IDENTIFICATION OF SINGLE NUCLEOTIDE POLYMORPHISMS AND THE FUNCTIONAL CHARACTERIZATION OF TWO APPLE *KIP RELATED PROTEINS*, *MdKRP4* AND *MdKRP5*

by

LISA KLIMA JOHNSON

(Under the Direction of Anish Malladi)

ABSTRACT

Fruit size in apple is determined through the combination of cell production and expansion. Cell cycle genes identified in apple include two that may negatively regulate cell production. These genes are cyclin dependent kinase inhibitors, referred to as *KRP*s (*Kip-Related Proteins*). Characterization of these genes is needed to determine how they influence fruit growth and size. Two approaches included identification of polymorphisms within the coding region of these *KRP*s in a population of *Malus* × *domestica* varying in fruit size, and transformation of *Arabidopsis thaliana* with *MdKRP4* and *MdKRP5*. One polymorphism identified in *MdKRP4* resulted in an amino acid substitution that correlated with small fruit size. Preliminary phenotypic observations of the transformants indicate smaller, serrated leaves and altered floral morphology, similar to plants overexpressing *A. thaliana KRP2*. These results indicate the *KRP*s have an important role in cell cycle regulation, potentially impacting cell production and fruit growth in apple.

INDEX WORDS: *Malus* × *domestica*, cell cycle, *KRP*, fruit growth

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DEDICATION

I dedicate this work to my son.

Work hard and you can accomplish anything.

I dedicate it also to my husband, who believes I can accomplish anything,

and to my parents who taught me that very important lesson.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Fruit production and fruit consumption continue to increase over time in the United States, which is positive for both the economic viability of growers and the overall health of consumers. About thirty years ago, in 1981, the non-citrus utilized production of fruit was worth over 3.8 billion dollars. In 2011, the same measurement represented over 13.9 billion dollars (USDA-ERS, 2012), a 3.5-fold increase. Over the same time period, the use of fruit other than citrus has increased from 161 pounds per capita per year in 1981, to 201.5 pounds per capita per year in 2011 (USDA-ERS, 2012). This shift could be due to a greater availability of seasonal fresh produce nationally because of an increase in imported produce, or an increase in popularity of processed fruit products like canned, frozen, or dried fruit, and juice.

The types of fruit available are vast, and vary with location, but there are several commonalities that contribute to successful commercial production. Optimum fruit growth requires a combination of the best cultural practices, an ideal location, and good genetic traits. Most fruit crops are perennial trees, vines, or shrubs, and therefore the yield and quality is distinctly affected by cropping, training, pruning, and thinning methods employed by growers. Similarly, irrigation and fertilization systems often contribute to the success or failure of fruit production operations. Environmental effects such as season length, bearing habits, chilling, and soil type determine whether a crop can be grown in a certain location. Area pressures due to fungal and bacterial diseases, insect

populations, and weed density affect fruit quality. Inherited traits are the most important contributors, being ultimately responsible for many commercially important fruit quality characteristics, including shape, size, color, shelf life, and flavor.

Fruit growth and development:

Fruit exists to protect the developing seeds, and later facilitate their dispersal (Srivastava and Handa, 2005). Fruit originate from flowers, and the different types of fruit are borne out of different parts of the flower. Fruit can be considered to be a pome like an apple, which arises from an ovary but the edible portion is non-ovarian tissue, or an accessory like a strawberry, which is also non-ovarian tissue (Rieger, 2006; Westwood, 1993). Other fruit are classified as drupes, berries, achenes, and nuts, for example. Many recent scientific discoveries on fruit growth, development, and ripening have been detailed using tomato, a berry, as a model fruit. It has been chosen as a model for fruit development because this species exhibits a wide range of fruit morphology (Grandillo et al., 1999; Tanksley, 2004). *Arabidopsis thaliana* is also used as a model plant, primarily because of its rapid life cycle, ease of culture, and transformation efficiency.

Simple fruit growth can be broken down into several phases (Fig. 1.1; Gillaspy et al., 1993; Westwood, 1993). The first phase of a simple sigmoidal fruit growth model involves fruit set, which is a result of successful flowering, followed by pollination and fertilization. Differences in fruit cell number can be significant as early as anthesis in some fruits (Bohner and Bangerth, 1988). The second phase is then triggered, which consists primarily of rapid cell division. This phase produces the majority of the cells

that will ultimately make up the fruit (Srivastava and Handa, 2005). The third phase consists of cell expansion, which usually contributes to a dramatic increase in fruit diameter. This phase makes up the rise of the sigmoidal curve (Fig. 1.1; Gillaspy et al., 1993; Westwood, 1993). Final fruit size is determined ultimately through a combination of cell division and cell expansion during fruit development (Chevalier, 2007; Harada et al., 2005). The final phase of fruit growth is maturation and ripening, including senescence. Fruit set, cell division, cell expansion, and maturation are regulated by the availability, concentration, signaling, and interaction of several hormones and growth regulators, and the availability of carbohydrates (Bohner and Bangerth, 1988; Cowan et al., 2001; Gillaspy et al., 1993; Ozga and Reinecke, 2003; Srivastava and Handa, 2005). Also, as found in most biological processes, many genes, transcription factors, and microRNAs (miRNAs), influence fruit development (Dash and Malladi, 2012; Sun et al., 2013; Tanksley, 2004; Yao et al., 2001).

Apple fruit growth and development:

Apple is a popular fruit in the United States and the crop is extremely important commercially. There are a wide variety of species and cultivars, and they are grown primarily in the northern part of the country with New York, Michigan, and Washington being top producers (USDA-ERS, 2012). Nationally, the utilized production for 2011 was estimated to be 9.31 billion pounds, which was worth over 2.7 billion US dollars (USDA-NASS, 2012). Americans have eaten close to the fresh weight equivalent of 50 pounds per person per year, for two decades (USDA-ERS, 2012). Apples are eaten fresh as well as processed into juice, cider, hard cider, applesauce, dried slices, apple butter,

jelly, baby food, vinegar, and other products. Apples have been cultivated in North America since the 1600's (Rieger, 2006).

Apple fruit size is a commercially important trait. Large apples of every variety are more valuable for the grower (USDA, 2012). Thinning the crop load is a popular technique for increasing apple size, and chemical methods are currently used in commercial production (Denne, 1960; Dennis, 2000; Forshey and Elfving, 1977; Link, 2000). Other cultural practices such as training, pruning, irrigation, and fertilization can also have an influence on the final fruit size (Chevalier, 2007; Denne, 1960; Ferree and Warrington, 2003; Forshey and Elfving, 1977; Skene, 1966). Environmental factors and maturity also help to determine final fruit size. Environmental conditions have been found to be important in determining the stability of fruit size quantitative trait loci in apple and other crops (Kenis et al., 2008). By far, the most important factor influencing apple fruit size is its genetic background.

The draft genome of *Malus* × *domestica* (Borkh.) 'Golden Delicious' became available in 2010, and with it came an increase in the available information about the general history and evolution of traits of the domesticated apple (Velasco et al., 2010). Marker assisted breeding has begun in apple; however, much of this approach is focused on disease resistance (Kumar et al., 2012). Some quantitative trait loci (QTL) mapping has been done in apple, but there is a lack of reliable data (Kumar et al., 2012). Major QTL for fruit diameter and weight have been identified across a few populations of apple, but it has been determined that they are not stable over time (Kenis et al., 2008; Devoghalaere et al., 2012). As previously stated, environmental conditions are a critical determinant for fruit quality characterization, which can vary significantly from year to

year (Kenis et al., 2008). Even though fruit size is a polygenic trait, there are typically a few genes that have a major influence (Tanksley, 2004). Apple is a highly heterozygous crop with an average polymorphism rate of 4.8 single nucleotide polymorphisms (SNPs) per kilobase within domestic cultivars (Velasco et al., 2010). Minor differences in gene sequence among genotypes could have a dramatic influence on traits such as final fruit size, depending on the SNP's functionality.

Larger apple size is the direct result of more cell production, and the following expansion of the higher number of cells (Bain and Robertson, 1950; Harada, et al., 2005). In apple, cell division starts prior to bloom, as the carpel/floral tube is increasing in diameter (Malladi and Johnson, 2011). After full bloom, cell division continues rapidly for several weeks, and a clear exit point from cell production is usually apparent (Bain and Robertson, 1951; Denne, 1960; Malladi and Hirst, 2010; Skene, 1966). The rate of cell production peaks around 15 days after full bloom, and the fruit cell number reaches a plateau around 30 days after full bloom (Malladi and Johnson, 2011). Cell expansion begins early in fruit development, and the cell size continues to increase until maturity (Bain and Robertson, 1951; Denne, 1960; Harada et al., 2005; Skene, 1966). Cell area in apple increases steadily from about 25 days after full bloom, until maturity (Malladi and Johnson, 2011). A slight increase in the number of cells can lead to a significant increase in fruit size (Bain and Robertson, 1950).

The cell cycle:

The final number of cells in a fruit is determined by the number of cells in the ovary before fertilization, and the number of cell divisions occurring throughout fruit

development (Gillaspy et al., 1993). Cell division is regulated by the plant cell cycle in apple, as in other plants. The cell cycle is characterized by four phases: G1, S, G2, and M (Fig. 1.2). The G1 phase is considered one of the gap phases. The two cells produced by mitosis during the previous phase are evaluated for complete and accurate division during the G1 phase. During the transition to S-phase, Cyclin-dependent kinases (CDKs) form complexes with cyclins, which then phosphorylate available substrates, triggering DNA replication, which mainly occurs within the S-phase (DeVeylder et al., 2003; Dewitte and Murray, 2003; Inze and DeVeylder, 2006; Wang et al., 2008). The G2 phase, another gap, therefore contains cells with double the DNA content found in a regular cell. During G2, accuracy of DNA replication is confirmed, enabling the cell cycle to move forward. Through the phosphorylation of substrates, the CDK-cyclin complexes again drive transition to the M-phase and the progression of mitosis (DeVeylder et al., 2003; Dewitte and Murray, 2003; Inze and DeVeylder, 2006; Wang et al., 2008).

The nature of the plant cell cycle is complex and there are many genes involved, only some of which have had their functions identified and confirmed. Key to the progress of the cell cycle is the availability of CDK and cyclin proteins. In Arabidopsis, 29 CDKs and 49 cyclins have been identified (Menges et al., 2005; Vandepoele et al., 2002). Specific relationships between CDKs and cyclins exist in various species, and their availability is important at different stages of the cell cycle. CDKs are considered to be inactive unless bound to a cyclin. CDKAs appear to regulate both transitions from the gap phases in the cell cycle, and CDKBs are important during the G2/M transition. Cyclin types, and their availability and destruction varies throughout the cell cycle (DeVeylder et al., 2003; Dewitte and Murray, 2003; Inze and DeVeylder, 2006; Wang et

al., 2008). In apple, 14 CDKs and 34 cyclins have been identified, confirming the complexity of the cell cycle at work (Malladi and Johnson, 2011). Kip-related proteins (KRPs) are also known as CDK inhibitors (CKIs). KRPs regulate the cell cycle by binding to CDK-cyclin complexes, and inhibiting their activity (Dewitte and Murray, 2003; Inze and DeVeylder, 2006; Wang et al., 1997, 2008). This may delay or result in exit from the cell cycle (Verkest et al., 2005*a*). The rate and the duration of cell production are determined by the availability and complex interactions of cyclins, CDKs, and KRPs, among other proteins. In apple, five *KRPs* have been identified (Malladi and Johnson, 2011).

An alternate cell cycle results in endoreduplication in many plants (Inze and DeVeylder, 2006). This occurs when the genome is replicated in the S phase, but the cell does not divide in the M phase, which results in multiple genome copies in the nucleus. It is thought that endoreduplication is a result of the inhibition of mitosis, and the event triggering it may happen in the G2 phase, or the G2/M transition of the cell cycle (Inze and DeVeylder, 2006). KRPs have been implicated in regulation of this cycle in some plants as well (Nafati et al., 2011; Schnittger et al., 2003; Verkest et al., 2005*b;* Weinl et al., 2005). Endoreduplication does not commonly occur in apple (Harada et al., 2005; Malladi and Hirst, 2010). However, in one case, the cultivar 'Grand Gala' was associated with increased ploidy due to endoreduplication, resulting in a larger fruit size. Cell production stopped earlier in 'Grand Gala' than in the control, so the larger fruit size was attributed to a greater than normal increase in cell size (Malladi and Hirst, 2010).

Cyclin-dependent kinase inhibitors (KRPs):

Seven KRP genes have been identified in Arabidopsis (DeVeylder et al., 2001). There is a conserved domain, sometimes broken down into three motifs, within the Arabidopsis KRP sequences, as well as other plant KRPs (Acosta et al., 2011). Deletion of a portion of this domain minimized or completely erased the effects of *KRP1* overexpression on Arabidopsis growth, suggesting that this region is responsible for binding and regulating CDK activity (Zhou et al., 2003*a*). DeVeylder et al. (2001) outline three motifs in particular which are conserved across all Arabidopsis KRPs. These motifs, 186-PLEGRYEW, 173-FIEKYNYD, and 156-ELEEFFAATE are conserved across many species in identified regions of cyclin-dependent kinase inhibitors (Acosta et al., 2011). Up to 31 residues in this region, which includes these three motifs, are conserved across many species, now considered to be required for KRP function, and it is now referred to as the CDK/cyclin interacting/inhibiting domain (Acosta et al., 2011; DeVeylder er al., 2001; Jasinski et al., 2002; Lui et al., 2000; Schnittger et al., 2003; Wang et al., 1997, 2008; Zhou et al., 2003*a*, 2003*b*). These three motifs are also found in the apple KRPs (Fig. 1.3).

A common technique used to determine gene function in plants is overexpressing that gene in a model system. When *KRP1* was overexpressed in Arabidopsis trichomes, endoreduplication within these cell types and the final trichome size were reduced (Schnittger et al., 2003). *KRP2* overexpression in Arabidopsis plants resulted in a reduction in leaf area, an increase in leaf thickness, leaf serration, reduced lateral roots, reduced cell number, increased cell size, and altered, and partially sterile flowers, some of which result from a reduction in the final cell number, indicating negative regulation

of cell division (DeVeylder et al., 2001). When *KRP*s are overexpressed strongly, CDK activity and cell division is decreased, but a weaker overexpression seems to induce endoreduplication (Verkest et al., 2005*a*; Weinl et al., 2005). These studies suggest that high KRP activity is associated with reduced organ size through an overall reduction in cell production.

In tomato, four *KRP* genes have been identified, and some of their functionally important domains have been characterized relative to fruit growth (Nafati et al., 2010). The overexpression of a *KRP* gene in tomato negatively influenced endoreduplication, but final fruit size was not affected, suggesting that endoreduplication can be regulated separately from cell growth (Nafati et al., 2011). By associating *KRP* expression with differentiating cells in tomato gel tissue, studies have shown that KRP is involved in regulating both the cell cycle, and endoreduplication (Bisbis et al., 2006). These studies further support the role of KRPs as important regulators of the cell cycle and possibly also endoreduplication.

Cell production regulation in apple:

During apple fruit development, a period of approximately 150 days, over 1900 genes show a change in expression, and their clustering has been coordinated with fruit development stages (Janssen et al., 2008). In apple, 71 cell cycle genes have been identified; thirty-four cyclins (CYC) have been identified in apple and grouped into nine classes, and 14 CDKs have been identified, and grouped into seven classes (Malladi and Johnson, 2011). From among the cell cycle genes identified, 14 were positively

associated with cell production, and five were negatively associated with cell production during various stages of fruit development (Malladi and Johnson, 2011).

In apple, five *KRP* genes have been identified (Malladi and Johnson, 2011). Among these, only *MdKRP4* and *MdKRP5* displayed expression patterns consistent with negative regulation of cell production during fruit development. MdKRP4 and MdKRP5 are more closely related to Arabidopsis KRP1 and KRP2 (Fig. 1.4). MdKRP4 displays 31% identity and 46% similarity with the Arabidopsis KRP1 and 30% identity and 48% similarity with Arabidopsis KRP2. MdKRP5 shares 32% identity and 48% similarity with Arabidopsis KRP1 and 32% identity and 48% similarity with Arabidopsis KRP2. In unpollinated apple flowers, the expression of these *KRP* genes increased by 2- to 4-fold, as early as 11 days after full bloom, coincident with a decline in cell production (Malladi and Johnson, 2011). The expression of these *KRP* genes in developing fruit increased by 3.9-fold and 5.3-fold at three days after shading (Dash et al., 2012). During the same period, cell production and fruit diameter were significantly decreased (Dash et al., 2012). Also, during the exit from mitotic cell production during normal fruit growth, the expression of these *KRP* genes increases by multiple-fold (Malladi and Johnson, 2011; Dash et al., 2013). Around this period, the rate of cell production in the developing fruit decreased sharply, and the overall fruit diameter was increasing primarily due to cell expansion (Malladi and Johnson, 2011).

These data suggest strongly that *KRPs* function as negative regulators of cell production in apple. As the final cell number is an important determinant of fruit size (Bain and Robertson, 1951), the *KRP*s may have an important role in final apple size determination. *KRP* genes appear to govern the rate and/or duration of cell production in

apple fruit, and their modification could result in altered fruit size (Malladi and Johnson, 2011). Detailed characterization of these genes is essential to better understand their functions in regulating apple fruit growth, and a comparison of these genes across multiple genotypes can provide a clearer picture of how variability in *KRP* sequence can influence fruit size variation.

Statement of research hypothesis and objectives:

Research hypothesis:

MdKRP4 and MdKRP5 are involved in determining the extent of cell production, and thereby fruit growth and final fruit size in apple.

Objective 1:

Perform functional characterization of *MdKRP4* and *MdKRP5*.

Objective 2:

Identify single nucleotide polymorphisms in *MdKRP4* and *MdKRP5* among apple genotypes

Research Significance:

If cell production in early fruit development of apple were allowed to continue for a longer period, larger fruit size may be achieved. If the apple *KRP4, KRP5* or both were involved in inhibiting cell division, then blocking their function may result in larger apple size and an economic benefit for growers. A characterization of their function and comparison of their sequences in a population of apples will provide more information about fruit growth in apple and the plant cell cycle.

Figures:

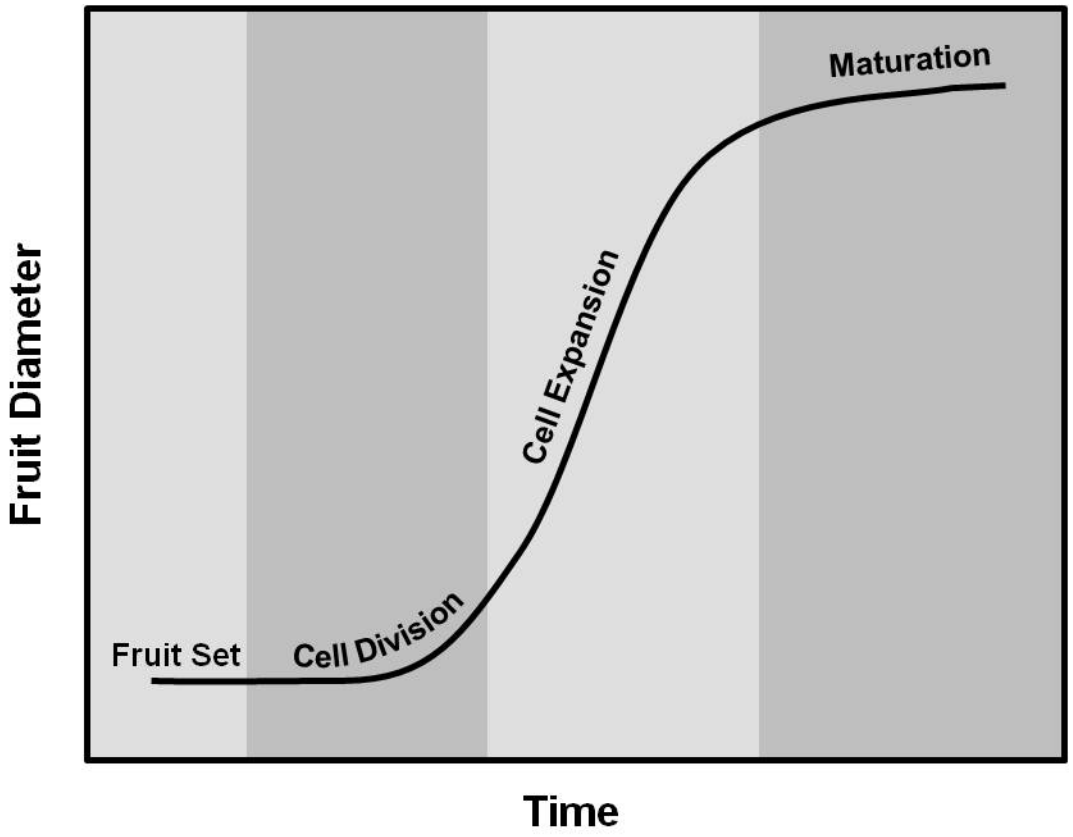

Figure 1.1. Sigmoidal curve representing general phases of simple fruit growth and development.

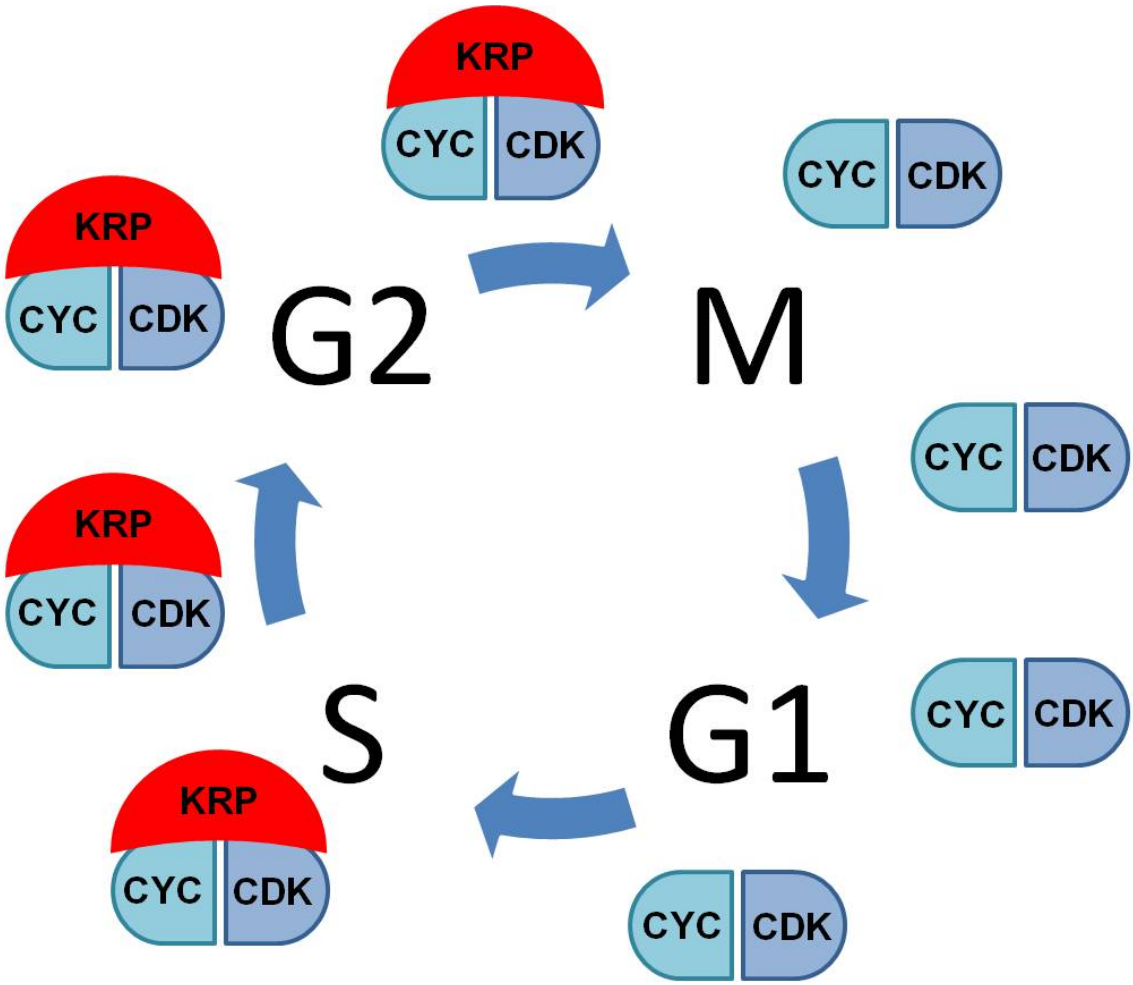

Figure 1.2. Simplified representation of KRPs at work in the plant cell cycle.

```
GALA_KRP_4                      MELARAATSANAVRKRKAGSADGESVELPSSSSYDVDQRKKPRRRVVVVRSAPKSEAEEE
GOLDEN_DELICIOUS_GENOME_KRP4    MELARAATSANAVRKRKAGSADGESVELPSSSSYDVDQRKKPRRRVVVVRSAPKXEAEXE
                                ************************************************** *** *

GALA_KRP_4                      SVRTSNDDFSTSDHAESSCCSSNGSSVLVEDRKFEFVDPEADESEQVESSTYNSSRDERR
GOLDEN_DELICIOUS_GENOME_KRP4    SVRTSNDDFSTSDHAESSCCSSNGSSVLVEDRKFEFVDPEADESEQVESSTYNSSRDERR
                                ************************************************************

GALA_KRP_4                      EMTAPTSEVRAEAESTAEPKEVESQRRSPPVVNVSELEEFFAATEKESQQKFIEKYNYDV
GOLDEN_DELICIOUS_GENOME_KRP4    EMTAPTSEVRAEAESTAEPKEVESQRRSPPVVNVSELEEFFAATEKESQQKFIEKYNYDV
                                ************************************************************

GALA_KRP_4                      VKDEPLEGRYEWIRLKP
GOLDEN_DELICIOUS_GENOME_KRP4    VKDEPLEGRYEWIRLKP
                                *****************


GALA_KRP_5                      MEVSRAATSATAAKKRKAGSADGESVELPSSYDVAQHKRPRRRVVVVSSAPKIKREAESV
GOLDEN_DELICIOUS_GENOME_KRP5    MEVSRAATSATAAKKRKAGSADGESVELPSSYDVAQHKRPRRRVVVVSSAPKIKREAESV
                                ************************************************************

GALA_KRP_5                      RTSNDGFWTSDHAESSCCSSNGSSELEDESDQVESWTYNSSRDERREMTAATSKVGAEAE
GOLDEN_DELICIOUS_GENOME_KRP5    RTSNDGFWTSDHAESSCCSSNGSSELEDESDQVESWTYNSSRDERREMTAXTSKVGAEAE
                                ************************************************ *********

GALA_KRP_5                      STARLKDESQRRSPTVVNASELEEFFAAMEKESQQKFKEMYNFDVAKDEPHEGRYEWVRL
GOLDEN_DELICIOUS_GENOME_KRP5    STARLKDESQRRSPTVVNASELEEFFAAMEKESQQKFKEMYNFDVAKDEPHEGRYEWVRL
                                ************************************************************

GALA_KRP_5                      KP
GOLDEN_DELICIOUS_GENOME_KRP5    KP
                                **
```

Figure 1.3. Conserved motifs in MdKRP4 and MdKRP5 in 'Gala' samples and the 'Golden Delicious' draft genome (Clustal Omega).

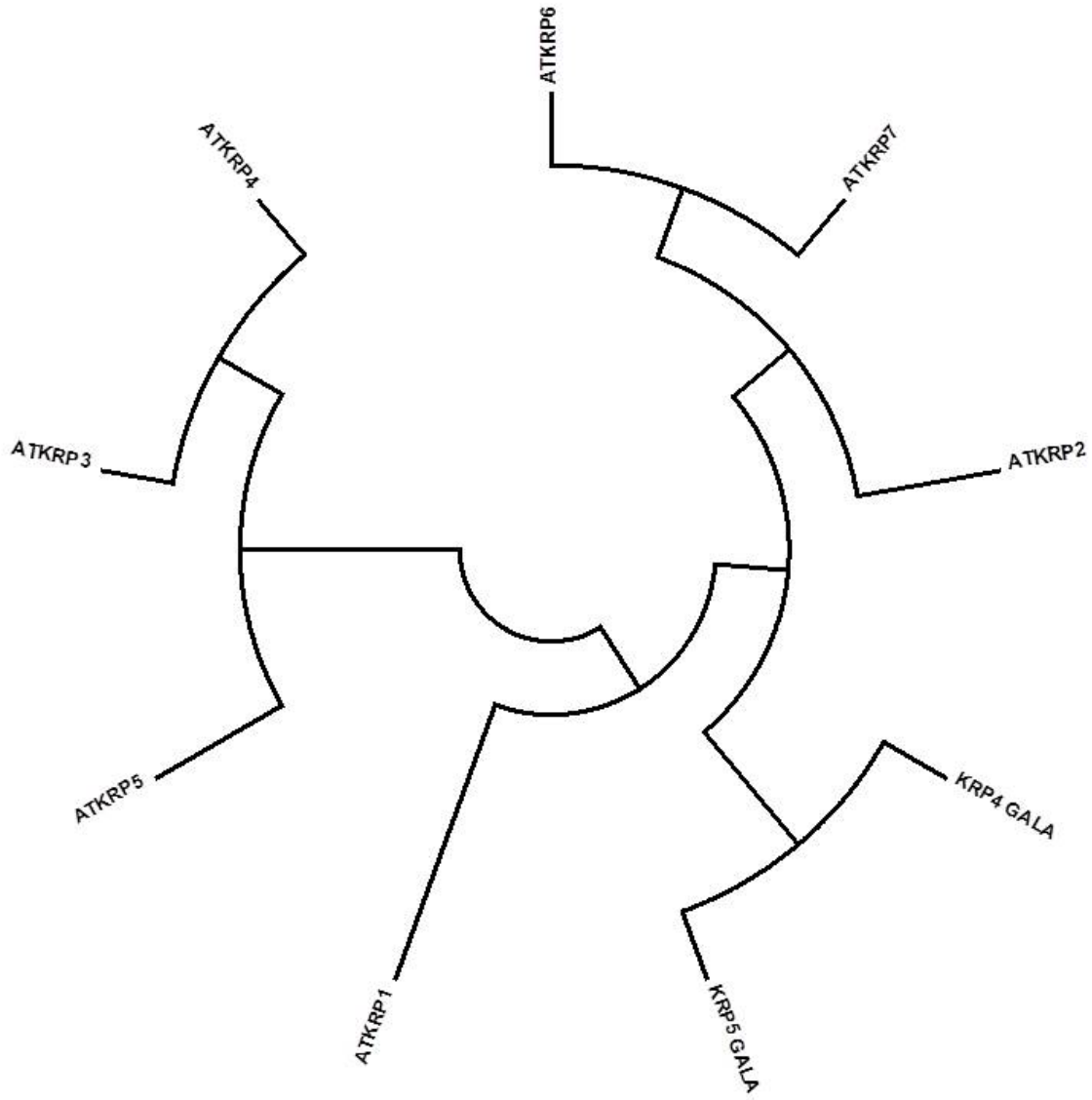

Figure 1.4. Phylogenetic tree (MEGA5) displaying the relationships between MdKRP4 and MdKRP5 and *Arabidopsis thaliana* KRPs.

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CHAPTER 2

FUNCTIONAL CHARACTERIZATION OF TWO APPLE *KIP RELATED PROTEINS*, *MdKRP4* AND *MdKRP5*

Introduction:

Final fruit size in apple is determined through a combination of the number of cells produced, and the subsequent expansion of those cells (Bain and Robertson, 1950; Harada, et al., 2005). Cell division begins before bloom in apple and continues for several weeks, then displays a clear exit from cell production and a transition to cell expansion (Bain and Robertson, 1951; Denne, 1960; Malladi and Hirst, 2010; Malladi and Johnson, 2011; Skene, 1966). Cell production in apple peaks around two weeks after bloom, and levels off around four weeks after bloom (Malladi and Johnson, 2011). Cell expansion in apple starts increasing about 25 days after bloom, and continues until maturity (Malladi and Johnson, 2011). The number of cells has a great influence over the final fruit size, and the rate and/or duration of the cell production period will determine that number. Increasing the cell number, even slightly, can lead to significantly larger fruit size (Bain and Robertson, 1950).

The number of cells produced in apple is regulated by the plant cell cycle. There are many genes involved, and not all of them have clearly identified functions yet. The cell cycle is known to have four phases. During the G1 phase, the two cells produced by mitosis during the previous phase are evaluated. Next, during the transition to the DNA replication phase (S-phase), Cyclin-dependent kinases (CDKs) form complexes with

cyclins, leading to the phosphorylation of substrates necessary to trigger DNA replication (DeVeylder et al., 2003; Dewitte and Murray, 2003; Inze and DeVeylder, 2006; Wang et al., 2008). During the G2 phase, the accuracy of DNA replication is assessed, and the cell cycle progression continues. The transition to M-phase also involves the CDK/cyclin complexes phosphorylating substrates, to drive mitosis during the M-phase (DeVeylder et al., 2003; Dewitte and Murray, 2003; Inze and DeVeylder, 2006; Wang et al., 2008). Although, the cell cycle in apple is expected to function similarly, little research has been undertaken thus far. The apple cell cycle is complex, which is evidenced by the recent identification of fourteen CDKs and 34 cyclins (Malladi and Johnson, 2011).

Contributing to the regulation of the plant cell cycle are CDK inhibitors, which are also known as Kip-Related Proteins (KRPs). KRPs regulate the cell cycle by binding to CDK-cyclin complexes, and inhibiting their activity (Dewitte and Murray, 2003; Inze and DeVeylder, 2006; Wang et al., 1997, 2008). Cells may exit the cell cycle or their progression may be delayed due to KRP activity (Verkest et al., 2005*a*). The availability of CDKs, cyclins, KRPs, and other cell cycle proteins, and the complex interactions among them, determine the rate and duration of cell production (DeVeylder et al., 2001; Dewitte and Murray, 2003; Inze, 2003; Inze and DeVeylder, 2006; Menges et al., 2005; Vandepoele et al., 2002; Verkest et al., 2005*b*; Wang et al., 2008).

Multiple *KRP* genes have been identified in the model plant *Arabidopsis thaliana* (DeVeylder et al., 2001) and other plants (Acosta et al., 2011). Up to 31 residues in the C-terminal region now considered to be required for KRP function, are conserved across many species, and it is now referred to as the CDK/cyclin interacting/inhibiting domain

(Acosta et al., 2011; DeVeylder er al., 2001; Jasinski et al., 2002; Lui et al., 2000; Schnittger et al., 2003; Wang et al., 1997, 2008; Zhou et al., 2003*a*, 2003*b*).

In apple, five *KRP* genes have been identified. Among these, only *MdKRP4* and *MdKRP5* displayed expression patterns consistent with negative regulation of cell production during fruit development (Malladi and Johnson, 2011). Several recent studies in apple indicate increased *MdKRP4* and *MdKRP5* expression during periods of low cell production. When apple flowers are unpollinated and cell production is reduced, *MdKRP4* and *MdKRP5* expression was increased by two- to four-fold (Malladi and Johnson, 2011). Their expression was increased during fruit development by 3.9-fold and 5.3-fold in response to shading, which reduced cell production and fruit diameter (Dash et al., 2012). Also, during normal fruit development, at a time coincident with exit from mitotic cell production, the expression of *MdKRP4* and *MdKRP5* was increased by over 14-fold and over 11-fold, respectively (Dash et al., 2012; Malladi and Johnson, 2011). These results suggest that MdKRP4 and MdKRP5 function as negative regulators of cell production in apple. The modification of the expression of these genes could have a strong influence over cell production, and therefore final fruit size in apple (Janssen et al., 2008; Malladi and Johnson, 2011).

Determining gene function is often achieved through expression of the gene in a model plant, like *Arabidopsis thaliana*. Comparison of phenotypic changes in response to the expression of the gene of interest in the model system, and the overexpression of the native gene in the model system can determine whether or not the function and molecular mechanisms at work are related. The family of *KRP* genes and the function of certain individual *KRP* genes have been investigated in multiple crops, including tobacco

(Jasinski et al., 2002), tomato (Bisbis et al., 2006; Nafati et al., 2010, 2011), rice (Barroco et al., 2006; Mizutani et al., 2010; Yang et al., 2011), potato (Campbell et al., 2012), and *A. thaliana* (DeVeylder et al., 2001; Jasinski et al., 2002; Jegu et al., 2013; Jun et al., 2013; Lui et al., 2000; Menges et al., 2005; Schnittger et al., 2003; Vandepoele et al., 2002; Verkest et al., 2005*a*; Wang et al., 1997, 2008; Weinl et al., 2005; Wen et al., 2013; Zhou et al., 2003). However, the functions of all seven of the *A. thaliana* KRPs (AtKRPs) are still not clear (Bird et al., 2007). The overexpression of several *AtKRP*s produced smaller leaves, indicating negative regulation of cell division (DeVeylder et al., 2001). The study of cell cycle genes in economic crops is limited. Research utilizing economic crops can allow for a deeper understanding of physiology, and at the same time, the application of physiological discoveries. The objective of this research is to perform a functional characterization of *MdKRP4* and *MdKRP5*. A better understanding of the role of these two genes in the regulation of the apple cell cycle may be gained from this research.

Materials and methods:

Identification of genes and sequences:

The identification of *MdKRP4* and *MdKRP5* genes in apple by Malladi and Johnson (2011) and the available *Malus* × *domestica* draft genomes enabled the isolation of these genes. The sequences were used for comparison with the seven identified *A.thaliana KRP* genes to determine which *AtKRP* would be useful for over-expression comparisons. Clustal Omega software was used for alignment and MEGA5 software was used for phylogenetic analysis. The published *Malus* × *domestica* 'Golden Delicious'

genome available from Fondazione Edmund Mach Istituto Agrario Di San Michele All'Adige Computational Biology Web Resources was used throughout this project to confirm the projected MdKRP4 and MdKRP5 sequences.

Plant tissue and growth conditions:

Fruit tissue from mature *Malus* × *domestica* 'Gala' trees grown at the UGA Mountain Research and Education Center, Blairsville, GA was collected at a time when *MdKRP4* and *MdKRP5* expression is known to be high, about 30 days after bloom. Tissue was immediately frozen in liquid nitrogen and stored at -80 °C. Mature leaf tissue from *Arabidopsis thaliana* ecotype Columbia was collected from 35-day old plants, and the tissue was frozen in liquid nitrogen and stored at -80 °C. *A. thaliana* plants were maintained in a growth chamber that provided continuous 21 °C, photoperiods over 12 hours, and photon flux density around 130 µmol m^{-2} s^{-1}.

RNA extraction, cDNA synthesis and amplification:

Total RNA was extracted from apple tissues using the protocol outlined in Malladi and Hirst (2010). A Trizol protocol modified from Chomczynski and Sacchi (1987) was used to extract total RNA from *A. thaliana* leaf tissue. Quality was visually confirmed using agarose gel electrophoresis, and the concentration was determined using a Nanodrop 8000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Complementary DNA was synthesized using 1 µg of total RNA, starting with DNase (Promega) treatment at 37 °C for 34 min. ImProm II Reverse Transcriptase (Promega) and oligo dT (Integrated DNA Technologies, Inc.) were used in a 20 µl reaction to reverse transcribe the RNA at 42 °C for 75 min, then 75 °C for 15 min. The cDNA was diluted five-fold and stored at -20 °C.

The *KRP* genes were amplified from the cDNA using primers including a region for cloning into a vector. The primer sequences were as follows: *MdKRP4F*: GCAGCACCATGGAACTGGCT, containing an NcoI region; *MdKRP4R*: CAGCATCTAGATCATGGCTTCAATCGAATCC, containing an XbaI region; *MdKRP5F*: GCAGGAAGACATCATGGAGGTGTCT, containing a BbsI region; *MdKRP5R*: CAGCATCTAGATCATGGCTTTAATCGAACCC, containing an XbaI region; *AtKRP2F*: TTAATACCATGGCGGCGGTTAGGAGA, containing an NcoI region; and *AtKRP2R*: GCATCTAGATCATGGATTCAATTTAACCCACTCG, containing an XbaI region. *A. thaliana KRP2* was chosen to represent overexpression of KRP because it resembled MdKRP4 and MdKRP5 most closely out of the seven Arabidopsis KRP genes. Phusion Hot Start II High Fidelity DNA polymerase was used for the amplification of one µl of cDNA template. The primer concentration used was 0.5 µM. Optimum annealing temperatures for the genes varied from *MdKRP4* at 52.1 °C, to *MdKRP5* at 56.8 °C, and *AtKRP2* at 60.3 °C. The PCR conditions were as follows: 98 °C for 30 sec; 35 cycles of 98 °C for 10 sec, annealing temperature for 30 sec, 72 °C for 1 min; 72 °C for 10 min, 4 °C hold. An Eppendorf Mastercycler EP gradient S thermal cycler (Eppendorf AG, Hamburg, Germany) was used for PCR. Products were electrophoresed in a gel containing 1.8% agarose, which was run at 83 V for 2 h. The products were extracted from the gel using an Omega Bio-tek gel extraction kit, and eluted with 30 µl sterile water.

Plasmid construction:

The pCambia 3300 N-R-OX vector was used. The vector contained a constitutive 35S promoter region, multiple linkage site, Nos terminator region, and a Basta herbicide

resistance gene. A plasmid prep was performed on the vector-containing bacteria colonies which were grown overnight in kanamycin-treated LB media. The three genes and vector were double digested using enzymes and buffers from New England Biolabs. *MdKRP4* was digested using 14 µl template, 1 µl each of NcoI and XbaI, and 2 µl of the NEB cutsmart buffer, in a 20 µl reaction. *MdKRP5* was digested using 14 µl template, 1 µl each of BbsI and XbaI, 2 µl 10X BSA, and 2 µl of NEB buffer 2 in a 20 µl reaction. *AtKRP2* was digested using 14 µl template, 1 µl each of NcoI and XbaI, and 2 µl of the NEB cutsmart buffer, in a 20 µl reaction. The vector was digested using 10 µl template, 1 µl each of NcoI and XbaI, and 1.5 µl of the NEB cutsmart buffer in a 15 µl reaction. All reactions were held at 37 °C for two hours and subsequently electrophoresed in a gel containing 1.2% agarose for 40 min at 95 V. The products were excised from the gel and cleaned up using the Omega Bio-tek gel extraction kit, and eluted with 15 µl sterile water. Ligation was performed in a 14 µl reaction, each containing approximately 150 ng of the desired gene product and 15 ng of the vector, along with 1.5 µl 10X buffer. Each reaction was held at 55 °C for 2 - 5 min prior to the addition of 1 µl T4 DNA ligase (Promega). The mixture was then held overnight at 16 °C. The construct was then transformed into JM109 competent cells using a heat shock treatment of 42 °C for 45 sec. 900 µl of LB media was added to each, and the cultures were shaken at 37 °C for two hours. The mixture was streaked out on kanamycin treated plates and incubated overnight at 37 °C. Colony PCR was performed to confirm the presence of the insert. The size of the inserts were: *MdKRP4* 594 bp, *MdKRP5* 549 bp, and *AtKRP2* 630 bp. Cultures of confirmed colonies were grown overnight in LB media at 37 °C, and plasmid preps were performed, providing two clones of each construct for transformation of

Agrobacterium tumefaciens. Sequencing was performed at the Georgia Genomics Facility to confirm the accuracy of the cloned fragments.

Transformation of *Agrobacterium tumefaciens* and *Arabidopsis thaliana*:

Fifty nano grams of each plasmid was added to 25 µl of electrocompetent GV3101 *A. tumefaciens* cells, which were electroporated and recovered with 1 ml of LB media. Samples were incubated for one hour at 30 °C, plated on kanamycin treated LB media plates, and incubated at 30 °C for two days. Two colonies were chosen and streaked on triple selection media plates which included rifampicin, gentamycin, and kanamycin, and incubated at 30 °C for two days. One streakout was used for a 20% glycerol stock, and 200 µl was plated on each of three plates and incubated for three days at 30 °C. The entire content of three plates for each construct was added to 500 ml infiltration solution (5% sucrose, 2.03% $MgCl_2$, 0.03% Silwet L-77), which was used to inoculate three 97-day old *A. thaliana* plants, using the floral dip method (Clough and Bent, 1998). The seeds were collected and dried. Fifteen ml of a 0.2% Basta herbicide solution (glufosinate ammonium) was used to imbibe 0.5 g of seed overnight at 4 °C. One flat was planted for each gene, held at 4 °C for two days, and placed in the growth chamber with the recommended conditions. Selection of Basta-resistant transformants was performed on the twelve-day old seedlings by spraying a 0.2% solution over all the seedlings to the point of drip. Four pots were un-treated to assess the viability of the selection. Sensitive plants were thinned out over the following days, and at 33-days old, transformants were transplanted into the aracon system (Betatech, Gent, Belgium) for isolation and seed harvest.

A single fully expanded leaf was removed from 36-day old plants for PCR analysis of transgene presence. The leaves were frozen in liquid Nitrogen and ground using a tissue lyser for 30 sec. DNA extraction was based on Edwards et al. (1991). PCR was performed using 1 µl of the DNA and the same primers that were used to amplify the cDNA initially were used. The PCR conditions were as follows: 94 °C for 3 min; 40 cycles of 94 °C for 30 sec, 60 °C for 30 sec, 72 °C for 1 min; 72 °C for 10 min, 4 °C hold. Again, the Eppendorf Mastercycler EP gradient S thermal cycler (Eppendorf AG, Hamburg, Germany) was used. Gel electrophoresis was performed using a 1.2% agarose gel to determine the band size.

Gene expression of transformants:

Ten PCR confirmed transformants were selected for initial gene expression. Two new, expanding leaves were harvested, frozen in liquid nitrogen, and ground using a tissue lyser for 15 sec. RNA was extracted using a modified Trizol method from Chomczynski and Sacchi, 1987. Quality was confirmed in an agarose gel, and the concentration determined using the Nanodrop 8000. Complementary DNA was synthesized using 500 ng of each sample, starting with DNase (Promega) treatment at 37 °C for 34 min. ImProm II Reverse Transcriptase (Promega) and oligo dT (Integrated DNA Technologies, Inc.) were used in a 20 µl reaction to reverse transcribe the RNA at 42 °C for 75 min, then 75 °C for 15 min. The cDNA was diluted fivefold and stored at -20 °C.

Two reference genes were selected from a group tested by Czechowski et al. (2005) based on their stability over the development period and under various environmental conditions. *AtSAND* and *AtTIP41L* were selected for normalization of

gene expression data. The Stratagene Mx3005P system (Agilent Technologies, Inc., Santa Clara, CA, USA) was used for quantitative Real-Time PCR. Ten Columbia wild type, ten *AtKRP2* overexpressing samples, ten *MdKRP4* expressing samples, and ten *MdKRP5* expressing samples, all with transgene confirmation were evaluated. Reactions were 12 µl, using 6 µl VeriQuest SYBR Green qPCR Master Mix with ROX (2X) (Affymetrix, Inc., Cleveland, OH, USA), 1 µl cDNA template, and 0.2 µM primer concentration. The thermal cycling conditions were 50 °C for 2 min, 95 °C for 10 min, then 40 cycles of 95 °C for 30 sec and 60 °C for 1 min. Primer specificity was confirmed using a melt curve analysis at the end of the qRT-PCR cycle. Reactions using water as a control template were included in each plate. Two samples from each of the *MdKRP* groups were excluded due to a low starting RNA concentration and poor cDNA quality. Baseline correction and determination of the efficiency of each gene was determined using LinregPCR software (Ramakers et al., 2003; Ruijter et al., 2009). Gene expression was calculated as described in Dash et al. (2012).

Results:

Sequence comparison and phylogenetic analysis revealed MdKRP4 and MdKRP5 protein sequences are closest to the *A. thaliana KRP2* (*AtKRP2*, Figure 2.1). This gene was selected to include in the transformation, providing a positive control for comparison.

MdKRP4 and *AtKRP2* were cloned into the pCAMBIA 3300N-S-OX vector using the NcoI and XbaI cloning sites, and *MdKRP5* was inserted using the BbsI and XbaI sites (Figure 2.2). Once the transformed bacteria had been incubated, colony PCR confirmed

each gene had been successfully inserted. The electroporation of competent cells and resulting *A. tumefaciens* growth provided enough inoculants to successfully carry out the floral dip method of *A. thaliana* transformation. The floral dip treatment of *A. thaliana* plants resulted in normal seed production. After harvesting the seeds from the treated plants (T_0 generation), the T_1 generation was germinated and treated with Basta herbicide, and there was a clear response from sensitive seedlings (Figure 2.3). Cotyledons turned light green in color and stopped expanding, indicating sensitivity, while transformants remained darker green and displayed continued growth.

The resulting plants displayed a variety of phenotypes. The effects of the expression of the two *MdKRP* genes appeared similar to that of the overexpression of *AtKRP2*. The fifth or sixth leaf in most cases was clearly different from wild-type (WT) Columbia (Figure 2.4). Representative samples shown in Figure 2.4 indicated smaller leaves, extensive leaf serration, and in some cases noticeable thickening and marked curvature of leaves. A variety of phenotypes was produced in each group of transformants, ranging from light to severe alteration of leaf morphology. Visual rating of the phenotype categorized the individuals as having light, moderate, or severe phenotypes. Plants considered to have light alteration of phenotype had some leaf serration, but leaf size was not dramatically different than that of the WT plants, and vigor was either unaffected or slightly increased. Plants considered to have moderate alteration had obvious leaf serration and smaller leaf size, but plant vigor was moderate. Plants considered to have a severe phenotype displayed leaf serration, smaller leaf size, in some cases twisted and/or thicker leaves, and greatly reduced vigor. Initial observations also revealed altered petal shape and earlier flowering. Genomic DNA samples extracted

from mature leaves of each sample confirmed the presence of the transgenes in the plants surviving the Basta treatment (Figure 2.5).

To choose lines for further characterization in subsequent generations, the relative expression of the inserted genes was determined on individual, confirmed transgenic plants. Ten samples from each group were evaluated. Normalization of gene expression was performed using two reference genes, *AtSAND* and *AtTIF41L*. Expression level data was compared to the sample displaying the least gene expression within each group. The WT plants displayed *AtKRP2* expression within a very small range (Figure 2.6). The sample showing the highest expression was 3.5-fold higher than that of the sample expressing the lowest level of expression. In the group over-expressing *AtKRP2*, the plant expressing the lowest level of *AtKRP2* nearly matched the level of natural expression in the highest control plants, around 3-fold over the lowest control plant (Figure 2.7). The *AtKRP2* transformants displaying a severe phenotype showed expression levels 20- to 30-fold higher than that observed in plants with a slight leaf alteration phenotype. The expression levels of *AtKRP2* in these lines included the expression of the native gene. The *MdKRP4* plants displaying a severe phenotype displayed 10- to 15-fold increase in expression of *MdKRP4* over the plants with a light phenotype (Figure 2.8). The plants with a severe phenotype in the *MdKRP5* group had expression levels at 5- to 10-fold over the plants with a light phenotype (Figure 2.9).

Discussion:

Over 120 *KRP*s have been identified and their sequences compared in more than 60 plant species (Acosta et al., 2011). However, the study of these genes and other cell cycle genes in economic crops is limited, but is essential to allow for a deeper

understanding of the application of new knowledge in this area. Interestingly, 1,4 Dimethylnapthlene (DMN) has been shown to induce KRP expression in potatoes, so certainly research will now advance further in this area (Campbell et al., 2012). In the current study, two *Malus* × *domestica* cyclin-dependent kinase inhibitors have been transformed into *A. thaliana,* in order to better understand their function in the apple cell cycle.

As MdKRP4 and MdKRP5 are closest in sequence to AtKRP2, this gene was used in this study for comparison. *AtKRP2* overexpression in has been studied in *A. thaliana* previously (DeVeylder et al., 2001). This overexpression has been repeated here, in order to compare directly, using the same constitutive promoter and conditions. DeVeylder et al. (2001) overexpressed *AtKRP2* in *A. thaliana* plants. A reduction in leaf area, an increase in leaf thickness, leaf serration, reduced lateral roots, reduced cell number, increased cell size, and altered, partially sterile flowers were observed as a result of its overexpression. *AtKRP2* was found to be most abundant in flowers. In the T_2 population, the transgene presence could be strictly identified by the leaf serration phenotype. The rate of cell production was affected by the overexpression of *AtKRP2*. The number of cells in the mature leaves of plants overexpressing AtKRP2 was 10-fold fewer than in wild type plants, although early in development, the numbers were only slightly different (DeVeylder et al., 2001). The average cell cycle duration doubled by maturity in *AtKRP2* overexpressing plants, stretching the period between one mitosis phase and the next (DeVeylder et al., 2001). The cell size was dramatically larger in the overexpressing lines, three-fold larger in palisade cells, and six-fold larger in adaxial epidermal cells. The increase in cell size was likely either due to 'compensation' or due to

endoreduplication. *AtKRP2* overexpression in particular clearly affects the cell cycle in *A. thaliana*.

The phenotypes of the rosettes and leaves of the T_1 generation of the transformed plants were visually rated for alteration, and compared among groups in the current study. The effects of the expression of the two *MdKRP* genes appeared similar to that of the overexpression of *AtKRP2*. The transgenic T_1 generation plants displayed an estimated reduction in leaf area, slight increase in leaf thickness, clearly serrated leaves, and altered flower morphology, all of which were seen in the previous study. The addition of *MdKRP4* and *MdKRP5* to the Arabidopsis transcript may have an effect on cell production, as was found in the previous study. The rosettes of the transformed plants in all groups varied in phenotype and were rated as being severe, moderate, or light in their alteration. The altered leaf phenotypes observed ranged from a severe reduction in vigor, estimated reduction in leaf number, and extensive leaf serration, to a slight increase in vigor over the control group, a possible increase in leaf number, and not much leaf serration (Figures 2.4, 2.7, 2.8, and 2.9). In previous *AtKRP2* overexpression, a dose-dependent response was seen, indicated by a weak overexpression resulting in rosettes similar to the control group, without much leaf serration or decrease in vigor, but having increased ploidy indicating endoreduplication (Verkest et al., 2005*a*). Strongly overexpressing *AtKRP2* plants displayed a decrease in endoreduplication coincident with smaller rosettes with reduced vigor (Verkest et al., 2005). Results from this study indicate a range of weak to strong *AtKRP2* overexpression phenotypes that are consistent with the previous studies. Moreover, the phenotypes of Arabidopsis plants expressing the apple *KRP* genes were largely similar to those over-expressing *AtKRP2*.

Leaf serration was observed in all groups of transformants in the fifth or sixth leaf, and was severe in some cases (Figures 2.4, 2.7, 2.8, and 2.9). In conjunction with the leaf serration, a reduction in leaf area was obvious. Increased leaf serration has been observed in the overexpression of many KRP genes, suggesting it is a symptom of reduced cell production more than the direct gene function (DeVeylder et al., 2001; Verkest et al., 2005*b*). In some of the more severe phenotypes, leaves were noticeably thicker, and in some cases twisted, indicating an effect on the cell cycle (Yang et al., 2011). In rice *ICK6* (*KRP4*) overexpression, the cell size was increased on the adaxial side of the leaf over the other side, suggesting also that the cell number was affected, the combination of the two effects resulted in the rolling leaf phenotype (Yang et al., 2011). A 30% increase in leaf thickness was observed in *A. thaliana KRP2* overexpressing plants due to cell expansion occurring primarily in the dorsoventral direction (DeVeylder et al., 2001).

Gene expression was determined for individuals in each group, in order to determine which lines will be further characterized (Figures 2.6, 2.7, 2.8, and 2.9). The *AtKRP2* overexpressing plant with the lowest relative expression level displayed about the same extent of gene expression as the highest native expression of *AtKRP2* in the WT. In most cases, the plants with the highest expression or overexpression displayed the most severe leaf and rosette phenotypes. The individuals with the highest expression will be further studied in future generations.

The phenotypes triggered by the overexpression of *AtKRP2* and the expression of *MdKRP4* and *MdKRP5* are very similar. Therefore, the function and molecular mechanisms may also be related. It is possible with a constitutive promoter at work, that

AtKRP2 overexpressing plants, as well as *MdKRP4* and *MdKRP5* expressing plants, are displaying the effects of the single gene, or the alteration may affect the function of other KRP genes at work in the cell cycle. Generally, when more KRPs are available, CDK activity is reduced, leading to a reduction in cell production (Verkest et al., 2005*b*). The five KRPs identified in apple may have slightly different functions, and would bind to different CDK/cyclin complexes that regulate the cell cycle at different phases or transition points. The possibility also exists that the alternate cell cycle of endoreduplication can be triggered by the influx of various KRPs (Jegu et al., 2013; Jun et al., 2013; Verkest et al., 2005).

Based on the observed phenotype of the T_1 generation, and the expression patterns of the expressed *KRP* genes, this study has demonstrated that *MdKRP4* and *MdKRP5* are likely important regulators of the apple cell cycle, and that their function resembles *AtKRP2* function in the cell cycle. *AtKRP2* displays increased expression when the cell cycle is inhibited (Menges et al., 2005). The increase in *MdKRP4* and *MdKRP5* expression during periods of low cell production further supports the role of negative regulation of the apple cell cycle by these two cyclin-dependent kinase inhibitors (Dash et al., 2012; Malladi and Johnson, 2011). Detailed characterization of later generations, and further study of the relationships between apple KRPs and other cell cycle genes will provide confirmation of the suggested roles of these two important apple genes.

Figures:

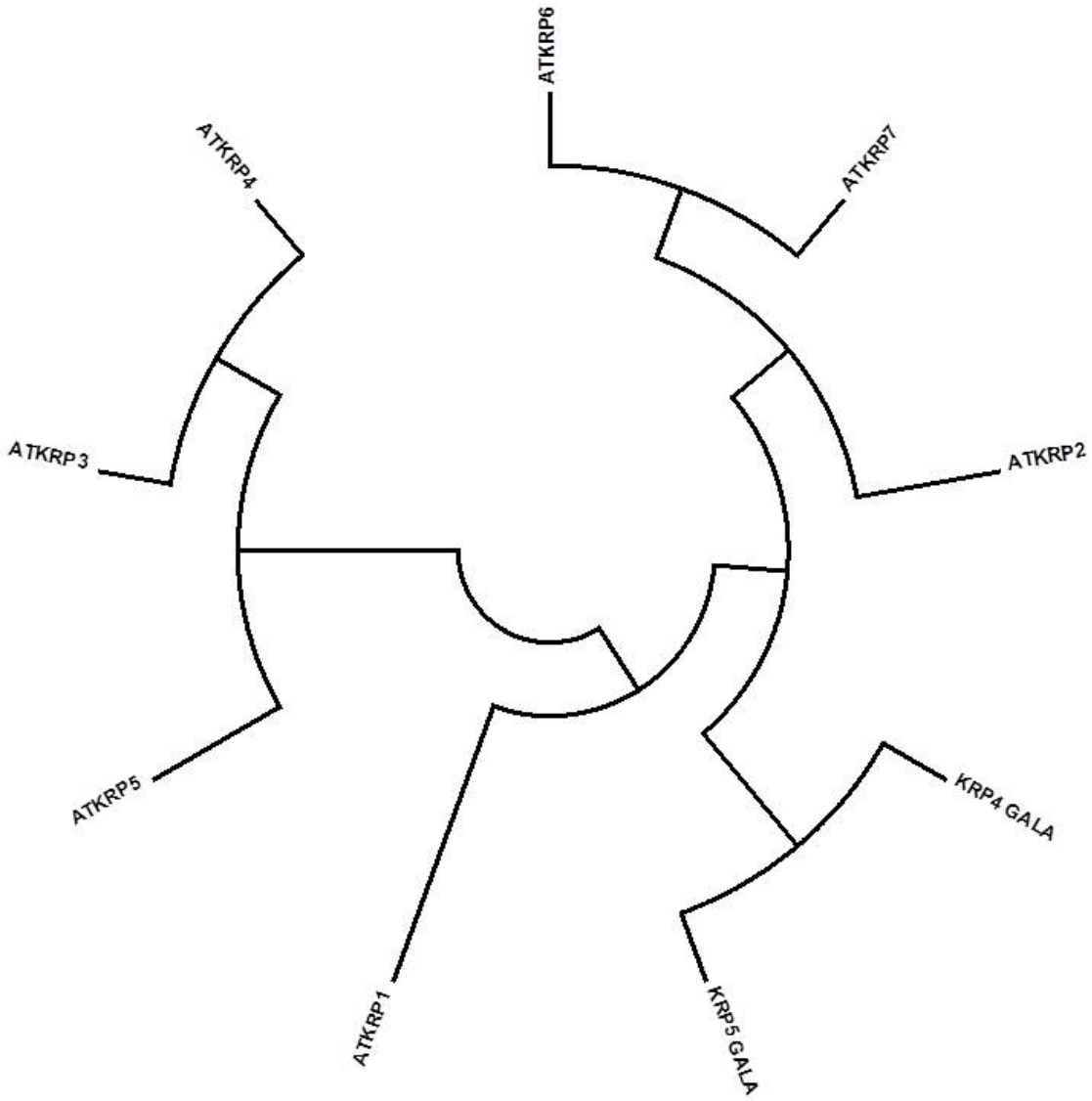

Figure 2.1. Phylogenetic relationships among MdKRP4 and MdKRP5 and seven *Arabidopsis thaliana* KRPs.

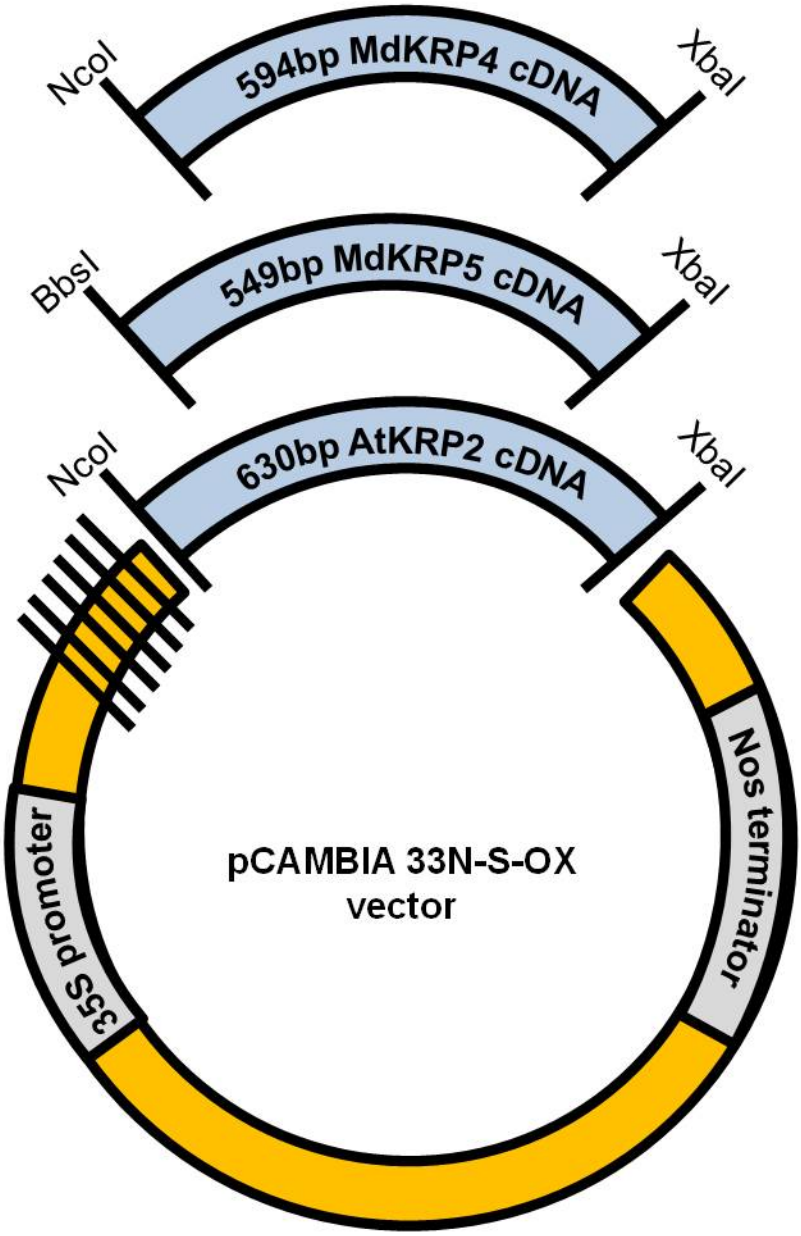

Figure 2.2. Representation of the pCambia 3300N-S-OX vector used to transform *A. thaliana* with *MdKRP4* and *MdKRP5*. It included a 35S promoter region, a Nos terminator region, a Basta-resistance gene, and a multiple cloning site. Inserts cloned individually into the vector were a 594 bp *MdKRP4* cDNA, a 549 bp *MdKRP5* cDNA, and a 630 bp *AtKRP2* cDNA. Cloning sites indicated represent the enzymes used to assemble the constructs.

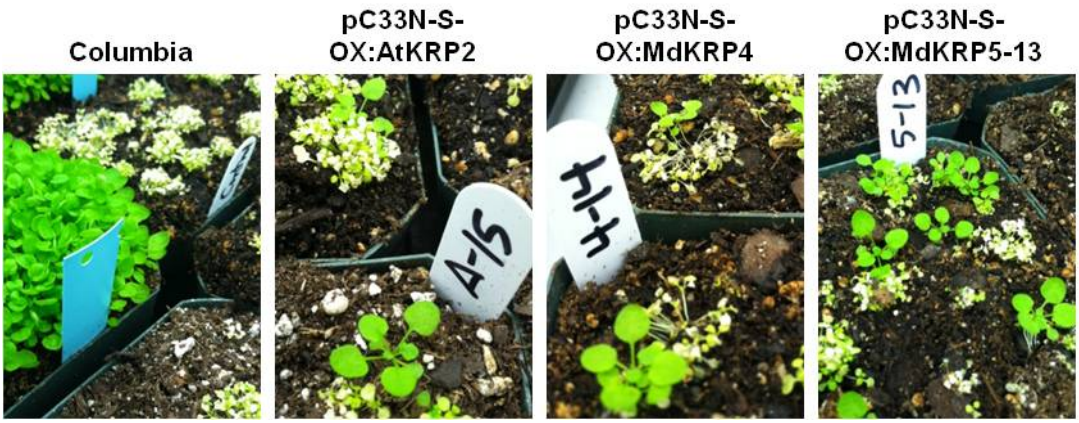

Figure 2.3. Basta herbicide selection of transgenic lines overexpressing *AtKRP2* or expressing the apple *KRP*s. Surviving seedlings were considered at this stage to be transformants.

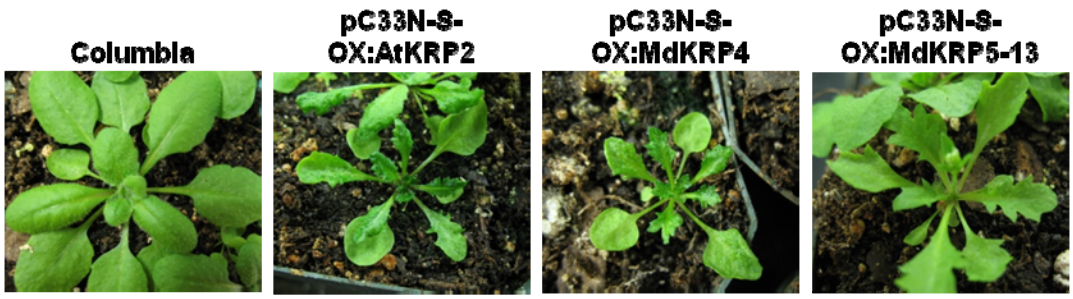

Figure 2.4. Representative plants from each group of *A. thaliana* KRP transformants. In many cases a clear phenotype was observed in the fifth or sixth leaf.

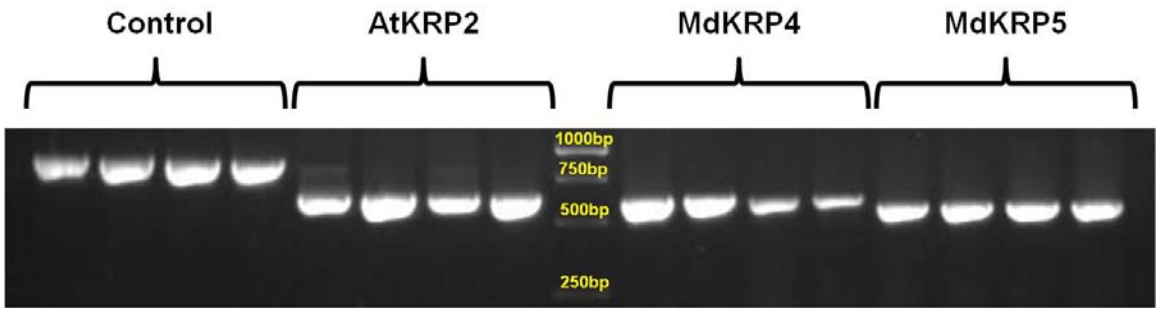

Figure 2.5. PCR confirmation of AtKRP2 in the Columbia wild type control plants and the AtKRP2 overexpressing plants, and MdKRP4 and MdKRP5 in plants expressing those transgenes. The primer amplified the genomic sequence, which included the introns, in the wild type plants.

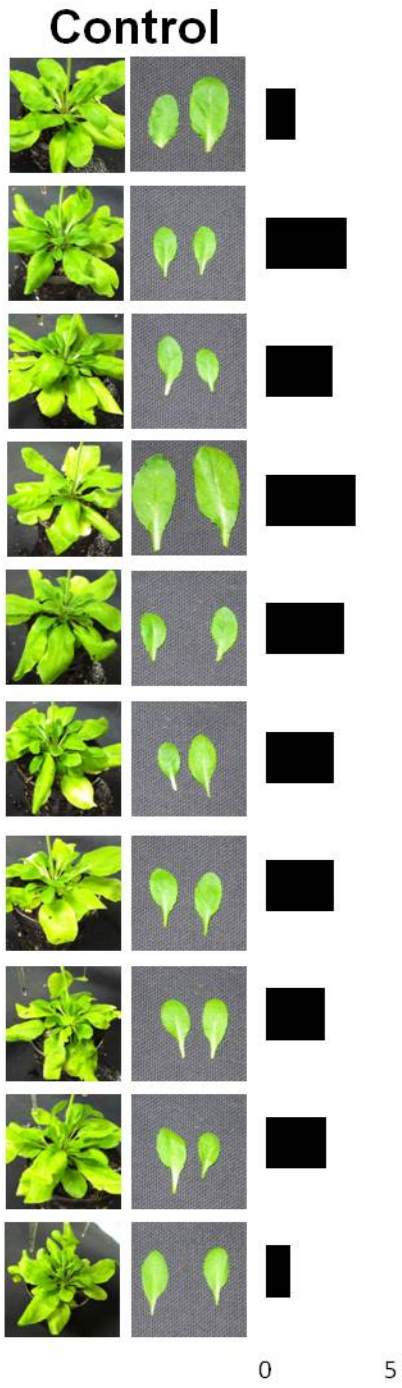

Figure 2.6. Relative expression of *AtKRP2* in Columbia wild type *Arabidopsis thaliana*. Rosette and leaves of individual plants are presented. The two leaves represent new, expanding leaves that were sampled for RNA extraction and determination of gene expression level. Expression of the AtKRP2 gene relative to the expression in the plant with the lowest expression is presented here. Gene expression was normalized using *AtSAND* and *AtTIP4*.

Figure 2.7. Relative expression of *AtKRP2* in *Arabidopsis thaliana* plants overexpressing *AtKRP2*. Rosette and leaves of individual plants are presented. The two leaves represent new, expanding leaves that were sampled for RNA extraction and determination of gene expression level. Expression of the AtKRP2 gene relative to the expression in the plant with the lowest expression is presented here. Gene expression was normalized using *AtSAND* and *AtTIP4*.The sample having the least relative expression displayed a similar expression level as those wild type plants expressing AtKRP2 at the highest level.

Figure 2.8. Relative expression of representative *Arabidopsis thaliana* plants expressing the *MdKRP4* transgene. Rosette and leaves of individual plants are presented. The two leaves represent new, expanding leaves that were sampled for RNA extraction and determination of gene expression level. Expression of the *MdKRP4* gene relative to the expression in the plant with the lowest expression is presented here. Gene expression was normalized using *AtSAND* and *AtTIP4*.

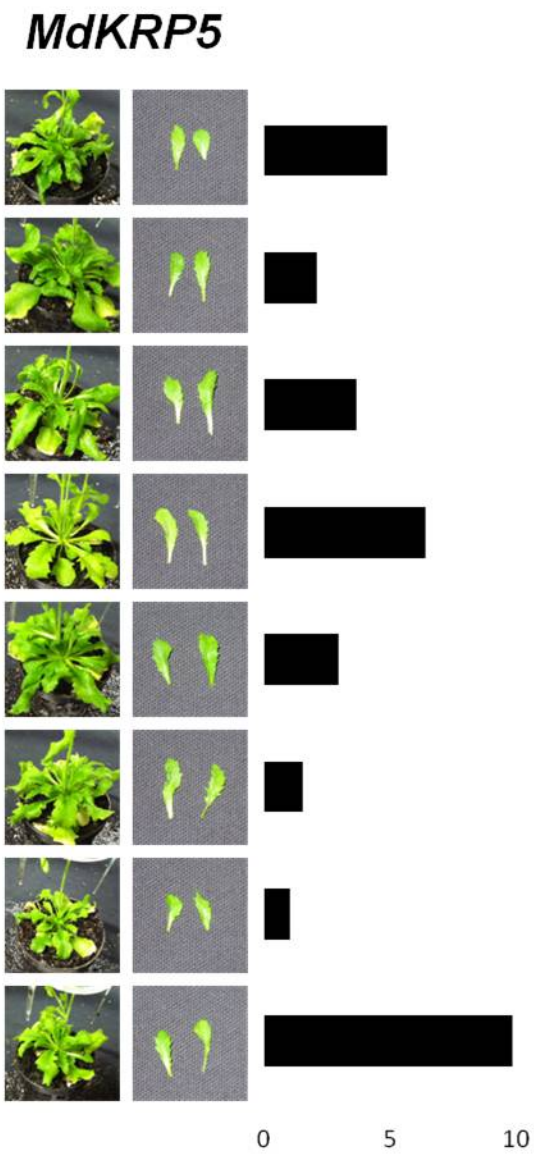

Figure 2.9. Relative expression of representative *Arabidopsis thaliana* plants expressing the *MdKRP5* transgene. Rosette and leaves of individual plants are presented. The two leaves represent new, expanding leaves that were sampled for RNA extraction and determination of gene expression level. Expression of the *MdKRP5* gene relative to the expression in the plant with the lowest expression is presented here. Gene expression was normalized using *AtSAND* and *AtTIP4*.

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CHAPTER 3

IDENTIFICATION OF SINGLE NUCLEOTIDE POLYMORPHISMS IN *MdKRP4* AND *MdKRP5* IN A *MALUS* × *DOMESTICA* POPULATION

Introduction:

Fruit size in apple, an important economic quality trait, is determined through a combination of cell production and cell expansion (Bain and Robertson, 1950; Harada, et al., 2005). A small increase in cell number can significantly change final fruit size (Bain and Robertson, 1950). Cell divisions in plants are regulated by the cell cycle, which is driven by the availability and combinations of several Cyclin-Dependent Kinases (CDKs) and Cyclins (CYCs; DeVeylder et al., 2003; Inze and DeVeylder, 2006). Kip-Related Proteins (KRPs) regulate the cell cycle by inhibiting CDK activity (Dewitte and Murray, 2003; Inze and DeVeylder, 2006; Wang et al., 1997, 2008). Progression of cells through the cell cycle, as well as cell cycle entry and exit involve complex co-ordinations of the activities of these proteins, and others.

Apple is an important economic crop in the United States. Information on apple genes is still limited, although many cell cycle genes have now been identified (Malladi and Johnson, 2011). The draft apple genome became available in 2010 (Velasco, et al., 2010). Major quantitative trait loci have been identified for fruit diameter and weight, but not confirmed. Also, marker assisted breeding has been used in apple but has primarily targeted disease resistance (Kenis et al., 2008; Kumar et al., 2012; Devoghalaere et al., 2012). Results from several studies involving cell cycle genes have revealed that *Malus* ×

domestica KRP4 (*MdKRP4*) and *MdKRP5* may negatively regulate cell production in apple (Dash et al., 2012; Malladi and Johnson, 2011).

Minor differences in gene sequence among genotypes can influence quality traits such as final fruit size. Often, SNPs do not lead to changes in the protein, due to degeneracy in the genetic code. Or, the SNP is located in an intron region, having no effect on the translated protein product. However, in some cases, the amino acid is altered. Often, this change will not have an effect on the protein, but some changes can be significant based on the properties, size, hydrophobicity, polarity, or affinities of the amino acid (Horton et al., 2006). Therefore, identification of SNPs can lead to a better understanding of diversity within populations, and development of SNP markers can contribute tools to breeding programs (Cho et al., 1999; Choi et al., 2007; Comai et al., 2004; Gilchrist et al., 2006; McNally et al., 2009; Zhang and Hewitt, 2003).

As final fruit size is determined by cell production, regulators of the cell cycle in apple are the focus of this study. Examination of the cell cycle in an economic crop allows for meaningful discovery in two areas simultaneously. New discoveries about plant physiology can be made, and the basis of the knowledge can be applied within the crop. The objective of this research is to identify SNPs in *MdKRP4* and *MdKRP5* genomic sequences that affect the amino acid sequence, and therefore may have an effect on the function of these important cell cycle regulators.

Materials and methods:

Plant material:

Leaf tissue samples from 173 accessions were obtained from the USDA-ARS

apple germplasm collection in Geneva, NY. Leaf tissue from 13 common *Malus* × *domestica* cultivars was collected at the University of Georgia Mountain Research and Education Center (Blairsville, GA). In all cases, the tissue was frozen in liquid nitrogen, and stored at -80˚C. Fruit width data was obtained from the United States Department of Agriculture, through the Agricultural Research Service Germplasm Resource Information Network (GRIN) database.

DNA extraction:

The tissue was ground in liquid nitrogen using a mortar and pestle, and genomic DNA was extracted from approximately 100 mg of the tissue, using the Omega Bio-tek Plant DNA kit (Omega Bio-tek, Norcross, GA). The quality of the DNA was analyzed using spectrophotometry, and the samples were diluted to a concentration of 10 ng/µl.

DNA amplification:

Polymerase chain reaction (PCR) was performed to amplify the the gene fragments, using Phusion Hot Start II high fidelity DNA polymerase (Thermo Scientific, Pittsburgh, PA) and a primer concentration of 0.5 µM. Six 25 µl reactions were performed for each sample, using 50 ng of template per reaction, with the reaction conditions of 98 °C for 30 sec; 35 cycles of 98 °C for 10 sec, 65 °C for 30 sec, and 72 °C for 1 min; 72 °C for 10 min. The primers were designed to amplify the genomic sequence of the required genes, using the published *Malus* × *domestica* 'Golden Delicious' genome available from Fondazione Edmund Mach Istituto Agrario Di San Michele All'Adige Computational Biology Web Resources. Primer sequences were as follows: *MdKRP4* forward CCATCATTATCGTCGTCATCGCACTC; reverse CGTACGCAGAACAAACTGCTGCT; *MdKRP5* forward

CCGTCCAACGGACTCGTCATC; reverse GCGAAGAACGGAACTTAATTAGGAGAACC. The expected genomic DNA product for *MdKRP4* was 1440 bp and the expected genomic DNA product for *MdKRP5* was 1317 bp.

The PCR products were loaded into a 1.8% agarose gel and electrophoresis was performed at 83 V for 90 min. Bands corresponding to the expected size were excised. The products were purified using the Omega Bio-tek MicroElute Gel Extraction kit (Omega Bio-tek, Norcross, GA). The samples were then diluted to a concentration of 20 ng/µl.

Sequencing:

The Big Dye Terminator v.3.1 Cycle Sequencing kit (Life Technologies Co., Grand Island, NY) was used to perform sequencing reactions using 40 ng of template and a primer concentration of 0.2 µM. The reaction conditions were as follows: 96 °C for 2 min; 39 cycles of 96 °C for 20 sec, 50 °C for 5 sec, and 60 °C for 2 min 15 sec; then 60 °C for 8 min. The samples were purified using columns of Sephadex g-50 fine powder (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA), and the resulting products were submitted to the Georgia Genomics Facility (Athens, GA) for Sanger sequencing using an Applied Biosystems 3730xl 96-capillary DNA Analyzer (Life Technologies Corporation, Carlsbad, CA, USA).

Data analysis:

The forward and reverse overlapping reads were assembled using Sequencher software and compared to the published apple genome sequence. The sequences were compared and translated into amino acid sequences using Sequencher and the exons

located from the published apple genome sequence. The amino acid sequences were compared using Clustal Omega and MEGA5 (Hall, 2013; Tamura et al., 2011). Phylogenetic analyses were performed using MEGA5. Further, Provean, NetPhos 2.0, and ePESTfind software programs were used to determine the potential effects of some of the amino acid changes on protein function.

Results:

The USDA apple germplasm collection in Geneva, NY provided 173 accessions identified here by six digit PI numbers, and 13 more genotypes were collected in north Georgia, totaling 186 genotypes available for evaluation. The genotypes ranged in fruit size from 37 to 99 mm (Figure 3.1). A total of seventy genotypes were successfully evaluated in this study. The DNA extraction, PCR amplification, purification and sequencing reactions resulted in complete, comparable sequences of *MdKRP4* for 59 genotypes, and sequences of *MdKRP5* for 38 genotypes.

The sequences used for *MdKRP4* corresponded to USDA genotypes having fruit width of 37 to 96 mm (Figure 3.1). The sequences used for *MdKRP5* corresponded to USDA genotypes having fruit width of 40 to 96 mm. Accessions with fruit width data from the USDA GRIN database up to 50 mm were considered small, from 50.5 to 70 mm were considered medium, and from 70.5 mm and up were considered to be large.
The full length genomic sequence of *MdKRP4* is 1099 bp, containing four exons. The coding region is 594 bp long and encodes a 198-aa protein product (Figure 3.2). The sequence of *MdKRP5* is 1030 bp long and contains four exons. The coding region is 549

bp and encodes a 183-aa protein product. The genomic sequences of the two genes compared within one genotype (‘Gala’) share 84% identity with each other.

*S*ingle nucleotide polymorphisms and amino acid changes in *MdKRP4*:

Within the genomic sequence of the fifty-nine genotypes studied and the published genome, *MdKRP4* features sixteen substitutions, eight insertions, and five deletions (Table 3.1). An analysis of these 29 modifications revealed that sixteen of the genotypes displayed these base changes. PI 158731 displayed two unique insertions as well as eight substitutions and two other insertions which were also observed in some other genotypes. PI 162716 displayed nine substitutions and three deletions. PI 589472 displays two unique deletions as well as nine substitutions and three other deletions. PI 589711 displays a unique substitution as well as nine other substitutions and three deletions. 'Red Fuji' displays a unique substitution, two unique insertions, as well as another substitution and two other insertions. PI 437038 displays two unique substitutions and a unique insertion, as well as seven other substitutions and two other insertions.

The locations of these modifications can determine whether or not they will translate into a protein product modification. None of the insertions or deletions was found to be within the exons of the *MdKRP4* sequence (Table 3.1). However, several substitutions were observed within the exons. Exon 1 included: a substitution at g.61G>A in three genotypes, g.164C>T in four genotypes (the draft genome sequence indicates a Y at this location), g.176A>C in five genotypes (the draft genome sequence indicates a M at this location), and g.205T>A in eight genotypes. Exon 3 included: a substitution at g.767A>G in seven genotypes, 'Red Fuji,' and the published genome, g.772C>G in two

genotypes, and g.854C>T in three genotypes and the published genome. Exon 4 displayed a substitution at g.1097G>A in three genotypes. The other substitutions, in additions to some insertions and deletions, were found in the intron portions of the *MdKRP4* sequences.

The translated and compared amino acid sequences for MdKRP4 display only a few substitutions (Figure 3.3). In the sequences for PI 162716, PI 589472, and PI 589711 there is a substitution of p.Ala21Thr. At p.Ser55Phe, a substitution is found in PI 158731, PI 162716, PI 589472, and PI 589711. A substitution at p.Glu59Ala is found in the same group, as well as in PI 437038. A substitution at p.Phe69Ile is found in the MdKRP4 protein sequences of PI 104799, PI 123960, PI 143180, PI 158730, PI 162544, PI 175545, PI 188606, and PI 589674. A substitution at p.Thr126Arg is found in PI 162716 and PI 589472. A p.Lys175Met substitution is found in PI 437038. A p.Arg194Gln substitution is found in PI 158731, PI 437038, and PI 589711. Analysis of the predicted protein structure using Provean protein suggested that only two of these amino acid variants may affect protein function, p.Ser55Phe and p.Lys175Met. The other substitutions were found to have neutral effects on the predicted protein structure. Phylogenetic analysis of MdKRP4 sequences using Mega5 displays the relationships among these genotypes (Figure 3.4).

Single nucleotide polymorphisms and amino acid changes in *MdKRP5*:

Within the genomic sequence of the thirty-eight genotypes studied and the draft genome sequence, *MdKRP5* features eleven substitutions and two insertions (Table 3.2). An analysis of these 13 modifications reveals thirty-four of the genotypes have been affected by the base changes. The *MdKRP5* sequence of 'Gala' contains a unique

insertion, as well as six substitutions and one other insertion. There were no insertions or deletions found in the exons of MdKRP5, only substitutions. Exon 1 included a substitution at g.99C>T, exon 2 included a substitution at g.396C>T, exon 3 included a substitution at g.690C>G, and exon 4 included a substitution at g.982G>C.

The translated and compared amino acid sequences for MdKRP5 display just one substitution (Figure 3.5). A p.Pro111Ala substitution is found in the sequences of PI 162722, 'Gala,' 'Golden Smoothee,' and 'Pink Lady.' Analysis of the predicted protein structure using Provean protein suggested that this variant may have a neutral effect on the predicted protein structure. Phylogenetic analysis of MdKRP5 sequences using Mega5 displays the relationships among the genotypes studied (Figure 3.6).

Discussion:

Plant KRP family proteins in plants bind to and inhibit the CDK complexes which are the driving force behind the cell cycle (Dewitte and Murray, 2003; Inze and DeVeylder, 2006; Wang et al., 1997, 2008). Specifically, AtKRP2 inhibits growth by negatively influencing the rate and/or duration of cell production (DeVeylder et al., 2001). *MdKRP4* and *MdKRP5* appear to have similar functions in apple (Malladi and Johnson, 2010). Minor changes in protein sequence can lead to alterations in function and stability.

A sequencing approach was used to identify single nucleotide polymorphisms in the genomic sequences of *MdKRP4* and *MdKRP5*. The number of changes revealed by the *MdKRP4* sequences was 26.4 per kb, and 12.6 per kb in *MdKRP5*. Both genes have well above the average number of SNPs in the published draft apple genome, which is 4.8 SNPs per kb (Velasco et al., 2010). The regions conserved across the evaluated

genotypes are also important. In the C-terminal region of MdKRP4, the most conserved motifs in the seven *A. thaliana KRP*s are also found intact. These motifs, 186-PLEGRYEW, 173-FIEKYNYD, and 156-ELEEFFAATE are conserved in identified regions of cyclin-dependent kinase inhibitors across many species (Acosta et al., 2011). When fifteen residues within this C-terminal region were removed in transgenic A. thaliana plants, the ability to bind to CDK was lost, suggesting this region is critical for its function (Zhou et al., 2003*a*). Up to 31 residues in this region are referred to as the CDK/cyclin interacting /inhibiting domain, are conserved across many species, and are considered to be required for KRP function (Acosta et al., 2011; DeVeylder er al., 2001; Jasinski et al., 2002; Lui et al., 2000; Schnittger et al., 2003; Wang et al., 1997, 2008; Zhou et al., 2003*a*, 2003*b*).

Seven out of the eight SNPs found in the coding region of *MdKRP4* were determined to be missense non-synonymous variations, leading to a change in amino acid sequence. One of these, the substitution at p.Ser55Phe, stands out as having the potential to alter KRP4 function. The SNP in exon 1 of MdKRP4, a substitution at g.164C>T in four genotypes leads to this change. This particular variation results in the loss of a potential phosphorylation site (Serine in location 55), based on analysis using NetPhos 2.0. The genotypes having this variation, PI 158731, PI 162716, PI 589472, and PI 589711, have in common a small fruit phenotype. Smaller fruit contains fewer cells, suggesting that this variation may lead to altered cell production. Phosphorylation of S55 may affect the regulation or stability of KRP expression and thereby alter cell production. Phosphorylation of AtKRP6 and AtKRP7 has been recently demonstrated to affect their functions, suggesting that it is an important mechanism of regulation of KRP activity

(Guerinier et al., 2013). Phosphorylation of a Serine in a similar sequence context within a CDK inhibitor, in yeast, disrupts coordination between cell expansion and production (Coccetti et al., 2004). The Serine is present within the motif SEAEEE, and the Phenylalanine variation alters this motif to FEAEAE, in the four small fruit genotypes.

Another modification, revealed by ePESTfind analysis, suggests that the typical MdKRP4 amino acid sequence, represented by 'Gala' in this case contains one predicted potential protein cleavage site, whereas the altered genotype represented by PI 589711 contains two predicted cleavage sites, known as PEST motifs (PEST score >7.0). The substitution in Exon 1 of g.61G>A, leading to p.Ala21Thr substitution in the three genotypes of PI 162716, PI 589472, and PI 589711 is responsible for this modification. These regions are known to be rich in Proline, Glutamic Acid, Serine, and Threonine, and this motif may target the protein for degradation (Rogers et al., 1986; Rechsteiner and Rogers, 1996). Proteolysis has been shown to regulate AtKRP2 abundance (Verkest et al., 2005). Degradation of MdKRP4 could allow for higher CDK activity, leading to a longer period of cell production, or an increased rate of cell production.

In exon 3 of MdKRP5, the substitution at g.690C>G is the only non-synonymous SNP, leading to a p.Pro111Ala substitution in the sequences of PI 162722, 'Gala,' 'Golden Smoothee,' and 'Pink Lady.' This substitution does not lead to the loss of a phosphorylation site, nor is it predicted to be deleterious. Also, there is no addition of a PEST motif. The similarity of sequences of MdKRP5 across the 38 genotypes in this study can indicate that the protein function or at least its stability is stably conserved (Horton et al., 2006).

These results provide novel information regarding the genomic and amino acid sequences of *MdKRP4* and *MdKRP5*. The identification of SNPs in the *MdKRP4* sequence of four small-fruited genotypes in a *Malus* × *domestica* population may provide a clue in determining its function in the cell cycle. Examination of predicted three-dimensional structure may provide further insights into MdKRP4 and MdKRP5 function.

Tables:

Table 3.1. Locations and details of variations in *MdKRP4* genomic DNA sequences of fifty-nine genotypes and the draft genome. Consensus sequence was used to determine what constituted a variation.

	Variation details and location	Accessions displaying variation in *MdKRP4*
Substitutions	g.61G>A	PI 162716, PI 589472, PI 589711
	g.164C>T	PI 158731, PI 162716, PI 589472, PI 589711, genome Y
	g.176A>C	PI 158731, PI 162716, PI 437038, PI 589472, PI 589711, genome M
	g.205T>A	PI 104799, PI 123960, PI 143180, PI 158730, PI 162544, PI 175545, PI 188606, PI 589674
	g.561A>C	'Red Fuji'
	g.596C>T	PI 158731, PI 162716, PI 589472, PI 589711
	g.648A>C	PI 589711
	g.767A>G	PI 158731, PI 162716, PI 392312, PI 437038, PI 589472, PI 589711, PI 589852, 'Red Fuji,' genome
	g.772C>G	PI 162716, PI 589472
	g.854C>T	PI 162716, PI 437038, PI 589472, genome
	g.919A>T	PI 437038
	g.935A>T	PI 158731, PI 162716, PI 437038, PI 589472, PI 589711
	g.954G>A	PI 158731, PI 162716, PI 437038, PI 589472, PI 589711, genome R
	g.963T>A	PI 437038
	g.998G>A	PI 158731, PI 437038, PI 589711, genome R
	g.1097G>A	PI 158731, PI 437038, PI 589711
Insertions	g.524_525GACACACACA	'Red Fuji'
	g.526_527CATA	genome
	g.526_527CACA	'Red Fuji'
	g.526_527TATA	PI 158731
	g.526_527CA	PI 437038
	g.557_558A	PI 104799, PI 123960, PI 143180, PI 158730, PI 158731, PI 162544, PI 175545, PI 188606, PI 437038, PI 589674, 'Red Fuji,' genome
	g.558_559T	PI 104799, PI 123960, PI 143180, PI 158730, PI 158731, PI 162544, PI 175545, PI 188606, PI 437038, PI 589674, 'Red Fuji,' genome
	g.946_947T	PI 158731
Deletions	g.372delG	PI 158731, PI 162716, PI 437038, PI 589472, PI 589711
	g.389delT	PI 158731, PI 162716, PI 589472, PI 589711
	g.556_558delATA	PI 162716, PI 589472, PI 589711
	g.708delT	PI 589472
	g.710delT	PI 589472

Table 3.2. Locations and details of variations in *MdKRP5* genomic DNA sequences of thirty-eight genotypes and the draft genome. Consensus sequence was used to determine what constituted a variation.

	Variation details and location	Accessions displaying variation in *MdKRP5*
Substitutions	g.99C>T	PI 123960, PI 161839, PI162724, PI 187352, PI 324523, PI 392312, PI 589472, PI 589565, PI 589588, PI 589650, PI 589674, PI 589688, PI 589692, PI 590144, 'Arkansas Black'
	g.310T>C	PI 162722, 'Gala,' 'Golden Smoothee,' 'Pink Lady,' genome Y
	g.314C>A	PI 162722, 'Gala,' 'Golden Smoothee,' 'Pink Lady,' genome M
	g.324A>G	PI 162722, 'Gala,' 'Golden Smoothee,' 'Pink Lady,' genome R
	g.396C>T	PI 123960, PI 161839, PI162724, PI 187352, PI 324523, PI 392312, PI 589472, PI 589565, PI 589588, PI 589650, PI 589674, PI 589688, PI 589692, PI 590144, 'Arkansas Black'
	g.476A>G	PI 123960, PI 161839, PI162724, PI 187352, PI 324523, PI 392312, PI 589472, PI 589565, PI 589588, PI 589650, PI 589674, PI 589688, PI 589692, PI 590144, 'Arkansas Black'
	g.690C>G	PI 162722, 'Gala,' 'Golden Smoothee,' 'Pink Lady,' genome S
	g.849G>T	PI 162722, 'Gala,' 'Golden Smoothee,' 'Pink Lady,' genome K
	g.856A>T	PI 123960, PI 161839, PI162724, PI 187352, PI 324523, PI 392312, PI 589472, PI 589565, PI 589588, PI 589650, PI 589674, PI 589688, PI 589692, PI 590144, 'Arkansas Black'
	g.863T>A/G	PI 162722, 'Gala,' 'Golden Smoothee,' 'Pink Lady,' genome K
	g.982G>C	PI 158730, PI 162716, PI 173986, PI 175545, PI 344551,PI 588799, PI 589689, PI 589852, PI 590133, 'Detroit,' 'Empire,' 'Honeycrisp,' 'Wild1,' 'Wild 2,' 'Wild 3'
Insertions	g.601_602CA	PI 162722, 'Gala,' 'Golden Smoothee,' 'Pink Lady,' genome
	g.663_664T	'Gala'

Figures:

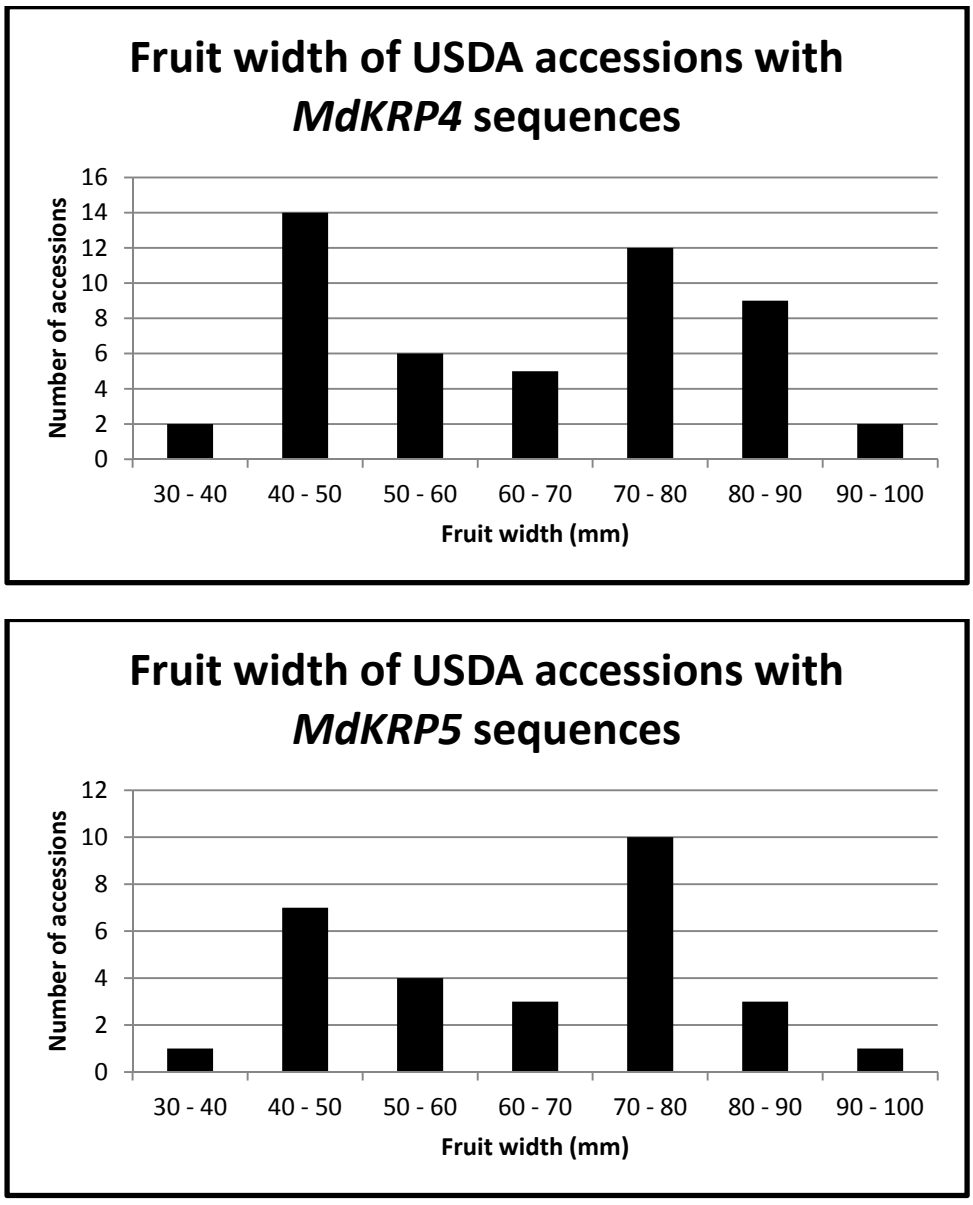

Figure 3.1. Number and fruit size of USDA accessions for which this study provided sequence data for *MdKRP4* and *MdKRP5*.

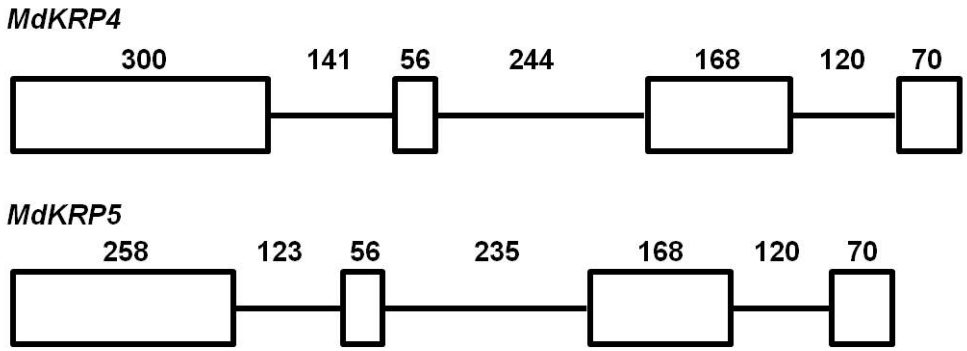

Figure 3.2. Representation of *MdKRP4* and *MdKRP5* including exon and intron regions. Open bars indicate exons.

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CLUSTAL O(1.2.0) multiple sequence alignment


PI162716              MELARAATSANAVRKRKAGSTDGESVELPSSSSYDVDQRKKPRRRVVVVRSAPKFEAEAE
PI589472              --LARAATSANAVRKRKAGSTDGESVELPSSSSYDVDQRKKPRRRVVVVRSAPKFEAEAE
PI158731              MELARAATSANAVRKRKAGSADGESVELPSSSSYDVDQRKKPRRRVVVVRSAPKFEAEAE
PI589711              MELARAATSANAVRKRKAGSTDGESVELPSSSSYDVDQRKKPRRRVVVVRSAPKFEAEAE
PI437038              MELARAATSANAVRKRKAGSADGESVELPSSSSYDVDQRKKPRRRVVVVRSAPKSEAEAE
GENOME_MDKRP4         MELARAATSANAVRKRKAGSADGESVELPSSSSYDVDQRKKPRRRVVVVRSAPKXEAEXE
PI589674              MELARAATSANAVRKRKAGSADGESVELPSSSSYDVDQRKKPRRRVVVVRSAPKSEAEEE
PI104799              MELARAATSANAVRKRKAGSADGESVELPSSSSYDVDQRKKPRRRVVVVRSAPKSEAEEE
PI123960              MELARAATSANAVRKRKAGSADGESVELPSSSSYDVDQRKKPRRRVVVVRSAPKSEAEEE
PI175545              MELARAATSANAVRKRKAGSADGESVELPSSSSYDVDQRKKPRRRVVVVRSAPKSEAEEE
PI143180              MELARAATSANAVRKRKAGSADGESVELPSSSSYDVDQRKKPRRRVVVVRSAPKSEAEEE
PI158730              MELARAATSANAVRKRKAGSADGESVELPSSSSYDVDQRKKPRRRVVVVRSAPKSEAEEE
PI162544              MELARAATSANAVRKRKAGSADGESVELPSSSSYDVDQRKKPRRRVVVVRSAPKSEAEEE
PI188606              MELARAATSANAVRKRKAGSADGESVELPSSSSYDVDQRKKPRRRVVVVRSAPKSEAEEE
PI162722              --LARAATSANAVRKRKAGSADGESVELPSSSSYDVDQRKKPRRRVVVVRSAPKSEAEEE
PI588943              --LARAATSANAVRKRKAGSADGESVELPSSSSYDVDQRKKPRRRVVVVRSAPKSEAEEE
PI589598              -ELARAATSANAVRKRKAGSADGESVELPSSSSYDVDQRKKPRRRVVVVRSAPKSEAEEE
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PI588998              MELARAATSANAVRKRKAGSADGESVELPSSSSYDVDQRKKPRRRVVVVRSAPKSEAEEE
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PI589689              MELARAATSANAVRKRKAGSADGESVELPSSSSYDVDQRKKPRRRVVVVRSAPKSEAEEE
PI590133              MELARAATSANAVRKRKAGSADGESVELPSSSSYDVDQRKKPRRRVVVVRSAPKSEAEEE
PI589852              MELARAATSANAVRKRKAGSADGESVELPSSSSYDVDQRKKPRRRVVVVRSAPKSEAEEE
PI589688              MELARAATSANAVRKRKAGSADGESVELPSSSSYDVDQRKKPRRRVVVVRSAPKSEAEEE
PI392312              MELARAATSANAVRKRKAGSADGESVELPSSSSYDVDQRKKPRRRVVVVRSAPKSEAEEE
PI590179              MELARAATSANAVRKRKAGSADGESVELPSSSSYDVDQRKKPRRRVVVVRSAPKSEAEEE
PI161846              MELARAATSANAVRKRKAGSADGESVELPSSSSYDVDQRKKPRRRVVVVRSAPKSEAEEE
PI264558              MELARAATSANAVRKRKAGSADGESVELPSSSSYDVDQRKKPRRRVVVVRSAPKSEAEEE
PI589692              MELARAATSANAVRKRKAGSADGESVELPSSSSYDVDQRKKPRRRVVVVRSAPKSEAEEE
PI589183              MELARAATSANAVRKRKAGSADGESVELPSSSSYDVDQRKKPRRRVVVVRSAPKSEAEEE
ARKANSAS_BLACK        MELARAATSANAVRKRKAGSADGESVELPSSSSYDVDQRKKPRRRVVVVRSAPKSEAEEE
DETROIT               MELARAATSANAVRKRKAGSADGESVELPSSSSYDVDQRKKPRRRVVVVRSAPKSEAEEE
GALA                  MELARAATSANAVRKRKAGSADGESVELPSSSSYDVDQRKKPRRRVVVVRSAPKSEAEEE
GOLDEN_SMOOTHEE       MELARAATSANAVRKRKAGSADGESVELPSSSSYDVDQRKKPRRRVVVVRSAPKSEAEEE
PINK_LADY             MELARAATSANAVRKRKAGSADGESVELPSSSSYDVDQRKKPRRRVVVVRSAPKSEAEEE
RED_FUJI              MELARAATSANAVRKRKAGSADGESVELPSSSSYDVDQRKKPRRRVVVVRSAPKSEAEEE
PI127311              MELARAATSANAVRKRKAGSADGESVELPSSSSYDVDQRKKPRRRVVVVRSAPKSEAEEE
PI131828              MELARAATSANAVRKRKAGSADGESVELPSSSSYDVDQRKKPRRRVVVVRSAPKSEAEEE
PI136243              MELARAATSANAVRKRKAGSADGESVELPSSSSYDVDQRKKPRRRVVVVRSAPKSEAEEE
PI136604              MELARAATSANAVRKRKAGSADGESVELPSSSSYDVDQRKKPRRRVVVVRSAPKSEAEEE
PI148503              MELARAATSANAVRKRKAGSADGESVELPSSSSYDVDQRKKPRRRVVVVRSAPKSEAEEE
PI161830              MELARAATSANAVRKRKAGSADGESVELPSSSSYDVDQRKKPRRRVVVVRSAPKSEAEEE
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PI158731              SVRTSNDDFSTSDHAESSCCSSNGSSVLVEDRKFEFVDPEADESEQVESSTYNSSRDERR
PI589711              SVRTSNDDFSTSDHAESSCCSSNGSSVLVEDRKFEFVDPEADESEQVESSTYNSSRDERR
PI437038              SVRTSNDDFSTSDHAESSCCSSNGSSVLVEDRKFEFVDPEADESEQVESSTYNSSRDERR
GENOME_MDKRP4         SVRTSNDDFSTSDHAESSCCSSNGSSVLVEDRKFEFVDPEADESEQVESSTYNSSRDERR
PI589674              SVRTSNDDISTSDHAESSCCSSNGSSVLVEDRKFEFVDPEADESEQVESSTYNSSRDERR
PI104799              SVRTSNDDISTSDHAESSCCSSNGSSVLVEDRKFEFVDPEADESEQVESSTYNSSRDERR
PI123960              SVRTSNDDISTSDHAESSCCSSNGSSVLVEDRKFEFVDPEADESEQVESSTYNSSRDERR
PI175545              SVRTSNDDISTSDHAESSCCSSNGSSVLVEDRKFEFVDPEADESEQVESSTYNSSRDERR
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PI588998              SVRTSNDDFSTSDHAESSCCSSNGSSVLVEDRKFEFVDPEADESEQVESSTYNSSRDERR
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PI590133              SVRTSNDDFSTSDHAESSCCSSNGSSVLVEDRKFEFVDPEADESEQVESSTYNSSRDERR
PI589852              SVRTSNDDFSTSDHAESSCCSSNGSSVLVEDRKFEFVDPEADESEQVESSTYNSSRDERR
PI589688              SVRTSNDDFSTSDHAESSCCSSNGSSVLVEDRKFEFVDPEADESEQVESSTYNSSRDERR
PI392312              SVRTSNDDFSTSDHAESSCCSSNGSSVLVEDRKFEFVDPEADESEQVESSTYNSSRDERR
PI590179              SVRTSNDDFSTSDHAESSCCSSNGSSVLVEDRKFEFVDPEADESEQVESSTYNSSRDERR
PI161846              SVRTSNDDFSTSDHAESSCCSSNGSSVLVEDRKFEFVDPEADESEQVESSTYNSSRDERR
PI264558              SVRTSNDDFSTSDHAESSCCSSNGSSVLVEDRKFEFVDPEADESEQVESSTYNSSRDERR
PI589692              SVRTSNDDFSTSDHAESSCCSSNGSSVLVEDRKFEFVDPEADESEQVESSTYNSSRDERR
PI589183              SVRTSNDDFSTSDHAESSCCSSNGSSVLVEDRKFEFVDPEADESEQVESSTYNSSRDERR
ARKANSAS_BLACK        SVRTSNDDFSTSDHAESSCCSSNGSSVLVEDRKFEFVDPEADESEQVESSTYNSSRDERR
DETROIT               SVRTSNDDFSTSDHAESSCCSSNGSSVLVEDRKFEFVDPEADESEQVESSTYNSSRDERR
GALA                  SVRTSNDDFSTSDHAESSCCSSNGSSVLVEDRKFEFVDPEADESEQVESSTYNSSRDERR
GOLDEN_SMOOTHEE       SVRTSNDDFSTSDHAESSCCSSNGSSVLVEDRKFEFVDPEADESEQVESSTYNSSRDERR
PINK_LADY             SVRTSNDDFSTSDHAESSCCSSNGSSVLVEDRKFEFVDPEADESEQVESSTYNSSRDERR
RED_FUJI              SVRTSNDDFSTSDHAESSCCSSNGSSVLVEDRKFEFVDPEADESEQVESSTYNSSRDERR
PI127311              SVRTSNDDFSTSDHAESSCCSSNGSSVLVEDRKFEFVDPEADESEQVESSTYNSSRDERR
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PI136604              SVRTSNDDFSTSDHAESSCCSSNGSSVLVEDRKFEFVDPEADESEQVESSTYNSSRDERR
PI148503              SVRTSNDDFSTSDHAESSCCSSNGSSVLVEDRKFEFVDPEADESEQVESSTYNSSRDERR
PI161830              SVRTSNDDFSTSDHAESSCCSSNGSSVLVEDRKFEFVDPEADESEQVESSTYNSSRDERR
PI173986              SVRTSNDDFSTSDHAESSCCSSNGSSVLVEDRKFEFVDPEADESEQVESSTYNSSRDERR
PI214080              SVRTSNDDFSTSDHAESSCCSSNGSSVLVEDRKFEFVDPEADESEQVESSTYNSSRDERR
PI588745              SVRTSNDDFSTSDHAESSCCSSNGSSVLVEDRKFEFVDPEADESEQVESSTYNSSRDERR
PI588986              SVRTSNDDFSTSDHAESSCCSSNGSSVLVEDRKFEFVDPEADESEQVESSTYNSSRDERR
PI589018              SVRTSNDDFSTSDHAESSCCSSNGSSVLVEDRKFEFVDPEADESEQVESSTYNSSRDERR
PI589167              SVRTSNDDFSTSDHAESSCCSSNGSSVLVEDRKFEFVDPEADESEQVESSTYNSSRDERR
PI589185              SVRTSNDDFSTSDHAESSCCSSNGSSVLVEDRKFEFVDPEADESEQVESSTYNSSRDERR
PI589362              SVRTSNDDFSTSDHAESSCCSSNGSSVLVEDRKFEFVDPEADESEQVESSTYNSSRDERR
PI589565              SVRTSNDDFSTSDHAESSCCSSNGSSVLVEDRKFEFVDPEADESEQVESSTYNSSRDERR
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PI590130              SVRTSNDDFSTSDHAESSCCSSNGSSVLVEDRKFEFVDPEADESEQVESSTYNSSRDERR
PI632625              SVRTSNDDFSTSDHAESSCCSSNGSSVLVEDRKFEFVDPEADESEQVESSTYNSSRDERR
WILD1                 SVRTSNDDFSTSDHAESSCCSSNGSSVLVEDRKFEFVDPEADESEQVESSTYNSSRDERR
WILD2                 SVRTSNDDFSTSDHAESSCCSSNGSSVLVEDRKFEFVDPEADESEQVESSTYNSSRDERR
WILD3                 SVRTSNDDFSTSDHAESSCCSSNGSSVLVEDRKFEFVDPEADESEQVESSTYNSSRDERR
ROME_BEAUTY           SVRTSNDDFSTSDHAESSCCSSNGSSVLVEDRKFEFVDPEADESEQVESSTYNSSRDERR
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PI158731              EMTAPTSEVRAEAESTAEPKEVESQRRSPPVVNVSELEEFFAATEKESQQKFIEKYNYDV
PI589711              EMTAPTSEVRAEAESTAEPKEVESQRRSPPVVNVSELEEFFAATEKESQQKFIEKYNYDV
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GENOME_MDKRP4         EMTAPTSEVRAEAESTAEPKEVESQRRSPPVVNVSELEEFFAATEKESQQKFIEKYNYDV
PI589674              EMTAPTSEVRAEAESTAEPKEVESQRRSPPVVNVSELEEFFAATEKESQQKFIEKYNYDV
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PI123960              EMTAPTSEVRAEAESTAEPKEVESQRRSPPVVNVSELEEFFAATEKESQQKFIEKYNYDV
PI175545              EMTAPTSEVRAEAESTAEPKEVESQRRSPPVVNVSELEEFFAATEKESQQKFIEKYNYDV
PI143180              EMTAPTSEVRAEAESTAEPKEVESQRRSPPVVNVSELEEFFAATEKESQQKFIEKYNYDV
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PI162544              EMTAPTSEVRAEAESTAEPKEVESQRRSPPVVNVSELEEFFAATEKESQQKFIEKYNYDV
PI188606              EMTAPTSEVRAEAESTAEPKEVESQRRSPPVVNVSELEEFFAATEKESQQKFIEKYNYDV
PI162722              EMTAPTSEVRAEAESTAEPKEVESQRRSPPVVNVSELEEFFAATEKESQQKFIEKYNYDV
PI588943              EMTAPTSEVRAEAESTAEPKEVESQRRSPPVVNVSELEEFFAATEKESQQKFIEKYNYDV
PI589598              EMTAPTSEVRAEAESTAEPKEVESQRRSPPVVNVSELEEFFAATEKESQQKFIEKYNYDV
PI161839              EMTAPTSEVRAEAESTAEPKEVESQRRSPPVVNVSELEEFFAATEKESQQKFIEKYNYDV
PI588998              EMTAPTSEVRAEAESTAEPKEVESQRRSPPVVNVSELEEFFAATEKESQQKFIEKYNYDV
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PI589689              EMTAPTSEVRAEAESTAEPKEVESQRRSPPVVNVSELEEFFAATEKESQQKFIEKYNYDV
PI590133              EMTAPTSEVRAEAESTAEPKEVESQRRSPPVVNVSELEEFFAATEKESQQKFIEKYNYDV
PI589852              EMTAPTSEVRAEAESTAEPKEVESQRRSPPVVNVSELEEFFAATEKESQQKFIEKYNYDV
PI589688              EMTAPTSEVRAEAESTAEPKEVESQRRSPPVVNVSELEEFFAATEKESQQKFIEKYNYDV
PI392312              EMTAPTSEVRAEAESTAEPKEVESQRRSPPVVNVSELEEFFAATEKESQQKFIEKYNYDV
PI590179              EMTAPTSEVRAEAESTAEPKEVESQRRSPPVVNVSELEEFFAATEKESQQKFIEKYNYDV
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PI589183              EMTAPTSEVRAEAESTAEPKEVESQRRSPPVVNVSELEEFFAATEKESQQKFIEKYNYDV
ARKANSAS_BLACK        EMTAPTSEVRAEAESTAEPKEVESQRRSPPVVNVSELEEFFAATEKESQQKFIEKYNYDV
DETROIT               EMTAPTSEVRAEAESTAEPKEVESQRRSPPVVNVSELEEFFAATEKESQQKFIEKYNYDV
GALA                  EMTAPTSEVRAEAESTAEPKEVESQRRSPPVVNVSELEEFFAATEKESQQKFIEKYNYDV
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PINK_LADY            EMTAPTSEVRAEAESTAEPKEVESQRRSPPVVNVSELEEFFAATEKESQQKFIEKYNYDV
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PI127311             EMTAPTSEVRAEAESTAEPKEVESQRRSPPVVNVSELEEFFAATEKESQQKFIEKYNYDV
PI131828             EMTAPTSEVRAEAESTAEPKEVESQRRSPPVVNVSELEEFFAATEKESQQKFIEKYNYDV
PI136243             EMTAPTSEVRAEAESTAEPKEVESQRRSPPVVNVSELEEFFAATEKESQQKFIEKYNYDV
PI136604             EMTAPTSEVRAEAESTAEPKEVESQRRSPPVVNVSELEEFFAATEKESQQKFIEKYNYDV
PI148503             EMTAPTSEVRAEAESTAEPKEVESQRRSPPVVNVSELEEFFAATEKESQQKFIEKYNYDV
PI161830             EMTAPTSEVRAEAESTAEPKEVESQRRSPPVVNVSELEEFFAATEKESQQKFIEKYNYDV
PI173986             EMTAPTSEVRAEAESTAEPKEVESQRRSPPVVNVSELEEFFAATEKESQQKFIEKYNYDV
PI214080             EMTAPTSEVRAEAESTAEPKEVESQRRSPPVVNVSELEEFFAATEKESQQKFIEKYNYDV
PI588745             EMTAPTSEVRAEAESTAEPKEVESQRRSPPVVNVSELEEFFAATEKESQQKFIEKYNYDV
PI588986             EMTAPTSEVRAEAESTAEPKEVESQRRSPPVVNVSELEEFFAATEKESQQKFIEKYNYDV
PI589018             EMTAPTSEVRAEAESTAEPKEVESQRRSPPVVNVSELEEFFAATEKESQQKFIEKYNYDV
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PI589185             EMTAPTSEVRAEAESTAEPKEVESQRRSPPVVNVSELEEFFAATEKESQQKFIEKYNYDV
PI589362             EMTAPTSEVRAEAESTAEPKEVESQRRSPPVVNVSELEEFFAATEKESQQKFIEKYNYDV
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PI589597             EMTAPTSEVRAEAESTAEPKEVESQRRSPPVVNVSELEEFFAATEKESQQKFIEKYNYDV
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PI632625             EMTAPTSEVRAEAESTAEPKEVESQRRSPPVVNVSELEEFFAATEKESQQKFIEKYNYDV
WILD1                EMTAPTSEVRAEAESTAEPKEVESQRRSPPVVNVSELEEFFAATEKESQQKFIEKYNYDV
WILD2                EMTAPTSEVRAEAESTAEPKEVESQRRSPPVVNVSELEEFFAATEKESQQKFIEKYNYDV
WILD3                EMTAPTSEVRAEAESTAEPKEVESQRRSPPVVNVSELEEFFAATEKESQQKFIEKYNYDV
ROME_BEAUTY          EMTAPTSEVRAEAESTAEPKEVESQRRSPPVVNVSELEEFFAATEKESQQKFIEKYNYDV
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PI589472             VKDEPLEGRYEWIRLKP
PI158731             VKDXPLEGRYEWIQLKP
PI589711             VKDEPLEGRYEWIQLKP
PI437038             VKDEPLEGRYEWIQLKP
GENOME_MDKRP4        VKDEPLEGRYEWIRLKP
PI589674             VKDEPLEGRYEWIRLKP
PI104799             VKDEPLEGRYEWIRLKP
PI123960             VKDEPLEGRYEWIRLKP
PI175545             VKDEPLEGRYEWIRLKP
PI143180             VKDEPLEGRYEWIRLKP
PI158730             VKDEPLEGRYEWIRLKP
PI162544             VKDEPLEGRYEWIRLKP
PI188606             VKDEPLEGRYEWIRLKP
PI162722             VKDEPLEGRYEWIRLKP
PI588943             VKDEPLEGRYEWIRLKP
PI589598             VKDEPLEGRYEWIRLKP
PI161839             VKDEPLEGRYEWIR---
PI588998             VKDEPLEGRYEWIRLKP
PI589025             VKDEPLEGRYEWIRLKP
PI324523             VKDEPLEGRYEWIRLKP
PI613818             VKDEPLEGRYEWIRLKP
PI589689             VKDEPLEGRYEWIRLKP
PI590133             VKDEPLEGRYEWIRLKP
PI589852             VKDEPLEGRYEWIRLKP
PI589688             VKDEPLEGRYEWIRLKP
PI392312             VKDEPLEGRYEWIRLKP
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PI161846             VKDEPLEGRYEWIRLKP
PI264558             VKDEPLEGRYEWIRLKP
PI589692             VKDEPLEGRYEWIRLKP
PI589183             VKDEPLEGRYEWIRLKP
ARKANSAS_BLACK       VKDEPLEGRYEWIRLKP
DETROIT              VKDEPLEGRYEWIRLKP
GALA                 VKDEPLEGRYEWIRLKP
GOLDEN_SMOOTHEE      VKDEPLEGRYEWIRLKP
PINK_LADY            VKDEPLEGRYEWIRLKP
RED_FUJI             VKDEPLEGRYEWIRLKP
PI127311             VKDEPLEGRYEWIRLKP
PI131828             VKDEPLEGRYEWIRLKP
PI136243             VKDEPLEGRYEWIRLKP
PI136604             VKDEPLEGRYEWIRLKP
PI148503             VKDEPLEGRYEWIRLKP
PI161830             VKDEPLEGRYEWIRLKP
PI173986             VKDEPLEGRYEWIRLKP
PI214080             VKDEPLEGRYEWIRLKP
PI588745             VKDEPLEGRYEWIRLKP
PI588986             VKDEPLEGRYEWIRLKP
PI589018             VKDEPLEGRYEWIRLKP
PI589167             VKDEPLEGRYEWIRLKP
PI589185             VKDEPLEGRYEWIRLKP
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PI589565             VKDEPLEGRYEWIRLKP
PI589597             VKDEPLEGRYEWIRLKP
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PI632625            VKDEPLEGRYEWIRLKP
WILD1               VKDEPLEGRYEWIRLKP
WILD2               VKDEPLEGRYEWIRLKP
WILD3               VKDEPLEGRYEWIRLKP
ROME_BEAUTY         VKDEPLEGRYEWIRLKP
                    *** *********:
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Figure 3.3. Amino acid alignment of 59 MdKRP4 sequences and the expected MdKRP4 of the draft genome using Clustal Omega. Underlined sequences represent conserved motifs also found in *Arabidopsis thaliana* KRP family sequences.

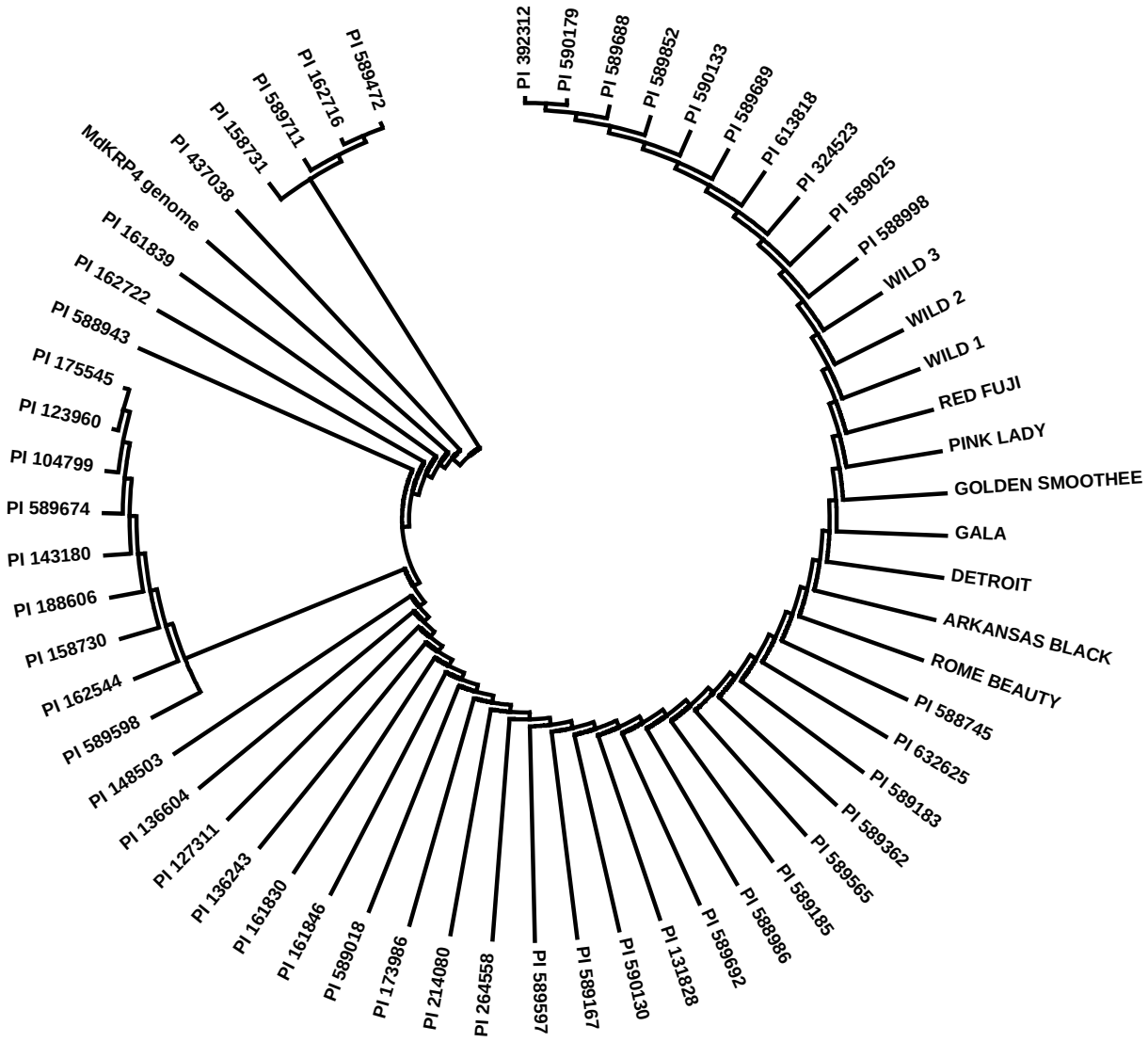

Figure 3.4. Phylogenetic tree representing the relationships among the MdKRP4 gene product of 59 genotypes studied, and the draft genome.

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CLUSTAL O(1.2.0) multiple sequence alignment


PI589689              --VSRAATSATAAKKRKAGSADGESVELPSSYDVAQHKRPRRRVVXVSSAPKIKREAESV
GALA                  MEVSRAATSATAAKKRKAGSADGESVELPSSYDVAQHKRPRRRVVVVSSAPKIKREAESV
GOLDEN_SMOOTHEE       MEVSRAATSATAAKKRKAGSADGESVELPSSYDVAQHKRPRRRVVVVSSAPKIKREAESV
PINK_LADY             MEVSRAATSATAAKKRKAGSADGESVELPSSYDVAQHKRPRRRVVVVSSAPKIKREAESV
PI162722              --------SATAAKKRKAGSADGESVELPSSYDVAQHKRPRRRVVVVSSAPKIKREAESV
GENOME_MDKRP5         MEVSRAATSATAAKKRKAGSADGESVELPSSYDVAQHKRPRRRVVVVSSAPKIKREAESV
PI589472              ------------------------SVELPSSYDVAQHKRPRRRVVVVSSAPKIKREAESV
PI392312              --------SATAAKKRKAGSADGESVELPSSYDVAQHKRPRRRVVVVSSAPKIKREAESV
PI324523              --------SATAAKKRKAGSADGESVELPSSYDVAQHKRPRRRVVVVSSAPKIKREAESV
PI589674              --------SATAAKKRKAGSADGESVELPSSYDVAQHKRPRRRVVVVSSAPKIKREAESV
PI589852              --------SATAAKKRKAGSADGESVELPSSYDVAQHKRPRRRVVVVSSAPKIKREAESV
PI590133              --------SATAAKKRKAGSADGESVELPSSYDVAQHKRPRRRVVVVSSAPKIKREAESV
PI175545              --------SATAAKKRKAGSADGESVELPSSYDVAQHKRPRRRVVVVSSAPKIKREAESV
PI123960              --------SATAAKKRKAGSADGESVELPSSYDVAQHKRPRRRVVVVSSAPKIKREAESV
PI161839              ---SRAATSATAAKKRKAGSADGESVELPSSYDVAQHKRPRRRVVVVSSAPKIKREAESV
ARKANSAS_BLACK        MEVSRAATSATAAKKRKAGSADGESVELPSSYDVAQHKRPRRRVVVVSSAPKIKREAESV
DETROIT               MEVSRAATSATAAKKRKAGSADGESVELPSSYDVAQHKRPRRRVVVVSSAPKIKREAESV
EMPIRE                MEVSRAATSATAAKKRKAGSADGESVELPSSYDVAQHKRPRRRVVVVSSAPKIKREAESV
HONEYCRISP            MEVSRAATSATAAKKRKAGSADGESVELPSSYDVAQHKRPRRRVVVVSSAPKIKREAESV
PI105524              MEVSRAATSATAAKKRKAGSADGESVELPSSYDVAQHKRPRRRVVVVSSAPKIKREAESV
PI158730              MEVSRAATSATAAKKRKAGSADGESVELPSSYDVAQHKRPRRRVVVVSSAPKIKREAESV
PI162724              MEVSRAATSATAAKKRKAGSADGESVELPSSYDVAQHKRPRRRVVVVSSAPKIKREAESV
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PI238028              MEVSRAATSATAAKKRKAGSADGESVELPSSYDVAQHKRPRRRVVVVSSAPKIKREAESV
PI344551              MEVSRAATSATAAKKRKAGSADGESVELPSSYDVAQHKRPRRRVVVVSSAPKIKREAESV
PI588799              MEVSRAATSATAAKKRKAGSADGESVELPSSYDVAQHKRPRRRVVVVSSAPKIKREAESV
PI589185              MEVSRAATSATAAKKRKAGSADGESVELPSSYDVAQHKRPRRRVVVVSSAPKIKREAESV
PI589565              MEVSRAATSATAAKKRKAGSADGESVELPSSYDVAQHKRPRRRVVVVSSAPKIKREAESV
PI589650              MEVSRAATSATAAKKRKAGSADGESVELPSSYDVAQHKRPRRRVVVVSSAPKIKREAESV
PI590144              MEVSRAATSATAAKKRKAGSADGESVELPSSYDVAQHKRPRRRVVVVSSAPKIKREAESV
WILD1                 MEVSRAATSATAAKKRKAGSADGESVELPSSYDVAQHKRPRRRVVVVSSAPKIKREAESV
WILD2                 MEVSRAATSATAAKKRKAGSADGESVELPSSYDVAQHKRPRRRVVVVSSAPKIKREAESV
WILD3                 MEVSRAATSATAAKKRKAGSADGESVELPSSYDVAQHKRPRRRVVVVSSAPKIKREAESV
PI588745              MEVSRAATSATAAKKRKAGSADGESVELPSSYDVAQHKRPRRRVVVVSSAPKIKREAESV
PI589692              MEVSRAATSATAAKKRKAGSADGESVELPSSYDVAQHKRPRRRVVVVSSAPKIKREAESV
PI589588              --------SATAAKKRKAGSADGESVELPSSYDVAQHKRPRRRVVVVSSAPKIKREAESV
PI589688              --------SATAAKKRKAGSADGESVELPSSYDVAQHKRPRRRVVVVSSAPKIKREAESV
PI162716              --------SATAAKKRKAGSADGESVELPSSYDVAQHKRPRRRVVVVSSAPKIKREAESV
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GALA                  RTSNDGFWTSDHAESSCCSSNGSSELEDESDQVESWTYNSSRDERREMTAATSKVGAEAE
GOLDEN_SMOOTHEE       RTSNDGFWTSDHAESSCCSSNGSSELEDESDQVESWTYNSSRDERREMTAATSKVGAEAE
PINK_LADY             RTSNDGFWTSDHAESSCCSSNGSSELEDESDQVESWTYNSSRDERREMTAATSKVGAEAE
PI162722              RTSNDGFWTSDHAESSCCSSNGSSELEDESDQVESWTYNSSRDERREMTAATSKVGAEAE
GENOME_MDKRP5         RTSNDGFWTSDHAESSCCSSNGSSELEDESDQVESWTYNSSRDERREMTAXTSKVGAEAE
PI589472              RTSNDGFWTSDHAESSCCSSNGSSELEDESDQVESWTYNSSRDERREMTAPTSKVGAEAE
PI392312              RTSNDGFWTSDHAESSCCSSNGSSELEDESDQVESWTYNSSRDERREMTAPTSKVGAEAE
PI324523              RTSNDGFWTSDHAESSCCSSNGSSELEDESDQVESWTYNSSRDERREMTAPTSKVGAEAE
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ARKANSAS_BLACK        RTSNDGFWTSDHAESSCCSSNGSSELEDESDQVESWTYNSSRDERREMTAPTSKVGAEAE
DETROIT               RTSNDGFWTSDHAESSCCSSNGSSELEDESDQVESWTYNSSRDERREMTAPTSKVGAEAE
EMPIRE                RTSNDGFWTSDHAESSCCSSNGSSELEDESDQVESWTYNSSRDERREMTAPTSKVGAEAE
HONEYCRISP            RTSNDGFWTSDHAESSCCSSNGSSELEDESDQVESWTYNSSRDERREMTAPTSKVGAEAE
PI105524              RTSNDGFWTSDHAESSCCSSNGSSELEDESDQVESWTYNSSRDERREMTAPTSKVGAEAE
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PI173986              RTSNDGFWTSDHAESSCCSSNGSSELEDESDQVESWTYNSSRDERREMTAPTSKVGAEAE
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PI344551              RTSNDGFWTSDHAESSCCSSNGSSELEDESDQVESWTYNSSRDERREMTAPTSKVGAEAE
PI588799              RTSNDGFWTSDHAESSCCSSNGSSELEDESDQVESWTYNSSRDERREMTAPTSKVGAEAE
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PI589692              RTSNDGFWTSDHAESSCCSSNGSSELEDESDQVESWTYNSSRDERREMTAPTSKVGAEAE
PI589588              RTSNDGFWTSDHAESSCCSSNGSSELEDESDQVESWTYNSSRDERREMTAPTSKVGAEAE
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PI589688            RTSNDGFWTSDHAESSCCSSNGSSELEDESDQVESWTYNSSRDERREMTAPTSKVGAEAE
PI162716            RTSNDGFWTSDHAESSCCSSNGSSELEDESDQVESWTYNSSRDERREMTAPTSKVGAEAE
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GALA                STARLKDESQRRSPTVVNASELEEFFAAMEKESQQKFKEMYNFDVAKDEPHEGRYEWVRL
GOLDEN_SMOOTHEE     STARLKDESQRRSPTVVNASELEEFFAAMEKESQQKFKEMYNFDVAKDEPHEGRYEWVRL
PINK_LADY           STARLKDESQRRSPTVVNASELEEFFAAMEKESQQKFKEMYNFDVAKDEPHEGRYEWVRL
PI162722            STARLKDESQRRSPTVVNASELEEFFAAMEKESQQKFKEMYNFDVAKDEPHEGRYEWV--
GENOME_MDKRP5       STARLKDESQRRSPTVVNASELEEFFAAMEKESQQKFKEMYNFDVAKDEPHEGRYEWVRL
PI589472            STARLKDESQRRSPTVVNASELEEFFAAMEKESQQKFKEMYNFDVAKDEPHEGRYEWVR-
PI392312            STARLKDESQRRSPTVVNASELEEFFAAMEKESQQKFKEMYNFDVAKDEPHEGRYEWVR-
PI324523            STARLKDESQRRSPTVVNASELEEFFAAMEKESQQKFKEMYNFDVAKDEPHEGRYEWVR-
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PI589852            STARLKDESQRRSPTVVNASELEEFFAAMEKESQQKFKEMYNFDVAKDEPHEGRYEWVR-
PI590133            STARLKDESQRRSPTVVNASELEEFFAAMEKESQQKFKEMYNFDVAKDEPHEGRYEWVR-
PI175545            STARLKDESQRRSPTVVNASELEEFFAAMEKESQQKFKEMYNFDVAKDEPHEGRYEWVR-
PI123960            STARLKDESQRRSPTVVNASELEEFFAAMEKESQQKFKEMYNFDVAKDEPHEGRYEWVR-
PI161839            STARLKDESQRRSPTVVNASELEEFFAAMEKESQQKFKEMYNFDVAKDEPHEGRYEWVR-
ARKANSAS_BLACK      STARLKDESQRRSPTVVNASELEEFFAAMEKESQQKFKEMYNFDVAKDEPHEGRYEWVRL
DETROIT             STARLKDESQRRSPTVVNASELEEFFAAMEKESQQKFKEMYNFDVAKDEPHEGRYEWVRL
EMPIRE              STARLKDESQRRSPTVVNASELEEFFAAMEKESQQKFKEMYNFDVAKDEPHEGRYEWVRL
HONEYCRISP          STARLKDESQRRSPTVVNASELEEFFAAMEKESQQKFKEMYNFDVAKDEPHEGRYEWVRL
PI105524            STARLKDESQRRSPTVVNASELEEFFAAMEKESQQKFKEMYNFDVAKDEPHEGRYEWVRL
PI158730            STARLKDESQRRSPTVVNASELEEFFAAMEKESQQKFKEMYNFDVAKDEPHEGRYEWVRL
PI162724            STARLKDESQRRSPTVVNASELEEFFAAMEKESQQKFKEMYNFDVAKDEPHEGRYEWVRL
PI173986            STARLKDESQRRSPTVVNASELEEFFAAMEKESQQKFKEMYNFDVAKDEPHEGRYEWVRL
PI187352            STARLKDESQRRSPTVVNASELEEFFAAMEKESQQKFKEMYNFDVAKDEPHEGRYEWVRL
PI238028            STARLKDESQRRSPTVVNASELEEFFAAMEKESQQKFKEMYNFDVAKDEPHEGRYEWVRL
PI344551            STARLKDESQRRSPTVVNASELEEFFAAMEKESQQKFKEMYNFDVAKDEPHEGRYEWVRL
PI588799            STARLKDESQRRSPTVVNASELEEFFAAMEKESQQKFKEMYNFDVAKDEPHEGRYEWVRL
PI589185            STARLKDESQRRSPTVVNASELEEFFAAMEKESQQKFKEMYNFDVAKDEPHEGRYEWVRL
PI589565            STARLKDESQRRSPTVVNASELEEFFAAMEKESQQKFKEMYNFDVAKDEPHEGRYEWVRL
PI589650            STARLKDESQRRSPTVVNASELEEFFAAMEKESQQKFKEMYNFDVAKDEPHEGRYEWVRL
PI590144            STARLKDESQRRSPTVVNASELEEFFAAMEKESQQKFKEMYNFDVAKDEPHEGRYEWVRL
WILD1               STARLKDESQRRSPTVVNASELEEFFAAMEKESQQKFKEMYNFDVAKDEPHEGRYEWVRL
WILD2               STARLKDESQRRSPTVVNASELEEFFAAMEKESQQKFKEMYNFDVAKDEPHEGRYEWVRL
WILD3               STARLKDESQRRSPTVVNASELEEFFAAMEKESQQKFKEMYNFDVAKDEPHEGRYEWVRL
PI588745            STARLKDESQRRSPTVVNASELEEFFAAMEKESQQKFKEMYNFDVAKDEPHEGRYEWVRL
PI589692            STARLKDESQRRSPTVVNASELEEFFAAMEKESQQKFKEMYNFDVAKDEPHEGRYEWVRL
PI589588            STARLKDESQRRSPTVVNASELEEFFAAMEKESQQKFKEMYNFDVAKDEPHEGRYEW---
PI589688            STARLKDESQRRSPTVVNASELEEFFAAMEKESQQKFKEMYNFDVAKDEPHEGRYEWVR-
PI162716            STARLKDESQRRSPTVVNASELEEFFAAMEKESQQKFKEMYNFDVAKDEPHEGRYEWVR-
                    ******************************************************

PI589689            S-
GALA                KP
GOLDEN_SMOOTHEE     KP
PINK_LADY           KP
PI162722            --
GENOME_MDKRP5       KP
PI589472            --
PI392312            S-
PI324523            S-
PI589674            S-
PI589852            S-
PI590133            S-
PI175545            S-
PI123960            S-
PI161839            S-
ARKANSAS_BLACK      KP
DETROIT             KP
EMPIRE              KP
HONEYCRISP          KP
PI105524            KP
PI158730            KP
PI162724            KP
PI173986            KP
PI187352            KP
PI238028            KP
PI344551            KP
PI588799            KP
PI589185            KP
PI589565            KP
PI589650            KP
PI590144            KP
WILD1               KP
WILD2               KP
WILD3               KP
PI588745            KP
PI589692            KP
```

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PI589588            --
PI589688            --
PI162716            --
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Figure 3.5. Amino acid alignment of 38 MdKRP5 sequences and the expected MdKRP5 from the draft genome using Clustal Omega.

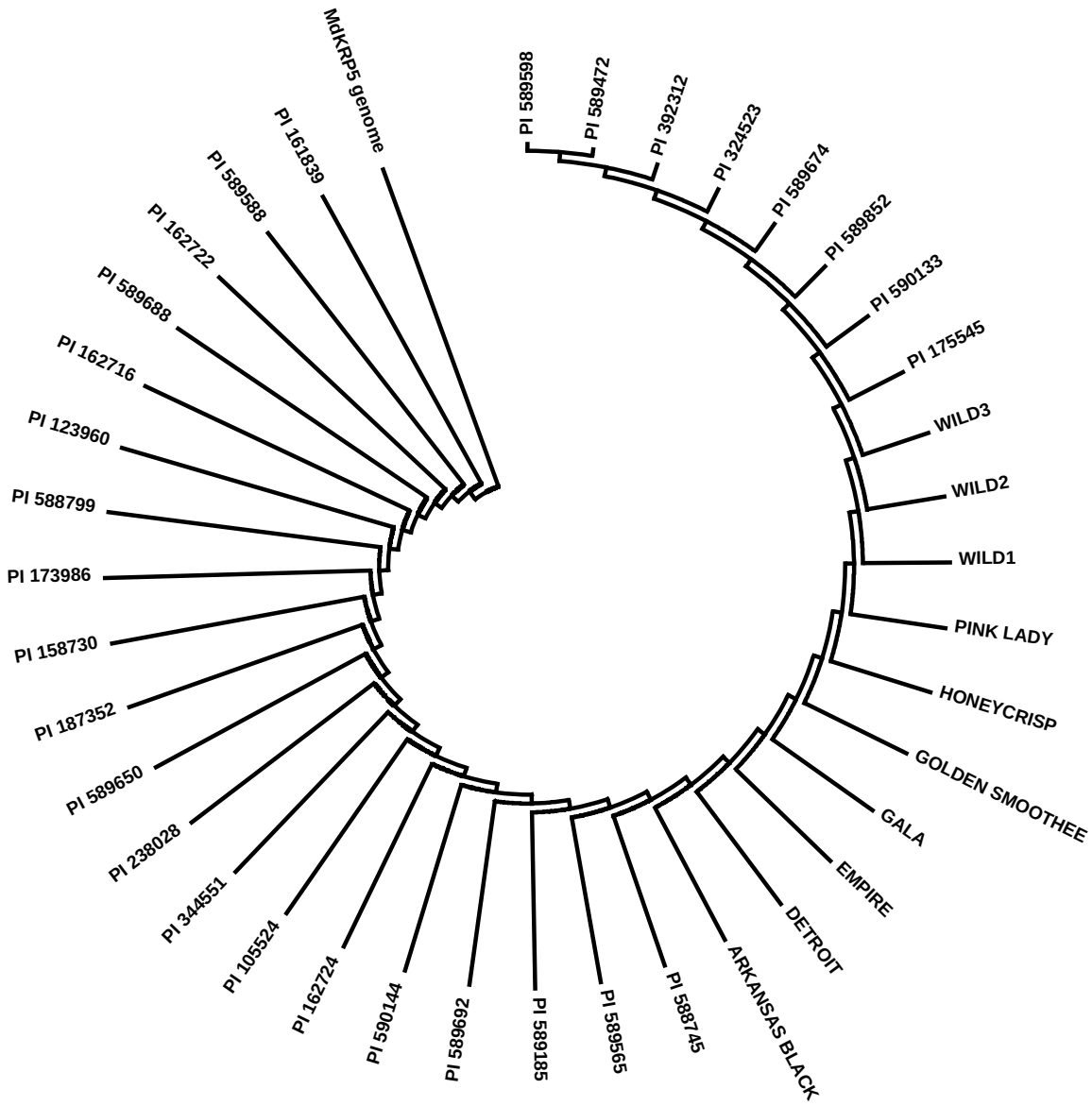

Figure 3.6. Phylogenetic tree representing the relationships among the MdKRP5 gene product of 38 genotypes studied, and the draft genome.

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CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

The aim of this research was to better understand how the two apple *Kip-related proteins*, *MdKRP4* and *MdKRP5*, regulate cell production in apple. Two approaches were used to achieve the objective.

Previous information about *MdKRP4* and *MdKRP5* in apple suggested an important role in regulating cell production, and *Arabidopsis thaliana* was transformed with these two genes, with the intent to learn more about their function. The transgenic T_1 generation displayed phenotypes that resembled the phenotypes seen when *AtKRP2* was overexpressed under the same promoter and growth conditions. The comparison suggests that the functions of MdKRP4 and MdKRP5 in apple are similar to that of AtKRP2 in *A. thaliana*. A detailed investigation of the leaf size, shape, thickness, cell number, cell size, and floral morphology will be performed in the T_3 generation to further confirm the above conclusion.

The single nucleotide polymorphism analysis of these two genes led to the discovery of a potentially important change in amino acid sequence, the substitution of Phenylalanine for Serine at position 55 of MdKRP4 in four small-fruited genotypes. Analysis of the normal and modified protein products is now needed to understand how the properties of this cell cycle regulator have been altered. In addition, now that this SNP has been identified and its location has been determined, higher throughput techniques such as allele-specific polymerase chain reaction can be used to screen larger

populations more rapidly, which can lead to the identification of this alteration in more genotypes. Further, functional analysis of the potential phosphorylation of the serine residue at this site and its effect on protein function may be performed in future studies to better understand the regulation of KRP activity.