTTGTCTGGCAAATCAGGTTGTCCATCCAGGAGATCTATGTTTT 480
                 **** **   * * ***    **** ** *** **** ** ** *  ***** ** **

4               AAATTGCCGGAAAACGTGAGTTTGGAGGAAGGGGCAATGTGTGAGCCCTTGAGTGTTGGG 522
9               AAATTGCCGGAAAACGTGAGTTTGGAGGAAGGGGCAATGTGTGAGCCCTTGAGTGTTGGG 522
5               AAGTTGCCGGAAAATGTGAGCTTGGAGGAAGGGGCAATGTGTGAACCCTTGAGTGTTGGG 422
8               AAATTGCCAAAAAACGTGAGTTTGGAGGAAGGGGCAATGTGTGAGCCCTTGAGTATTGGG 422
6               AAGCTGCCAGAGAATGTGAGCTTGGAGGAAGGGGCAATGTGCGAGCCCTTGAGTGTTGGA 422
2               AAATTGCCGGAAAATGTGAGCTTGGAAGAAGGGGCAATGTGTGAGCCCTTGAGTGTTGGG 524
7               AAATTGCCGGAAAATGTGAGCTTGGAAGAAGGGGCAATGTGTGAGCCCTTGAGTGTTGGG 422
3               AAACTGCCCGAAAACGTGAGCTTGGAGGAAGGAGCAATGTGTGAGCCCTTGAGTGTTGGG 422
1               AAACTGCCAGACAATGTGAGTTTGGAGGAAGGCGCGATGTGTGAGCCCTTAAGTGTTGGT 540
                **  ****  * ** ***** ***** ***** ** ***** ** ***** *** ****

4               GTTCATGCTTGTCGGCGAGCCAATGTTGATCCCGAGACAACTGTCCTGATCATCGGTGCA 582
9               GTTCACGCTTGTCGGCGAGCCAATGTTGGTCCCGAGACAACTGTCCTGATCATCGGAGCA 582
5               GTTCACGCTTGTCGGCGAGCCAATGTTGGTCCTGAAACAACTGTTCTGATCATCGGCGCA 482
8               GTTCACGCTTGTCGGCGAGCCAATGTTGGTCCCGAAACAACTGTTCTGATCACCGGAGCA 482
6               GTTCATGCATGTCGGCGAGCCAATGTTGGTCCCGAAACAACTGTTCTGATCATCGGAGCA 482
2               GTTCACGCTTGTCGGCGAGCCAATGTTGGTCCCGAAACAACTGTTCTGATCGTCGGCGCA 584
7               GTTCACGCTTGTCGGCGAGCCAATGTTGGTCCCGAAACAACTGTTCTGATCGTCGGCGCA 482
3               GTTCACGCTTGTCGGCGAGCCAATGTTGGTCCCGAAACAACTGTTCTGATCGTCGGTGCA 482
1               ATTCATGCTTGTCGCCGGGCAAATGTCTGTCAAGAAACAAATGCCTTGGTCGTGGGAGCA 600
                 **** ** ***** ** ** *****   **  ** **** **   ** **   ** ***

4               GGGCCTATTGGTCTCGTTTCAGTTTTAGCCGCTCGTGCTTTCGGGGCACCAAGAATTGTG 642
9               GGGCCTATTGGTCTCGTTTCAGTTTTAACCGCTCGTGCTTTCGGGGCACCAAGAATTGTG 642
5               GGGCCTATTGGTCTGGTCTCAGTTTTGGCCGCTCGTGCTTTCGGGGCACCAAGAATTGTT 542
8               GGGCCTATTGGTCTCGTTTCAGTTTTAGCCGCTCGTGCTTTCGGGGCACCAAGAATTGTG 542
6               GGGCCTATTGGTCTGGTTTCAGTTTTGGCCGCTCTTGCTTTCGGGGCACCAAGAATTGTC 542
2               GGGCCGATCGGGCTGGTTTCCGTGCTGGCTGCTCGTGCTTTCGGAGCACCAAGAATTGTC 644
7               GGGCCGATCGGGCTGGTTTCCGTGCTGGCTGCTCGTGCTTTCGGAGCACCAAGAATTGTC 542
3               GGGCCGATTGGGCTGGTTTCCGTGCTCGCTGCTCGTGCTTTCGGAGCACCAAGAATTGTC 542
1               GGACCTATAGGACTTGTTACACTGCTAGCCGCTCGTGCTTTTGGGGCGCCCCGAATTGTC 660
                ** ** ** ** ** **  *  *  *  * **** ****** ** ** **  *******
```

```
4               ATTGTGGATATGGATGACAAGCGTCTAGCAATGGCAAAGTCTCTCGGTGCTGATGAAGCC 702
9               ATTGTGGATATGGATGACAAGCGTCTAGCAATGGCAAAGTCTCTCGGTGCTGATGAAGCC 702
5               ATAGTGGATATGGATGACAAGCGTCTAGCGATGGCAAAGTCTCTCGGGGCTGATGACACC 602
8               ATTGTGGATATGGATGACAAGCGTCTAGCAATGGCAAAGTCTCTCGGTGCTGATGAAACC 602
6               ATTGTGGATATGGACGACAAGCGTTTAGCCATGGCAAAGTCTCTCGGCGCTGATGGCACC 602
2               ATCGTAGATATGGATGACAGGCGTTTAGCCATGGCAAAGTCTCTCGGCGCCGATGGCACG 704
7               ATCGTAGATATGGATGACAGGCGTTTAGCCATGGCAAAGTCTCTCGGCGCCGATGGCACG 602
3               ATCGTAGATATGGATGATAAGCGTTTAGCCGTGGCCAAGTCTCTGGGAGCTGATGGAACC 602
1               ATTGCGGATGTGAATGACGAGCGTTTGTTGATTGCAAAGAGTCTTGGCGCAGATGAAGTC 720
                ** *  *** ** * **   **** *     * ** ***  *** ** ** ****

4               GTGAAAGTTTCGACAAAAATGGAGGATTTAGATGATGAAGTTGCCGAAATTAAAGAAGCC 762
9               GTGAAAGTTTCGACAAAAATGGAGGATTTAGATGATGAAGTTGCCGAAATTAAAAAAGCC 762
5               GTCAAAGTTTCGACAAAAATGGAGGATTTAGATGATGAAGTTGCCGAAATTAAAAAAGCC 662
8               GTGAAAGTTTCGACAAAAATGGAGGACTTAGATGATGAAGTTGCTGAAATTAAAAAAGCA 662
6               GTCAAAGTTTCAACAAAAATGGAGGATTTGGATGACGAAGTTGCCAAGATTAAAGAAGCA 662
2               GTCAAAGTTTCGACAAAAATGGAGGATTTAGATGACGAAGTTGCCAAGATTAAAGAAGCT 764
7               GTCAAAGTTTCGATAAAAATGGAGGATTTAGATGACGAAGTTGCCAAGATTAAAGAAGCT 662
3               GTCAAAGTTTCAGCAAAAATGGAGGATTTAGATGACGAAGTTGCCAAGATTAAAGAAACC 662
1               GTTAAGGTTTCAACAAATATTGAGGATGTAGCTGAAGAAGTGGCTAAGATACAAAAGGTT 780
                ** ** *****   *** ** *****  * * *** ***** **  * **  ** *

4               ATGATCTCCGAAGTGGATGTGACCTTCGATTGTGTGGGTTTCAACAAAACCGTGTCGACC 822
9               ATGATCTCCGAAGTGGATGTGACCTTCGATTGTGTGGGTTTCAACAAAACCATGTCGACC 822
5               ATGATCTCCGAAGTGGATGTGACCTTCGATTGTGTGGGTTTCAACAAAACCATGGCGACC 722
8               ATGGAATCGGAGGTGGATGTGACCTTTGATTGTGTGGGTTTTAATAAAACCATGTCGACG 722
6               ATGGAATCGGAGGTGGATGTGACCTTTGATTGTGTGGGTTTTAATAAAACCATGTCGACG 722
2               ATGGGATCCGAAGTTGATGTGACCTTCGACTGTGTTGGCTTCAACAAAACCATGTCTACG 824
7               ATGGGATCCGAAGTTGATGTGACCTTCGACTGTGTTGGCTTCAACAAAACCATGTCTACG 722
3               ATGGGAGCCGAAGTTGATGTGACCTTCGACTGTGTGGGTTTCAACAAAACCATGTCTACG 722
1               TTGGAAAATGGAGTGGATGTAACCTTCGACTGTGCAGGCTTTAACAAAACCATAACAACA 840
                 **      *  ** ***** ***** ** ****  ** ** ** ****** *  * **

4               GGCCTCAATGCTACTCGTCCCGGCGGAAAAGTCTGCCTTGTAGGAATGGGACACGGCGTG 882
9               GGCCTCAATGCTACTCGTCCCGGCGGAAAAGTCTGCCTTGTAGGAATGGGACACGGTGTG 882
5               GGCCTCAATGCTACTCGTCCTGGCGGAAAAGTCTGCCTTGTCGGAATGGGACACGGCCTG 782
8               GGTCTCAACGCCACACGCCCCGGCGGCAAAGTCTGCCTTGTCGGAATGGGACACGGCATG 782
6               GGTCTCAACGCCACACGCCCCGGCGGCAAAGTCTGCCTTGTCGGAATGGGACACGGCATG 782
2               GGCCTCAATGCCACTCGTCCTGGCGGCAAAGTCTGCCTTGTCGGAATGGGACACGGGGTG 884
7               GGCCTCAATGCCACTCGTCCTGGCGGCAAAGTCTGCCTTGTCGGAATGGGACACGGGGTG 782
3               GGCCTCAATGCTACTCGTCCCGGCGGCAAAGTTTGCCTTGTAGGAATGGGGCACAGCATG 782
1               GCTTTGAGTGCTACTCGTCCCGGAGGCAAAGTTTGCCTTGTGGGAATGGGTCAGAGAGAG 900
                *   * *  ** ** ** ** ** ** ***** ******** ******** **  *   *

4               ATGACCGTGCCTCTGACTCCCGCTGCTGCTAGGGAGGTTGACGTTGTTGGAGTTTTCCGA 942
9               ATGACGGTGCCTCTGACTCCCGCTGCTGCTAGGGAGGTTGACGTTGTTGGAGTTTTCCGA 942
5               ATGACAGTGCCTCTCACCCCTGCTGCTGCTAGGGAGGTCGACGTTGTTGGAGTTTTCAGA 842
8               ATGACAGTGCCTCTCACTCCAGCTGCTGCCAGGGAGGTTGATGTGGTTGGTGTTTTCCGG 842
6               ATGACAGTGCCTCTCACTCCAGCTGCTGCCAGGGAGGTTGATGTGGTTGGTGTTTTCCGG 842
2               ATGACAGTCCCTCTCACTCCGGCTGCTGCCAGGGAGGTTGACGTGGTTGGAGTTTTTCGT 944
7               ATGACAGTCCCTCTCACTCCGGCTGCTGCCAGGGAGGTTGACGTGGTTGGAGTTTTTCGT 842
3               ATGACAGTCCCTCTGACACCGGCTGCAGCCAGGGAGGTTGACGTGGTTGGAGTTTTCCGG 842
1               ATGACTCTCCCTCTC---------GCTACCAGAGAGATTGATGTAATTGGAATTTTCCGA 951
                *****  * *****          **  * ** *** * ** **  ****  ****  *

4               TACCAGAAAACATGGCCGCTTTGCCTCGAGTTTTTGAGAAGTGGGAAGATTGACGTGAAG 1002
9               TACCAGAACACATGGCCGCTTTGCCTCGAGTTTTTGAGAAGTGGGAAGATTGACGTGAAG 1002
5               TACAAGAACACATG---------------------------------------------- 856
8               TACAAGAACACATGGCCACTTTGCCTCGAGTTTTTGAGAAGTGGGAAGATCGACGTGAAA 902
6               TGCAAGAACACATGGCCACTTTGCCTCGAGTTTTTGAGAAGTGGGAAGATCGACGTGAAG 902
2               TACAAGAACACATGGCCGCTTTGCCTTGAGTTTTTGAGAAGCGGGAAGATCGACGTGAAG 1004
7               TACAAGAACACATGGCCGCTTTGCCTTGAGTTTTTGAGAAGCGGGAAGATCGACGTGAAG 902
3               TATAAGAACACATGGCCTCTTTGCCTTGAGTTTTTGAGAAGCGGGAAGATCGACGTGAAG 902
1               TACCAGAACACATGGCCGCTGTGCCTTGAGTTTCTGAGAAGTGGTAAGATTGATGTGAAG 1011
                *   **** *****
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4               CCGCTTATCACACACCGTTTTGGATTTACTGAGAAGGAGGTGGAAGAAGCGTTTGCAACA 1062
9               CCGCTTATCACACACCGTTTTGGATTTACTGAGAAGGAGGTGGAAGAAGCGTTTGCAACA 1062
5               ------------------------------------------------------------
8               CCGCTTATCACGCACCGTTTTGGATTCACGGAGAAGGAGGTGGAAGAAGCAAGCTTGGAA 962
6               CCGCTTATTACTCACCGGTTTGGATTTACCGAGAAGGAGGTGGAAGAAGCAAGCTTG--- 959
2               CCGCTTATTACCCACCGGTTTGGTTTTACCGAGAAGGAGGTGGAAGAAGCTTTTGCAACC 1064
7               CCGCTTATTACCCACCGGTTTGGTTTTACCGAGAAGGAGGTGGAAGAAGCA--AGCTTGG 960
3               CCGCTTATTACCCACCGGTTTGGATTTACCGAGAAGGAGGTGGAAGAAGCA--AGCTTGG 960
1               CCCCTCATAACACATCGGTTTGGATTTTCTCAGAAGGAGGTGGAAGAAGCCTTTGAAACC 1071


4               AGTGCTCGTGGGGGTAATGCCATCAAGGTGATGTTCAAATTGTAA 1107
9               AGTGCTCGTGGGGGTAATGCCATCAAGGTGATGTTCAAATTGTAA 1107
5               -------------------------------------------
8               TTC-------------------------------------------- 965
6               -------------------------------------------
2               AGTGCTCGGGGGGGCAACGCCATTAAAGTCATGTTTACTCTATA- 1108
7               AATTCT----------------------------------------- 966
3               AATTCT----------------------------------------- 966
1               AGTGCTCGCGGAGGCAATGCCATTAAGGTCATGTTTAACCTGTAA 1116
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