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DECIPHERING THE MOLECULAR MECHANISMS OF ROOTSTOCK INDUCED DWARFING IN CHERRIES (*Prunus spp.*)

By

Constantinos Prassinos

A DISSERTATION

Submitted to
Michigan State University
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ABSTRACT

DECIPHERING THE MOLECULAR MECHANISMS OF ROOTSTOCK INDUCED DWARFING IN CHERRIES (*Prunus spp.*)

By

Constantinos Prassinos

Rootstock induced dwarfing has been one of the major breakthroughs in orchard management in the twentieth century. The majority of the cherry rootstocks have been produced in the last 30 years. Nevertheless, breeding of new rootstocks has proven challenging, due to the lack of understanding of the dwarfing phenomenon. This project explores the phenotypic and genetic differences between cherry graft combinations that exhibit varying degrees of vigor. Growth data have indicated a consistent cessation of shoot growth across rootstocks of varying vigor and across growing seasons. The initial rate of shoot elongation was the same for all graft combinations tested, but dwarfing graft combinations showed faster cessation of shoot growth. The same pattern was observed for the number of nodes added during the elongation of the shoot. The average metamer length though was not affected between grafts, indicating that cessation of growth is due to reduced cell growth and expansion at the apical meristem. This was further confirmed by the absence of significant difference in the size or number of cells within the metamer, between grafts. Complementary DNA Amplified Fragment Length Polymorphism of shoot and graft union samples revealed a high degree of co-regulation in gene expression between the dwarfing 'Bing'/Gi5 and semi-vigorous 'Bing'/Gi6 graft combinations. Few genes showed differential expression between the two graft combinations. Forty-three of those genes were differentially expressed in the shoot samples and 56 in the graft union

samples. The differentially expressed genes had a variety of functions with the most interesting being a group of genes previously involved in brassinosteroid signaling. The analysis of gene expression also revealed the presence of the Cherry Virus A in the dwarfing combination 'Bing'/Gi5. Screening of rootstocks that confer different degrees of vigor did not show any correlation between the presence of the virus and the vigor of the rootstock. Also, the absence of the virus from some rootstocks is circumstantial rather than due to resistance, which was shown by screening different scions grafted on the same rootstock variety. The current study provides an initial cataloging of genes that may be involved in the process of rootstock-induced dwarfing. No certain conclusion can be drawn from these results and further study will be necessary to identify which of these genes contribute significantly to this phenomenon.

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DEDICATION

To my parents

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LITERATURE REVIEW

Historical background

Early reports on the use of rootstocks came from ancient Greece and Rome (Hedrick, 1915). Nevertheless, the interest at that time was concentrated in the acquisition of new cherry varieties for the two cherry species that are in commercial use up to this day, namely *Prunus avium* L. and *Prunus cerasus* L. The most extended report of cherry varieties was performed by Pliny, who described the varieties collected by the wealthy Roman Lucullus (DeCandole, 1890). For many centuries and through the middle ages, cherry cultivation and varieties did not develop further (Hedrick, 1915). It was only after the 16th and 17th centuries that cherry cultivation started to develop once again. Nevertheless, it was only at the level of fruit varieties that progress was made. In the beginning of the 20th century, there were two main choices for rootstocks. These were the *'Mazzard'* (*P. avium*) and the *'Mahaleb'* (*P. mahaleb* L.), both propagated through seedlings. In an early comparison of rootstock performance between *'Mazzard'* and *'Mahaleb'*, Hendrick (1915) list of the advantages of the second over the first: a) cold hardiness, b) dwarfing ability, c) precocity, d) no change in fruit size and e) better adaptation to diverse soils and the disadvantages; a) weaker graft union, b) shorter life cycle and c) lower yield. Hedrick (1915) discusses a number of other rootstocks that were less popular and used in special environments that mainly had to do with low temperatures or low water availability. These were the Russian Cherry which was used in cold regions of the United States, the pigeon cherry *Prunus pensylvanica* L.f. (Linnaeus filius) in cold regions with the potential to dwarf scions, the sand cherry *Prunus pumila* L. in cold and dry areas and a Japanese variety of *Prunus pseudocerasus* L. used in Japan for its cold hardiness and ability to root easily. Hedrick also refers to the ability of

rootstocks to dwarf trees, with '*Morello*' (*P. cerasus*) producing dwarf and '*Mahaleb*' very dwarf trees. Nevertheless, dwarfness could be accomplished only through the appropriate pruning and working of the crown.

During most of the 20th century *'Mahaleb'* and *'Mazzard'* remained the predominant cherry rootstocks (Perry, 1987). They were propagated through seed and only the clone F12/1 of *'Mazzard"* was clonally propagated. Other rootstocks that have been used in a smaller extent are the '*Stockton Morello*' and '*Colt'* (*P. avium* x *P. pseudocerasus*). Since the 80's cherry rootstock breeding has been accelerated through the production of interspecific hybrids. Some of the most important rootstock breeding programs were developed in Germany with Weiroot, Gisela and Pi-Ku series, in Italy the CAB series and in Denmark the DAN series (Hrotko, 2005). Individual rootstocks with considerable success were also produced such as Edabriz, Victor, Damil and Camil (Hrotko, 2005).

Significance of rootstocks and rootstock induced dwarfing (RID)

One of the most important cultural advances in temperate tree fruit production has been the development and adoption of dwarfing rootstocks. Trees on dwarfing rootstocks can exhibit several economically important traits, including precocious flowering, increased yield, reduced tree height and disease/virus resistance (Lang *et al.* 1997; Webster, 1998; Atkinson and Else, 2001). The coexistence of two organisms of the same or different species background, in a single plant structure requires a high degree of co-regulation in molecular, biochemical and physiological processes. The rootstock is the

source of nutrients that are transported to the scion to be metabolized, and the scion is the source of photoassimilates, which will partially reach the rootstock for maintenance. Also, the rootstock is the receiver of many signals from the soil environment, while the scion is the receiver of the signals from the open air. Survival of the grafted tree depends largely on the ability of the rootstock and the scion to communicate effectively.

Reduced tree height, also known as dwarfism, conferred by the rootstock to the scion remains a scientific mystery.

The apple system: hypotheses and advances

Significant progress in the understanding of RID has been made through research performed in the apple rootstocks. Apple has a longer history of rootstock breeding and an extended array of rootstock varieties.

Early attempts to explain the dwarfing phenomenon were summarized in 1956 by Beakbane, who listed five theories for the mechanism of the rootstock effect. The first theory suggested that competition for resources between various parts of the grafted tree results in the restriction of scion growth. According to the author dwarfing rootstocks tend to attract more resources due to higher content in living tissue. The second theory suggested that transport of water and metabolites differs between rootstocks due to the differences in vessel element diameter. Dwarfing rootstocks contain smaller vessels compared to vigorous rootstocks thus having lower transport capacity. The third theory suggests that the ratio of living tissue to plant surface affects the amount of oxygen that is available for respiration. Dwarf trees with a high ratio tend to absorb less oxygen than

necessary thus having reduced growth as a result. The fourth hypothesis involves the ability of the rootstock to transport ions, based on the percentage of live tissue. Finally, a fifth theory postulates that each rootstock variety has different capacity to form elaborated compounds, such as phenolics. A year later Rogers and Beakbane (1957) reduced the number of mechanisms to three. These were nutrient availability, nutrient transport and auxin metabolism. In 1967, Tubbs proposed four mechanisms to explain the phenomenon, with the assumption that these mechanisms are not exclusive, but each contributes partially to the phenomenon. The mechanisms were i) nutrient availability, ii) variation in metabolism between tissues, iii) effect of morphogenesis in resource production and allocation and iv) growth regulators, such as growth promoting hormones and growth inhibitors. These theories have been revised or rejected through time. More sophisticated chemical and physiological analysis methods have lead to more detailed and substantiated hypotheses. These are described in the following paragraphs.

The first hypothesis proposes that auxin, which is produced in the aerial parts of the grafted tree, is transported at different concentrations between grafts on different rootstock genotypes, thus affecting cytokinin production in the root, which further causes differences in shoot growth (Lockard and Schneider, 1981; Webster, 1998). Lockard and Schneider (1981) proposed that auxin is degraded during its flow from the scion to the rootstock. Auxin is degraded by enzymes such as IAA oxidase, peroxidase and phenols. Concentration of these compounds differs between graft combinations, which results in differences in auxin concentration that reach the root. Some support for this hypothesis has been provided in recent years for the apple rootstocks (Soumelidou *et al.* 1994a; Kamboj *et al.* 1997). Auxin was found to be transported at lower rates in shoots of the

dwarfing rootstock M.9, than the vigorous MM.111 (Soumelidou *et al.* 1994a). During active growth of apple shoots, auxin is taken-up much faster in the vigorous roostocks MM.104 and MM.111 than in the dwarfing M.9 and M.27 (Kamboj *et al.* 1997). Nevertheless, measurements of endogenous auxin in the bark of rootstock stems did not show significant differences in concentration across rootstocks of varying vigor and across the growing season. In contrast, ABA concentration was found to be higher in shoots of the M.9 and M.27 dwarfing rootstocks than in the vigorous MM.106 and MM.111 (Kamboj *et al.* 1999a). ABA injections have been shown to have higher impact on shoot growth retardation of dwarfing apple trees (Robitaille and Carlson, 1971). In another report, Jones (1986) gives cytokinin a more important role in the control of scion growth, through rootstocks or interstocks. Cytokinin in the form of zeatin and zeatin riboside is present at higher concentration in the xylem sap of invigorating MM.106 rootstocks than in dwarfing M.27 and M.9 rootstocks (Kamboj *et al.* 1999b). The concentration of these two cytokinins does not change between shoots of grafted or ungrafted rootstocks (Kamboj *et al.* 1999b), excluding thus an effect of the graft union in the acropetal transport of these hormones. Nevertheless, when the variety "Fiesta" was used as a scion it grew more on M.9 and M.27 rootstocks compared to the shoots of the ungrafted rootstocks, but grafted trees were significantly shorter than on MM.106 rootstocks (Kamboj *et al.* 1999b).

The second hypothesis assumes that phenols accumulating at the graft union reduce tissue viability and perhaps the rate of auxin break down (Lockard and Schneider, 1981). This hypothesis has been based on observations from heterologous systems and in few reports on apple rootstocks. A number of phenolic compounds have been found to

act synergistically or antagonistically to IAA (Lockard and Schneider, 1981). The most abundant of these compounds is phloridzin, which accumulates in the phloem of apple stems and acts antagonistically to IAA. It was found to comprise 9.2% and 7.7% of the dry weight in MM.111 and M.26 rootstocks respectively (Lockard and Schneider, 1981). During active growth of the shoots, phloridzin concentration drops and increases again at the onset of dormancy. Even though phloridzin has been given an important role in this hypothesis, the concentrations mentioned above show that it is present in higher concentration in the vigorous rootstocks. Even though its seasonal concentration correlates well with inhibition of growth it has not been proven that phloridzin has a regulatory role in this phenomenon.

The last hypothesis postulates that reduced tree size conferred by dwarfing rootstocks is caused by reduced solute transport across the graft union (Atkinson *et al.* 2003; Basile *et al.* 2003). In support of this hypothesis, Atkinson *et al.*, (2003) demonstrated that hydraulic conductance in apples increased with the vigor of the rootstocks. In peach, reduced stem extension rates were significantly correlated with lower stem water potential when comparing dwarfing and invigorating rootstock/scion combinations (Basile *et al.* 2003). This difference in transport can be caused by vessel diameter and number. It was found that vessel elements at the graft union of dwarf trees on the M9 rootstock were initially few, but larger than those of trees grafted on the semi dwarfing MM106 (Soumelidou *et al.* 1994b). A year later though, vessel elements became smaller in the dwarfing combination, thus suggesting a more unstable auxin supply compared to the semi-dwarfing trees that had normal vessel development.

Nevertheless, current knowledge is still inconclusive on which is the exact mechanism of RID in apples. All of the above hypotheses have produced a number of important findings on the biology of grafted trees. Undoubtedly, the findings portray the differences between grafts of varying vigor. What remains to be explored is the series of events; a separation of primary and secondary effects.

In recent years research has focused on the genetic aspect of the dwarfing phenomenon. Map based cloning was used by Rusholme *et al.* (2004) to screen a population of rootstocks generated by crossing the dwarf apple rootstock M.9 and the vigorous rootstock Robusta 5. The population segregated for the dwarfing trait and was used in a bulked segregant analysis with RAPD markers. Trees were grouped to four categories based on visual assessment of dwarfness and trunk circumference. These two phenotypic characters though were not always co-segregating. Thus, trees considered dwarf based on height were vigorous in terms of trunk circumference and vice versa, indicating the influence of more than one loci. Four RAPD loci were found to be linked to the dwarfing phenotype. Detailed mapping identified a 2.5cM region named *Dw1* containing the dwarfing locus. Nevertheless, 15 individuals from the cross between M.9 to R.5 that were classified as vigorous also contained M.9 alleles from the *Dw1* locus. This is expected since the dwarfing trait probably involves more than one gene or genetic loci as mentioned above. This is the first attempt to directly target the genes involved in the dwarfing phenomenon and it has a promising future for the identification of dwarfing related genes in apple.

Jensen *et al.* (2004) applied a genomics approach in apple to compare the dwarfing rootstock M.9 to the vigorous M.7 rootstock. The analysis identified 92 genes

that were differentially expressed between the shoots of the two rootstocks. Of those, 56 were up-regulated in M.9, the most important of which were cell cycle and signaling related genes. This approach provided information on the gene expression differences between dwarf and vigorous trees, but more work is needed to identify which of these genes act at the initial stages of growth control.

Even though the above hypotheses and new advances are presented individually, they can all be interrelated and orderly placed in the process of dwarfing. In the effort to answer the complex question of RID in apples and other systems it is important to reduce the complexity as much as possible. Thus it would be wise to focus on specific group of genotypes and later expand the knowledge obtained to a larger collection of genotypes. The apple system is and will continue to be the pioneer system in the quest for signals that promote RID.

Rootstock Induced Dwarfing in other systems

The advances achieved towards the understanding of the dwarfing phenomenon in apples can be used as guides for explaining dwarfing in other fruit tree systems. Nevertheless, one should be cautious when relying on the apple system to study RID in other tree species. Even within the same species the causes of dwarfism may differ between genotypes.

Response of cherry trees to grafting

Similarities and discrepancies between the apple and cherry models are discussed below in an attempt to compare the two mechanisms. It is critical to understand how the two systems work, to avoid transfer of knowledge that will complicate either system more than it is in reality. Unfortunately, current research in cherry RID is focusing heavily on knowledge obtained in apples. Thus, more data are needed to establish a model for RID in cherries.

Interstock contribution to RID

As discussed previously, rootstock varieties that have been used as interstocks in apple grafts respond in the same fashion as when they are used as rootstocks. In cherries there is conflicting evidence about the role of interstocks in the control of scion vigor. Some report the inability of interstocks to dwarf scions (Jones, 1986; Webster, 1995, 1998), while others report marginal to significant changes in vigor. In a sixteen year trial using 14 different rootstocks as interstocks, grafted on *P. avium* or *P. mahaleb* seedlings, the TCSA and crown volume of 'Van' or 'Büttner's Red' scions were statistically different from control trees without interstocks (Rozpara *et al.* 1998). Nevertheless, the vigor of the rootstocks without interstocks was not reported. Thus, it cannot be concluded whether these genotypes behave in a similar fashion when used as rootstocks or interstocks. Furthermore, yield efficiency of the trees does not correlate well with tree vigor, suggesting a putative effect of the age of these trees. In another trial when sour cherry varieties were used as interstocks on *P. mahaleb* seedlings, there was no change in vigor of 'Van' and 'Germersdorfi óriás' scions, while a *P. fruticosa* interstock caused a

significant reduction in vigor (Hrotkó *et al.* 1997,1998). Perry (1987) reported several instances in which interstocks caused subtle reduction in tree vigor, and in only a few combinations, did the reduction reach 20-30% under certain conditions. These results suggest that the genotype of the plant material used in interstock grafting is the critical factor affecting vigor of the resulting tree. This effect of the interstock does not seem to be proportional to the size controlling ability of the same genotype when used as rootstock. It should be further pursued whether such a response is the result of compatibility or the physiology of the grafted material.

Budding height effect on the control of vigor

Budding height in cherries, as in the case of interstocks, is not clearly defined as a vigor controlling technique. Webster (1998) reported that budding height has no effect on the degree of dwarfness achieved, in contrast to apple, where increases in budding height decrease in scion vigor (Lockard and Schneider, 1981). More recently though, Santos *et al.* (2004) identified budding height as a significant contributor to vigor control in five different rootstocks grafted with three sweet cherry varieties. Not all rootstock to scion combinations responded the same to differences in budding height. 'Summit' sweet cherry showed the most significant difference in TCSA between 10 and 60 cm budding. Edabriz, Gisela5 and Cab11E were the rootstocks with the most significant difference in TCSA between 10 and 60 cm budding. The effect though of the rootstocks to the control of vigor contributed 80% of the difference, while budding height only 4%. Also, the reduction in vigor conferred by the increase of budding height was proportional for all rootstocks, suggesting a physiological rather than genotypic cause for this phenomenon.

Webster (1998), based on the inability of cherry interstocks and budding height to confer dramatic change in scion vigor, proposed that the source of the dwarfing signal must originate in the roots. In contrast, in the apple system the signal originates in the stem. This hypothesis is supported by the significant dwarfing ability of interstocks and budding height (Webster, 1998).

Phenolics in grafted cherry trees

Extended analyses in the phenolics content of various cherry graft combinations have been performed over many years by W. Feucht and his coworkers in Germany. This is perhaps the most extended study on the interaction between rootstock and scion in cherries. His group analyzed many different rootstock and scion varieties for phenolic and protein content in relation to tree vigor and graft compatibility. Credit should be given to other researchers as well, who have contributed to the enrichment of our knowledge on the effect of phenolic compounds in cherry biology.

Catechin is the most abundant phenol in cherry stems and more specifically in the phloem and the cambium (Treutter and Feucht, 1991; Usenik and Štampar, 2001). Results, however, are conflicting on the concentration of catechin between graft combinations of varying vigor. Treutter and Feucht (1991) found a higher concentration of cathechin in the phloem in comparison to the cambium in two year old stems of ungrafted 'Van' and 'Werdersche Braume' grafted on 'Stockton Morello'. Catechin concentration was higher in shoot tips of ungrafted and vigorous graft combinations and furthermore promoted larger callus formation when applied to excised shoots compared to non-treated explants, suggesting a role in cell division (Feucht and Nachit, 1977;

Treutter and Feucht, 1991). In contrast, Usenik and Štampar (2001) showed that catechin concentration is higher in graft unions of dwarfing graft combinations. The effect of catechin on cell divisions may explain the higher swelling observed in the callus of the graft union as the dwarfing ability of the rootstock increases (Wagner and Gruppe, 1985)

Another phenolic compound that accumulates in the stems of cherry trees is prunin. In contrast to catechin, prunin accumulates at higher concentrations in the cambium region rather than the phloem. Like catechin its concentration is higher above the graft union of dwarfing combinations (Treutter and Feucht, 1991; Usenik and Štampar, 2001). Prunin has been found to inhibit growth of callus cells and prevent xylogenesis (Feucht *et al.* 1988). This is in contrast to the effect of catechin that promotes cell divisions. Prunin, when applied to shoot calluses or shoots, promotes its synthesis and that of flavan-3-ols, such as catechin (Yuri *et al.* 1990). Based on the previous observations a balance between prunin and flavan-3-ols determines callus or shoot growth rates.

Other phenolic compounds that have been found in cherry tissues are dihydrowogonin-7-glucoside (DWG) and chlorogenic acids. DWG accumulates at higher concentrations in the phloem rather than the cambium (Treutter and Feucht, 1991). It is also found at higher levels above the graft union of dwarfing graft combinations in comparison to vigorous ones (Usenik and Štampar, 2001). Chlorogenic acids accumulate in the phloem and their concentration is higher in the rootstock directly below the graft union compared to above it (Feucht and Schmid, 1979). An effect of DWG and chlorogenic acid in growth has not been studied.

As presented before there is no strong correlation between phenolic content and dwarfism, but rather an indication that phenolics may affect the rate of division of callus cells at the graft union. Current research relates the accumulation of phenolic compounds in the graft union as a response to stress produced by the joining of two different genotypes (Treutter and Feucht, 1991; Usenik and Štampar, 2001). Further research is necessary to test any direct effect of phenolic compounds on the growth of grafted cherry trees.

Hormonal control of growth

Differences in growth between genotypes of the same plant species unavoidably lead to the investigation of hormone levels. As it was mentioned before, auxin has been implicated in the dwarfing phenomenon in apples, thus making it the first candidate for the control of RID in cherries. Leaf indole content has been found to differ significantly between ungrafted cherry rootstocks of varying vigor (Hrotkó, 1996). Interestingly, indole content was inversely proportional to rootstock vigor. Sweet cherry varieties 'Van' and 'Germersdorfi óriás' had the highest indole concentration in comparison to the ungrafted rootstocks. Nevertheless, when these two cherry varieties were grafted on rootstocks of varying vigor there was no significant difference in the indole content of leaves.

Hydraulic conductance in grafted trees

Transport rates of water and solutes between rootstock and scion have been hypothesized to be one of the factors involved in RID. In apple and peach stem hydraulic

conductance has been shown to be lower in the more dwarfing rootstocks compared to the invigorating ones (Atkinson *et al.* 2003; Basile *et al.* 2003). Differences in vessel diameter due to grafting have been implicated in this differentiation in water transport (Soumelidou *et al.* 1994b). In newly established cherry grafts, vessel element size was largely affected by the graft combination (Olmstead *et al.* 2006). It was more variable in interspecific graft combinations where the dwarfing rootstocks produced vessels of smaller diameter (Olmstead *et al.* 2006a). In 2-year old grafts, vessel diameter was smaller in the dwarfing combination 'Lapins'/Gi5 compared to the more vigorous 'Lapins'/Colt (Olmstead *et al.* 2006b). In the same study it was shown that translocation of the dye Safranin O through the graft union was slower in dwarfing trees, suggesting a slower water conductance. Nevertheless, differences in leaf water potential in 7-year-old grafted cherry trees were attributed to flow resistance within the rootstock-to-shoot pathway rather than graft union resistances (Schmitt *et al.* 1989). Similar results have been obtained for peach trees, where hydraulic resistance fluctuated mainly between the rootstock and the scion rather than the graft union, in combinations of varying vigor (Solari *et al.* 2006). Dwarf trees had smaller hydraulic conductance mainly exerted by the higher hydraulic resistance of the rootstock (Solari *et al.* 2006). According to the above observations lower hydraulic conductance in dwarf trees can be attributed to the smaller vessel diameter in the rootstock rather than the barrier of the graft union. Such lower rates of solute transport are expected to have an impact on growth rates of the grafted trees.

According to the above mentioned responses of the cherry trees to grafting on size controlling rootstocks or interstocks, any transfer of knowledge from the apple system to cherry should be performed with caution. Physiological changes that occur as a result of

the dwarfing phenomenon may be similar between the two systems as secondary responses, but the leading causes seem to be different.

Long distance transport of macromolecules

The plant body consists of a large number of cells that perform different functions. Cells of the same or synergistic function are organized into tissues that support certain physiological processes in the plant. Tissues are combined to form higher order structures called the organs. As a result plants have developed mechanisms to support this complex and highly organized structure. Communication between cells and even organs is necessary for the transmission of information critical to the survival of the organism. Very early in the history of plant biology it was identified that plants uptake nutrients from the soil and distribute them across the plant body to promote its expansion and reinforcement. The distribution occurs either through the symplast or the apoplast. Symplastic transport is performed by cell-to-cell transport through plasmodesmata, while apoplastic transport is performed through the vascular system, which is comprised of xylem and phloem. The discovery of plant hormones added another factor in the array of substances that can be transported through the vascular system to exert their action. Viruses were another group of molecules that were identified to move through the vascular system, even spreading to uninfected stock material grafted to infected stocks. Other signals affecting plant growth and development were identified early in the history of plant biology, such as the "florigen" responsible for the promotion of flowering (Zeevaart, 2006). Such signals were identified only through elegant experiments that

involved the grafting of tissues that had undergone different treatments (Zeevaart, 2006), without determining the nature of the signal though. Only recently the detection of such molecules has been made possible with the advent of new technologies and techniques (Hoffman-Benning *et al.* 2002; Huang *et al.* 2005).

Viral movement through the vascular system occurs via transport of nucleic acids, either DNA or RNA, and proteins. The transport is made possible by specialized proteins called Movement Proteins (MPs) that bind to the viral nucleic acids (Leisner and Turgeon, 1993; Fujiwara *et al.* 1993; Lee and Lucas, 2001; Itaya *et al.* 2002). The nucleoprotein complex that forms can move through the phloem and enter destination tissues by passing through plasmodesmata. This is accomplished by the increase of the size exclusion limit (SEL) in plasmodesmata by the MPs. The ability of viral proteins and nucleic acids to move through the vascular system triggered the initiation of research on long distance transport of native proteins and nucleic acids. Furthermore, the use of plant species that yield significant amounts of phloem sap has permitted the isolation of many proteins and RNAs. As a result in the past decade we have seen major advances in the identification and understanding of macromolecular trafficking through the phloem (Lough and Lucas, 2006).

Several proteins have been identified to move non-cell autonomously, by cell-to-cell movement or through the phloem translocation stream (Lough and Lucas, 2006). Some of the proteins belong to the translocation machinery, facilitating the movement of other proteins or RNAs, while others function as long distance signals involved in developmental control. Important components of this transport pathway are the plasmodesmata, which form pores between neighboring cells of the phloem tissue for

intracellular communication (Zambryski and Crawford, 2000). Transport facilitators have similar properties as the virus movement proteins, allowing them to dilate plasmodesmata increasing their SEL and allow translocation of large proteins in the phloem translocation stream. Some of these proteins such as CmPP16 and CmHSC70-1 or -2 cause an increase in the SEL of plasmodesmata and facilitate their own transport (Xoconostle-Cazares *et al.* 1999; Aoki *et al.* 2002). CmPP16 shares some conserved domains with the movement protein of the Red Clover Necrotic Mosaic Virus (Xoconostle-Cazares *et al.* 1999), while CmHSC70-1 and -2 are heat shock cognate chaperones that use a conserved C-terminal domain to interact with the plasmodesmata and move through the phloem (Aoki *et al.* 2002). But probably the most significant effect of long distance transport is exerted by mRNAs involved in plant development. The *Mouse ears* (*Me*) leaf phenotype of tomatoes is caused by a fusion between a *KNOTTED1-like* gene *LeT6* and the *PYROPHOSPHATE-DEPENDENT PHOSPHO-FRUCTOKINASE (PFP)* (Kim *et al.* 2001). Even though the fused transcripts can be translocated to wild type scions after grafting, either protein cannot perform its function, producing a visible leaf phenotype. Another gene found to be transported through the phloem is *CmNACP*, which encodes a NAC domain protein (Ruiz-Medrano *et al.* 1999). The mRNA of *CmNACP* was translocated through the graft union from pumpkin stocks to cucumber scions and was localized in the apical meristem. Other regulatory sequences were also found to be transported through the graft union and accumulate in the shoot apical meristem of the interspecific scion (Ruiz-Medrano *et al.* 1999). One of these genes, *CmGAIP* is involved in gibberellin signaling and over-expression or dominant negative mutation causes alteration in the leaf phenotype of wild type scions in tomato (Haywood *et al.* 2005). Finally, the mRNA of the *FLOWERING*

LOCUS T gene in *Arabidopsis* has been shown to promote flowering by being expressed in the companion cells of the phloem and move systemically as a protein from the leaf to the shoot apex, where it is expressed, to the shoot apex and induce flowering, probably one of the most significant recent discoveries in plant biology (Huang *et al.* 2005; Jaeger *et al.* 2007; Mathieu *et al.* 2007). The property of these RNAs to be transported at long distances through interspecific graft unions shows that the latter do not impede movement through the vasculature. Nevertheless, the systems studied involve herbaceous plants and not trees. It is expected though that a similar system would apply to trees as well. Allelic variation between rootstocks may result in graft union transmissible RNAs that are perceived differently by the scion meristems or tissues. A notable discovery is that mRNA also can be transported between host and parasitic plants. Dodder (*Cuscuta pentagona* Engelm.) growing on tomato plants contained mRNA from the latter, some of which have been shown to move systemically in the phloem (Roney *et al.* 2007). One such gene was *LeGAI*, a homolog of *CmGAIP* that moves systemically into the phloem as discussed previously. Other unknown tomato sequences also were identified. Such a discovery suggests that mRNAs may not be targeted by the silencing machinery that exists in these plants. A similar response should be expected in the interspecific grafts as well, excluding the possibility of incompatibility. Nevertheless, such a hypothesis has to be tested further.

Long distance signals also move systemically in the form of peptides (Matsubayashi and Sakagami, 2006). The first such peptide was identified in tomato and induced systemic acquired resistance to herbivory and was thus named systemin. It induces resistance by activating the jasmonic acid signaling pathway (Ryan and Pearce,

2003). Another peptide signal is CLAVATA3 (CLV3) involved in shoot apical meristem determination of cell fate (Matsubayashi and Sakagami, 2006). CLV3 is expressed in layers L1 and L2 of the SAM and moves extracellularly to the central zone to activate the membrane bound receptors CLV1/2 (Rojo *et al.* 2002). The role of these peptides coincides with that of mobile RNA molecules, which act at the destination tissue or cell type. In contrast, phloem mobile proteins, as discussed before, are acting as helper molecules in macromolecular transport rather than signaling molecules.

In conclusion, all of these factors can affect communication between heterologous grafts through reduced interaction affinities. Identification of such signals though is a challenge for species that produce small amounts of phloem sap. Development of new techniques and use of the already known homologs from model species can advance significantly our understanding on the function of these molecules in diverse plant species and a putative role in RID.

Hypothesis and objectives

Previous research has shown that a significant factor in RID is the genotype of the rootstock and to some extent of the scion. Current advances in molecular techniques are allowing the investigation of the genetic differences in non-model plant systems. The hypothesis to be tested in this study is that "Long distance signaling between the rootstock and the scion has an effect on gene expression in both genotypes. Eventually these differences should lead to differential growth between graft combinations of varying vigor". Identification of genes responsible for the differences in growth between

combinations of varying vigor should eventually lead to the signals that cause dwarfing. The objectives of this study were: 1) To identify the most informative measures of growth capable of showing differences in vigor within a growing season. These measures will be used as guides for the analysis of gene expression, 2) Screen samples of dwarfing and vigorous combinations to identify differentially expressed genes. Samples will originate from tissues that have the most important contribution in the control of vigor.

The long term aim of this study is the production of genetic markers that can be used in future rootstock breeding programs. The long generation time and the difficulties in crossing due to self incompatibility make cherry a difficult fruit crop to breed. Any marker that can easily discriminate the desirable traits is of great need to the cherry breeder. Nevertheless, the range of rootstock vigor suggests a quantitative trait locus rather than a single or a few genes, adding to the difficulty of breeding the desirable rootstocks. Furthermore, dwarfness in the currently established rootstocks is not necessarily linked to the same QTLs.

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CHAPTER 1

GROWTH CHARACTERISTICS OF DWARF AND SEMI-VIGOROUS CHERRY GRAFT COMBINATIONS

INTRODUCTION

Characterization of a rootstock according to its vigor is not strictly defined. The most common method of categorizing rootstock vigor makes use of the trunk cross sectional area (TCSA) or trunk circumference in comparison to a reference vigorous rootstock. Reference rootstocks are those used traditionally before the breeding of size reducing rootstocks. In apple the reference rootstock is MM111, in peaches 'Nemaguard' and in cherry *P. avium* 'Mazzard' seedlings. Caution is needed though in this categorization due to the unparallel expansion of the trunk and elongation of the stems. Rusholme *et al.* (2004) in an attempt to map the dwarfing locus in apple, have shown that a population of rootstocks segregating for the dwarfing phenotype exhibited varying vigor, but were ordered differently whether TCSA or tree height was used. Other measures of growth include total tree height, current year shoot elongation and spread of canopy. It is not yet established which growth size indicator of a tree should be used to define its vigor in relation to other rootstocks.

One of the most important cherry rootstock breeding programs was developed in Giessen, Germany in the 1960's through 1980's under Professor Werner Gruppe (1985a). The program made use of an extended collection of *Prunus* species that are compatible and of smaller size than the standard sweet (*P. avium*) and sour cherry (*P. cerasus*) trees. Several interspecific crosses were tested which produced an array of rootstocks with varying vigor (Gruppe, 1985c). The most promising rootstocks were those whose parents belonged to the *P. cerasus, P. avium, P. canescens* Bois. and *P. fruticosa* Pall. species. The selection process involved testing the bred rootstocks for vigor control, precocity, yield, suckering, graft-incompatibility and winter hardiness (Gruppe, 1985b; Strauch and

Gruppe, 1985). Vigor in the local conditions ranged from 80% to 15% of standard rootstocks, when measured as shoot length (Seif and Gruppe, 1985). Sizes used for the determination of vigor were TCSA, shoot length, tree height and spread of canopy (Gruppe, 1985c; Franken-Bembenek, 1985; Wagner and Gruppe, 1985; Seif and Gruppe, 1985). Bud set was also measured in both grafted and ungrafted rootstocks. Shoot growth cessation was shown to be the driving force for height control (Seif and Gruppe, 1985). Dwarfing rootstocks tended to cease growing earlier than vigorous ones and as a result final shoot length was smaller. When the sweet cherry cultivar 'Hedelfingen' was used as scion, shoot growth cessation occurred significantly earlier than the ungrafted rootstocks, even for the vigorous ones (Table 1.1).

Perhaps one of the most successful rootstocks produced in this project was named Gisela 5 (Gi5) for Giessen Selection A 5 with an initial code 148/2. Gi5 has been tested extensively in rootstock trials and has proven to be precocious, dwarfing and high yielding. Several rootstock trials have incorporated Gi5 into their tests, which showed a 30-50% reduction in vigor compared to standard rootstocks (Facteau *et al.* 1996; Franken-Bembenek, 1996; Webster and Lucas, 1997; Lichev, 2001; Santos *et al.* 2006). The rootstock that most resembles Gi5, but was produced independent of the Giessen program, is Tabel® Edabriz that belongs to the *P. cerasus* species. Edabriz shows the same vigor and precocity as Gi5, but is less productive than Gi5 (Santos *et al.* 2006). It would be interesting to see whether this similarity in phenotype is due to the *P. cerasus* background of both rootstocks.

Table 1.1: Growth measurements of rootstocks produced in the Giessen cherry rootstock breeding program (adapted from Seif and Gruppe, 1985). Letters in shoot length indicate significant similarities.

Rootstock origin	Clone No.	Shoot length(cm)		Cessation of shoot growth (days from bud break)	
Cross		Ungrafted	Grafted	Ungrafted	Grafted
P.avium (control)	F12/1	142.7 a	37.7 a	102	61
P.cerasus x *P.fruticosa*	154/4	62.1 g	25.7 de	82	55
	154/7	54.1 g	25.9 de	81	55
P.cerasus x *P.canescens*	148/1(Gi6)	84.5 de	35.8 ab	81	61
	148/2(Gi5)	59.8 g	29.4 bd	69	55
	148/8	80.0 de	34.1 ab	81	61
	148/9	97.7 cd	22.9 de	90	47
P.fruticosa x *P.avium*	172/3	81.6 de	23.5 de	74	47
	172/9	20.0 h	11.9	54	47
P.fruticosa x *P.cerasus*	173/5	62.2 fg	20.3 ef	79	55
	173/9	64.9 fg	22.7 de	82	40
P.canescens x *P.avium*	196/4	96.1 c	26.6 cde	85	55
	196/13	93.3 cd	27.1 cde	95	55
P.canescens x *P.cerasus*	195/1	110.5 b	24.5 de	89	47
	195/2	73.8 ef	29.6 bd	84	47

Another promising rootstock that was produced in the Giessen program is Gi6 (148/1). It is more vigorous than Gi5, reaching 70-80% of standard rootstocks, but it is precocious and high yielding (Franken-Bembenek, 1996). Since both Gi5 and Gi6 have the same genetic background (*P. cerasus* x *P. canescens*) it will be interesting to see which locus is responsible for vigor control.

The objective pursued in this chapter is to identify the most informative measures of growth capable of showing differences in vigor within a growing season. These measures will be used as guides for the analysis of gene expression in the following chapters. The rootstocks Gi5, Gi6 and Tabel® Edabriz were used in this study due to their genetic and phenotypic similarities.

MATERIALS AND METHODS

Plant material

The trees used in this experiment were purchased from commercial nurseries. The graft combinations were 'Bing'/Gi5 and 'Bing'/Gi6 in which the scion was 1-year-old and the rootstock 2-years-old when purchased. 'Bing' is a commercial sweet cherry cultivar while Gi5 and Gi6 rootstocks are triploid F1 progeny from an interspecific cross between the tetraploid sour cherry (*P. cerasus* L. cv. *Schattenmorelle*) and the diploid greyleaf cherry (*P. canescens* Bois.) (Franken-Bembenek, 1996). The trees for each graft combination were planted in the spring of 2001 in two rows of 50 trees or in spring 2004 in one row of 50 trees, each with 6 meter row spacing and a 2 meter tree spacing with a North to South orientation at the MSU Clarksville Horticultural Experiment Station,

Clarksville, Michigan. Trees of the graft combination 'Bing'/Edabriz were planted in 2002 in two rows of 50 trees at the same location and the same planting distances with the other two combinations. Pruning was performed every spring prior to bud break so that only the main trunk was retained above ground. Flowers were removed before pollination.

Measuring

Ten trees for each graft combination were selected for the measurements. The trees were selected with the following criteria: healthy trunk before bud break devoid of canker or freezing damage and intact apical bud. The morphology of the plot consisted of a wet, fertile north part and a sandy, dry south part. The selected trees were equally distributed across the plot, to include both plot conditions. For the trunk diameter measurements, the trunk of the trees was marked initially with a tape and later with paint 10cm above the graft union. Measurements were taken with a digital caliper (VWR) which was always placed in the marked position with the same direction to avoid fluctuations due to positioning since the trunk is not a perfect cylinder. Main shoot length measurements were taken with a tailor's meter. The zero point was placed in the base of the shoot and the apical meristem was the end. Arching shoots were straightened during measuring. Shoots that were damaged by wind or deer were removed from the analyses. Node number was measured by counting the number of buds present on the growing shoot. The basal buds were not considered in the measurements. Counting started from the first bud that would form a metamer. The node formed in the shoot apex by the first fully unfolded leaf was considered the last node. In September 2002 after the leaves had

dropped all trees were measured for shoot length and node number. Those with damaged shoots were excluded from further analysis.

Laser scanning confocal microscopy of pith and epidermal cells

Cell number and approximate cell size within a specific internode length were determined from main shoot sections taken from the sixth internode on the 3rd of July 2002 or the tenth internode on the 29th of July. Pith cell measurements were obtained from radial sections of a 1 cm portion of the internode that were obtained manually with a razor blade. Epidermal cell measurements were obtained from a thin layer of epidermal cells that was obtained in tangential sections from the same portion of the internode with a razor blade. The sections were washed in 100% ethanol for 4 hr to reduce browning and remove the chlorophyll. Sections were then washed twice in distilled water for 30 min to remove the ethanol. Staining was performed in 0.01% acrydine orange for 12 hr followed by a wash in distilled water for 5 min to remove excess dye. The Zeiss Axiophot Laser Scanning Microscope (LSM) was used for fluorescent imaging. A laser line of 488 nm was induced by an argon laser, passed through a primary dichroic mirror at 488 nm and the produced fluorescence was filtered through a secondary dichroic mirror at 545 nm. Emission was viewed with a band pass filter at 505-530 nm. LSM pictures were analyzed using the software accompanying the microscope. Vertical lines of known size were drawn parallel to the shoot axis in the pictures of pith and epidermal cells. The number of cells falling within the line was counted and the average cell size was determined as (linear length)/(cell number). Cells forming continuous files were counted. One or more measurements were obtained for two or more sections from three independent

shoots/trees. Data were analyzed using ANOVA for unequal number of replications with sub-samples at α=0.05. Epidermal cells on the 29th of July were embedded in a thick cuticle, preventing imaging of the cells.

Statistical analysis

Statistical analysis was performed using the statistical software SAS v8.0. The appropriate test statistics were used where applicable.

RESULTS

Monitoring tree growth in three growing seasons

The objective of this project was the identification of critical points in growth of dwarf and vigorous trees within the growing season. Establishment of those critical points would allow in depth and more focused analysis of the changes in the biology between trees of different vigor. Measurements were taken for trunk circumference, main shoot elongation and node number. Two or three year old scions of 'Bing' sweet cherry grafted on Gisela5, Gisela6 or Edabriz were used for the measurements. Vegetative growth was reduced to a single growing point by removal of the side branches and the flowers before pollination. This action limited resources to those stored in the main stem and gave all graft combinations a common initiation point. Any growth potential of the pruned trees would be supported by a single stem compared to multiple stems in unpruned trees. The removal of flowers was aimed at eliminating any effect that a strong sink such as fruit, would have on vegetative growth. 'Bing'/Gi5 trees exhibit significant

differences in shoot elongation between fruit thinned, un-thinned and trees without fruit (Whiting and Lang, 2004). The latter showed the most vigorous and homogenous growth, while shoots on trees with fruit exhibited reduction in the elongation rates at stage III of fruit growth that were restored after harvest (Whiting and Lang, 2004).

Plantings occurred in 2001 and 2004 for 'Bing'/Gi5 and 'Bing'/Gi6 trees and in 2002 for 'Bing'/Edabriz. Measurements were taken weekly following bud break in the years 2002, 2003 and 2005. In the first year (2002) measurements were taken twice a week at the end of the growing season to have a more detailed monitoring of tree growth cessation.

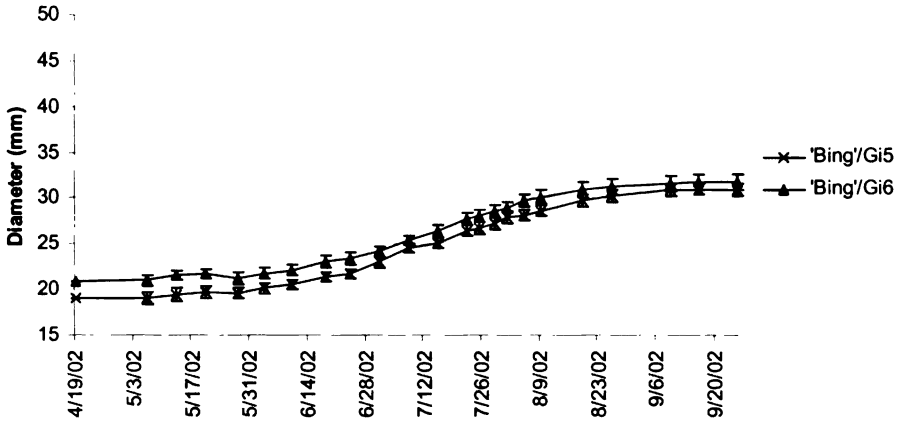
Expansion of trunk girth does not differ significantly between graft combinations

The traditional measure of tree vigor is trunk circumference that can also be presented as Trunk Cross Sectional Area (TCSA), if assumed that the trunk is circular. It is measured in trees of 6 to 10 years of age, when the difference in vigor is more distinct between graft combinations, since trunk circumference expansion is additive through the years. In this study we further measured the progress of trunk expansion within the growing season to detect any discrepancies between growth of dwarfing and vigorous rootstocks. The trees were very young with a thin trunk, thus measuring the diameter was more accurate than measuring the trunk circumference. Values of trunk diameter were taken 10cm above the graft union to avoid the effect of the graft union swelling. The trunk diameter of 'Bing'/Gi6 trees was consistently larger than that of 'Bing'/Gi5 and 'Bing'/Edabriz trees, but the difference depended on the age of the trees (Figure 1.1, Table 1.2). Two year old trees (2002, 2005) did not show significant difference in

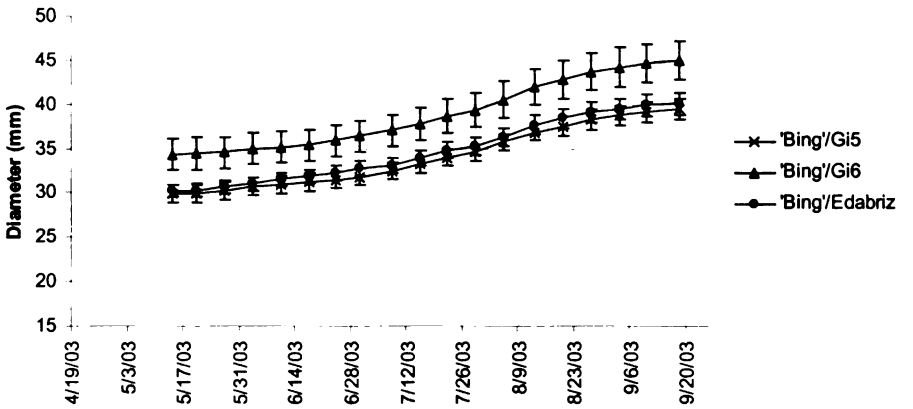
diameter, but in three year old trees (2003) 'Bing'/Gi6 trees had larger diameter than 'Bing'/Gi5. Expansion of the trunk for all the grafts was parallel throughout the growing season, which is illustrated in detail by the expansion rate of the trunk (Figure 1.2). In all years examined the expansion rate was higher in the end of July, after the cessation of shoot growth as it is shown later. In 2003 the trunk diameter of ungrafted Gi5 and Gi6 trees was measured. Expansion in these trees is more stable throughout the growing season (Figure 1.3) and follows a similar expansion rate as the two year old 'Bing' scions in 2002 and 2005 (Figure 1.2A, C). The expansion rate of the trees in all growing seasons is significantly affected by the environmental conditions rather than the age of the trees. This conclusion is supported by the highly synchronized growth rate within each year between graft combinations but also the changes in growth rate within each year due to changes in the environment (Figure 1.2).

Figure 1.1: 'Bing' cherry tree trunk diameter growth across three growing seasons and across three graft combinations. A. Growing season of 2002, B. Growing season of 2003, C. Growing season of 2005. In 2002 and 2005 measurements were taken from 2-year old trees, while in 2003 measurements were taken from 3-year old trees. Error bars indicate standard error.

A


Diameter (mm)
50
45
40
35
30
25
20
15
4/19/02
5/3/02
5/17/02
5/31/02
6/14/02
6/28/02
7/12/02
7/26/02
8/9/02
8/23/02
9/6/02
9/20/02
'Bing'/Gi5
'Bing'/Gi6

B


Diameter (mm)
50
45
40
35
30
25
20
15
4/19/03
5/3/03
5/17/03
5/31/03
6/14/03
6/28/03
7/12/03
7/26/03
8/9/03
8/23/03
9/6/03
9/20/03
'Bing'/Gi5
'Bing'/Gi6
'Bing'/Edabriz

C

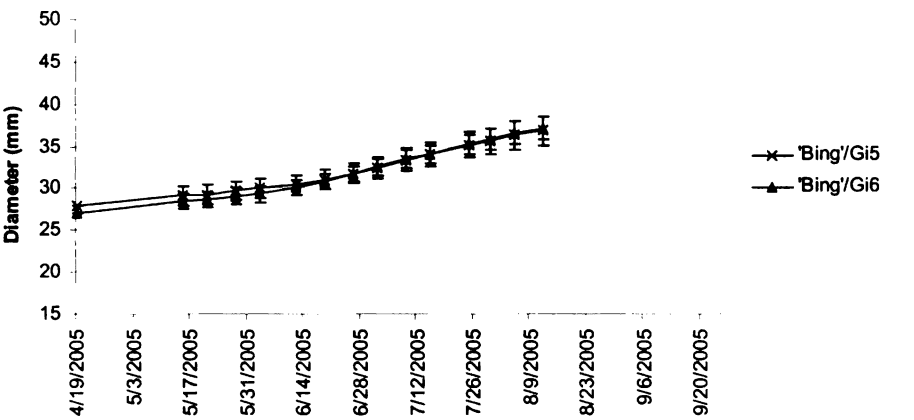
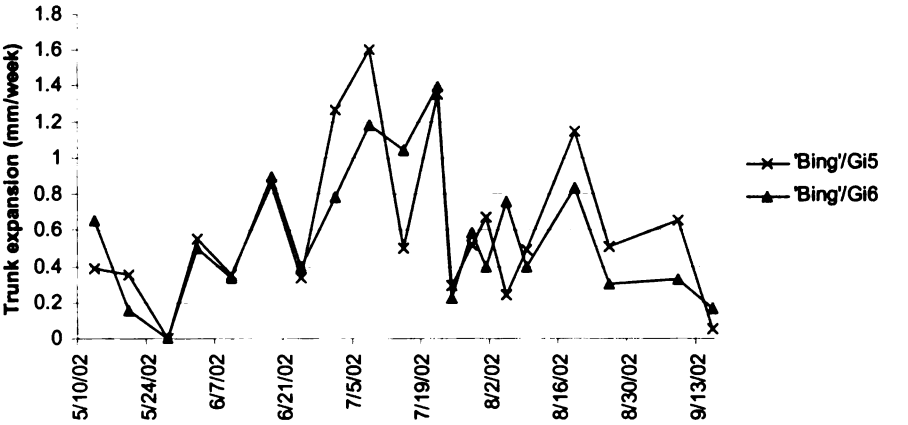
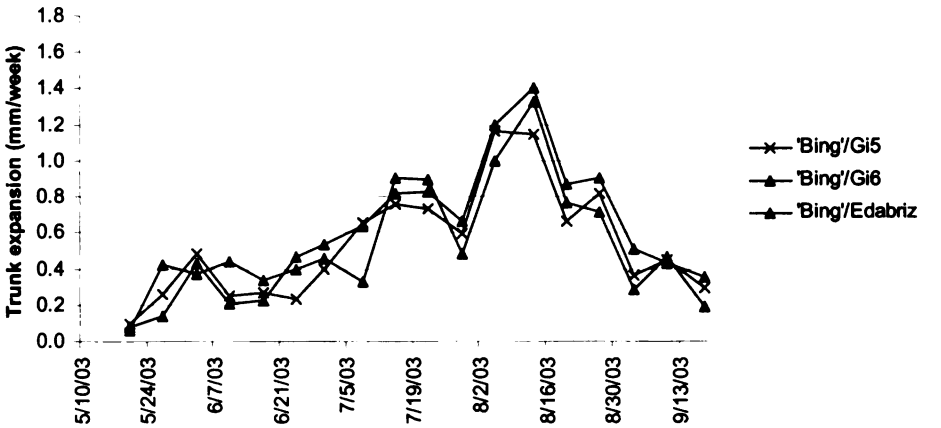

Diameter (mm)
50
45
40
35
30
25
20
15
4/19/2005
5/3/2005
5/17/2005
5/31/2005
6/14/2005
6/28/2005
7/12/2005
7/26/2005
8/9/2005
8/23/2005
9/6/2005
9/20/2005
'Bing'/Gi5
'Bing'/Gi6

Figure 1.2: Trunk diameter growth rate in 'Bing'/Gi5 and 'Bing'/Gi6 cheery trees in 2002 (A), 2003 (B) and 2005(C). In 2003 trunk expansion rate was calculated also for 'Bing'/Edabriz trees. Expansion rate was calculated as the ratio of weekly trunk diameter increase divided by the number of days between measurements. In 2002 and 2005 measurements were taken from 2-year old trees, while in 2003 measurements were taken from 3-year old trees.

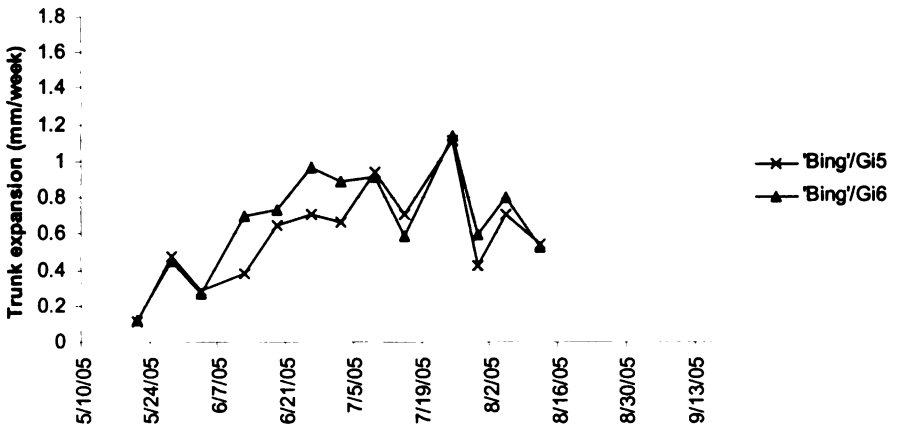
A


Trunk expansion (mm/week)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
5/10/02
5/24/02
6/7/02
6/21/02
7/5/02
7/19/02
8/2/02
8/16/02
8/30/02
9/13/02
'Bing'/Gi5
'Bing'/Gi6

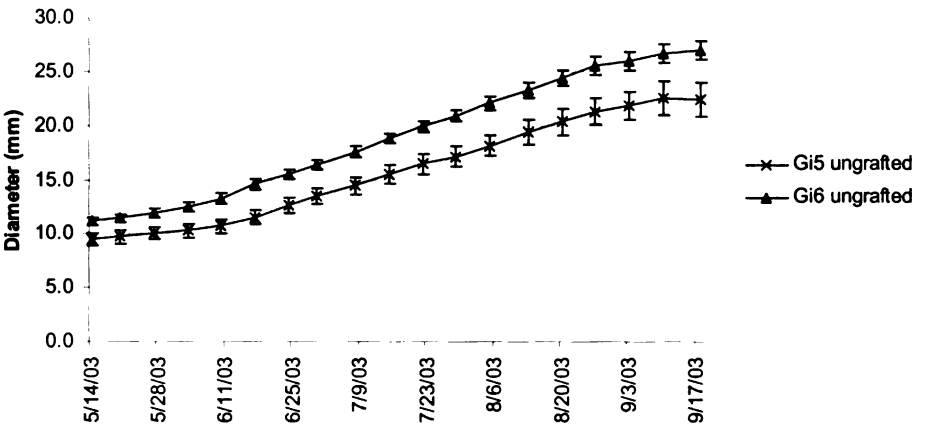
B


Trunk expansion (mm/week)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
5/10/03
5/24/03
6/7/03
6/21/03
7/5/03
7/19/03
8/2/03
8/16/03
8/30/03
9/13/03
'Bing'/Gi5
'Bing'/Gi6
'Bing'/Edabriz

C


Trunk expansion (mm/week)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
5/10/05
5/24/05
6/7/05
6/21/05
7/5/05
7/19/05
8/2/05
8/16/05
8/30/05
9/13/05
'Bing'/Gi5
'Bing'/Gi6

A



B

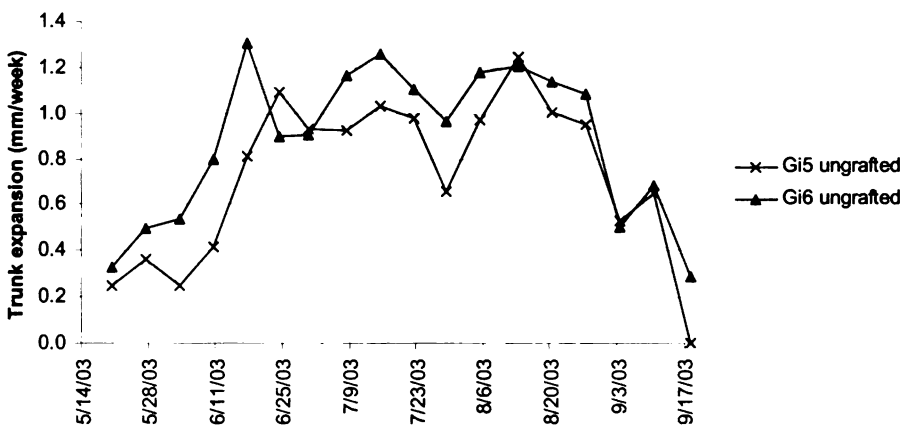


Figure 1.3: (A) Trunk diameter of ungrafted Gi5 and Gi6 rootstocks measured in 2003, (B) Trunk expansion rate of the same trees expressed as the ratio of weekly trunk expansion divided by the number of days between measurements. Error bars indicate standard error.

Table 1.2: Growth characteristics of cherry 'Bing' scions grafted on Gi5 and Gi6 rootstocks across three growing seasons.In 2002 and 2005 measurements were taken from 2-year old trees, while in 2003 measurements were taken from 3-year old trees. Letters denote significant difference at an a=0.05. (n=9 except for Trunk diameter where n=10)

	Final shoot length (cm)			Final node number			Metamer length (mm)			Trunk diameter (mm)		
	2002	2003	2005	2002	2003	2005	2002	2003	2005	2002	2003	2005
Bing'/Gi5	49.1a	46.1a	42.4a	27a	26a	23a	18.2a	17.5a	18a	31.0a	39.1a	36.8a
Bing'/Gi6	66.9b	57.3a	57.9b	37b	34b	29b	17.9a	16.8a	19.9b	31.8a	44.6b	37.1a

Main-shoot elongation in three growing seasons and different graft combinations

As a measure of vegetative growth and a major contributor to tree height, we monitored the elongation of the main shoot in the same trees as those used for measuring trunk diameter. All the trees, irrespective of the rootstock, broke bud simultaneously in mid April consistently for all three years. Shoot elongation followed a sigmoidal curve for the 'Bing' scions and the ungrafted Gi6 rootstocks, but followed a more linear curve for the ungrafted Gi5 rootstocks (Figure 1.4, 1.6A). Elongation was slow in the first 6 weeks, but remained equal for all grafted trees (Figure 1.4). During the 7^{th} or 8^{th} week, elongation differentiated between the semi-vigorous 'Bing'/Gi6 trees and the dwarfing 'Bing'/Gi5 or 'Bing'/Edabriz, which is shown in Figures 4 and 5, by the separation of the curves. In 2003 and 2005 this differentiation coincided with the highest elongation rate (Figure 1.5B,C), but in 2002 it occurred at a stage when elongation rate was not maximum (Figure 1.5A). Following shoot growth cessation, bud set occurred 1 to 3 weeks earlier for 'Bing'/Gi5 and 'Bing'/Edabriz trees compared to 'Bing'/Gi6 trees (Figure 1.7), with the exception of 2005 when bud set was completed the same week. 'Bing'/GI5 trees showed consistently earlier bud set compared to 'Bing'/Gi6 in all three years. At the end of the growing season 'Bing'/Gi5 shoots grew at 73%, 79% and 73% of the 'Bing'/Gi6 shoots in 2002, 2003 and 2005 respectively (Table 1.2). It has to be noted that 'Bing'/Gi5 trees sustained frost damage in the winter of 2004-2005, which led to the loss of many trees. Many of the trees that survived showed bleeding from the trunk. 'Bing'/GI6 showed much lower damage.

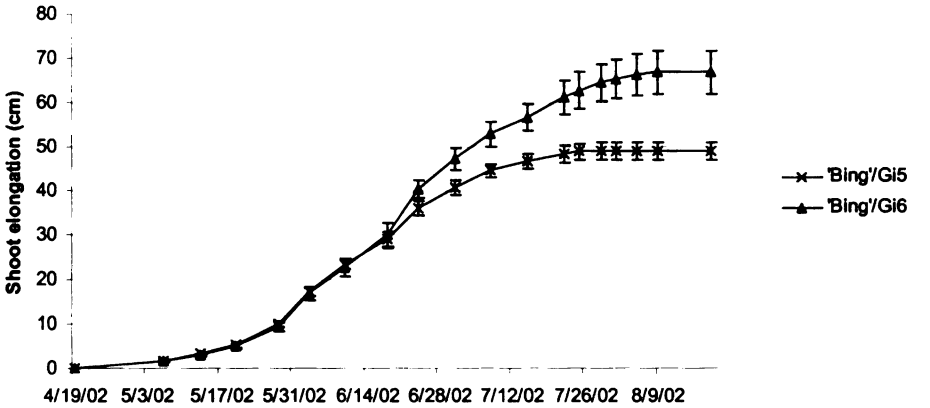
In contrast, ungrafted trees had a slower shoot elongation, which though lasted for a longer duration, until the middle of September (Figure 1.6). This is 7 to 8 weeks later

than the grafted trees, but is largely due to 10-20% of the trees that did not set bud (Figure 1.7B). Elongation rate for Gi5 was stable across the growing season, in contrast the elongation rate of Gi6 which reached a maximum in the beginning of July, 8 weeks after bud break (Figure 1.6B). Nevertheless, 100% bud set occurred simultaneously for the two rootstocks. Final shoot length was less for Gi5 compared to Gi6, which is consistent with the results in the grafted trees.

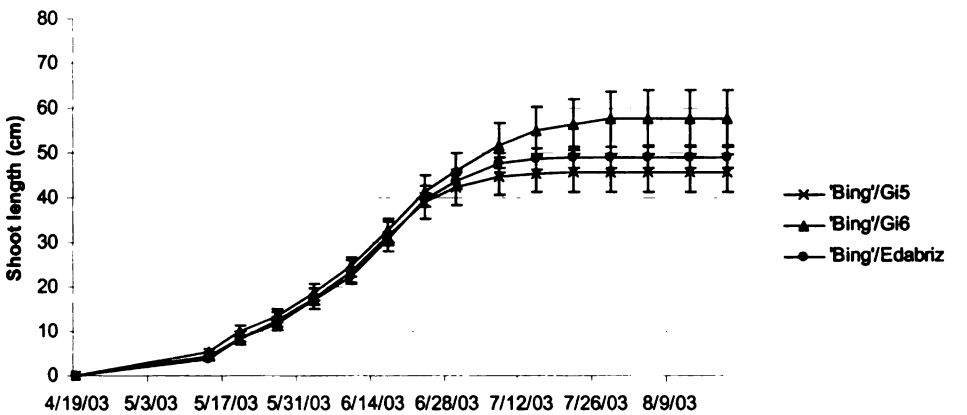
As it was observed for trunk diameter, the weather seemed to affect the temporal changes in elongation rate, depicted by the parallel changes in the elongation rate shown in Figure 1.5.

Figure 1.4: Main shoot length of 'Bing'/Gi5, 'Bing'/Gi6 cherry trees taken in 2002 (A), 2003 (B) and 2005 (3). Measurements were taken from bud break to bud set. Measurements were also taken for 'Bing'/Edabriz trees in 2003. In 2002 and 2005 measurements were taken from 2-year old trees, while in 2003 measurements were taken from 3-year old trees. Error bars indicate standard error.

A


Shoot elongation (cm)
80
70
60
50
40
30
20
10
0
4/19/02 5/3/02 5/17/02 5/31/02 6/14/02 6/28/02 7/12/02 7/26/02 8/9/02
'Bing'/Gi5
'Bing'/Gi6

B


Shoot length (cm)
80
70
60
50
40
30
20
10
0
4/19/03 5/3/03 5/17/03 5/31/03 6/14/03 6/28/03 7/12/03 7/26/03 8/9/03
'Bing'/Gi5
'Bing'/Gi6
'Bing'/Edabriz

C

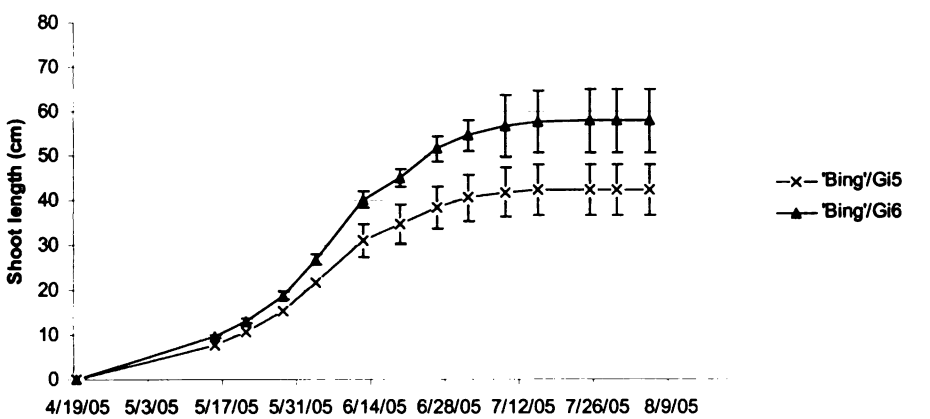
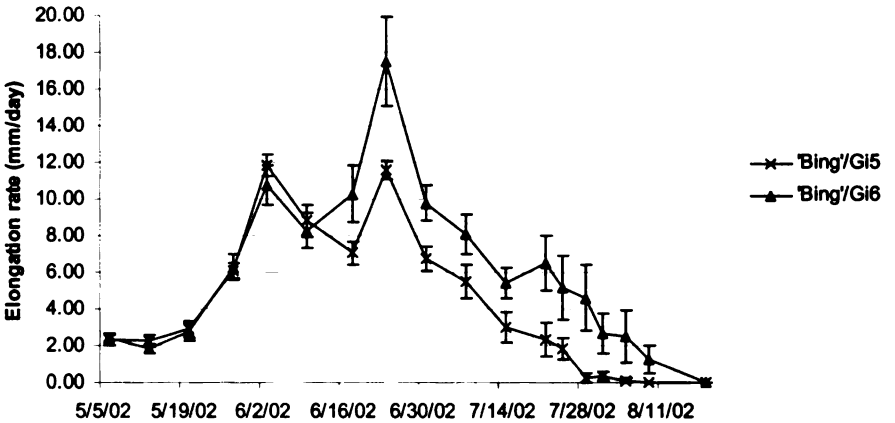
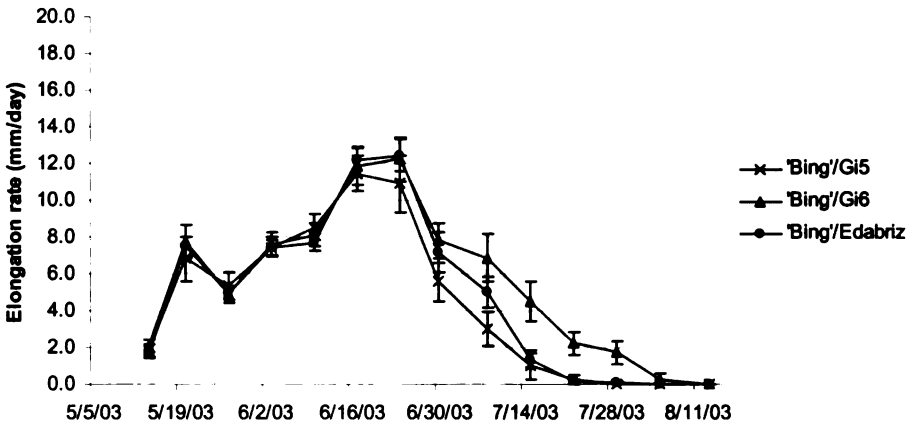

Shoot length (cm)
80
70
60
50
40
30
20
10
0
4/19/05 5/3/05 5/17/05 5/31/05 6/14/05 6/28/05 7/12/05 7/26/05 8/9/05
'Bing'/Gi5
'Bing'/Gi6

Figure 1.5: Main shoot elongation rate in 'Bing'/Gi5 and 'Bing'/Gi6 cherry trees in 2002 (A), 2003 (B) and 2005(C). In 2003 shoot elongation rate was calculated also for 'Bing'/Edabriz trees. Expansion rate was calculated as the ratio of weekly shoot elongation divided by the number of days between measurements. In 2002 and 2005 measurements were taken from 2-year old trees, while in 2003 measurements were taken from 3-year old trees. Error bars indicate standard error.

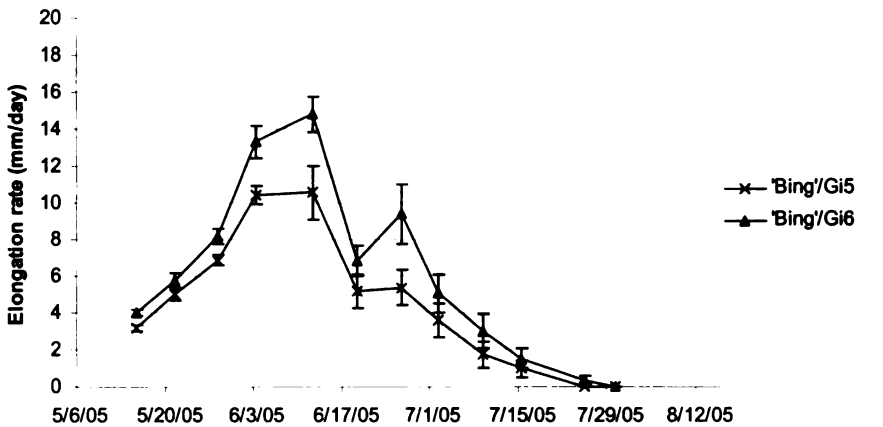
A


Elongation rate (mm/day)
20.00
18.00
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
5/5/02
5/19/02
6/2/02
6/16/02
6/30/02
7/14/02
7/28/02
8/11/02
'Bing'/Gi5
'Bing'/Gi6

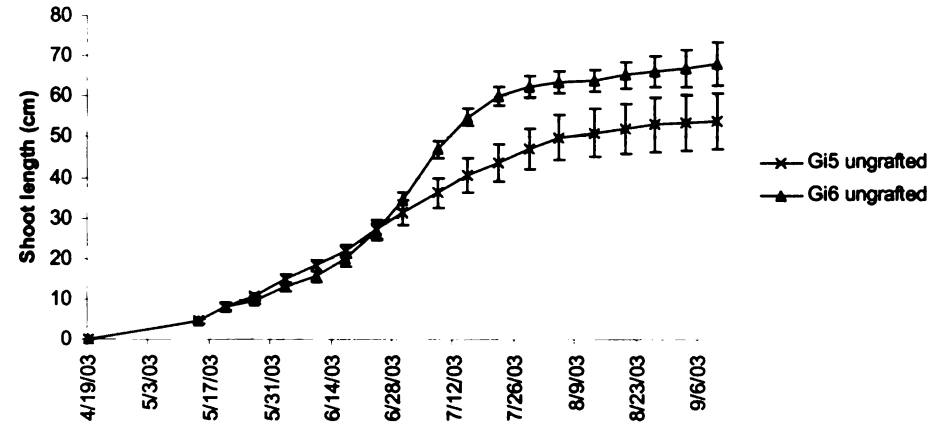
B


Elongation rate (mm/day)
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
5/5/03
5/19/03
6/2/03
6/16/03
6/30/03
7/14/03
7/28/03
8/11/03
'Bing'/Gi5
'Bing'/Gi6
'Bing'/Edabriz

C


Elongation rate (mm/day)
20
18
16
14
12
10
8
6
4
2
0
5/6/05
5/20/05
6/3/05
6/17/05
7/1/05
7/15/05
7/29/05
8/12/05
'Bing'/Gi5
'Bing'/Gi6

A



B

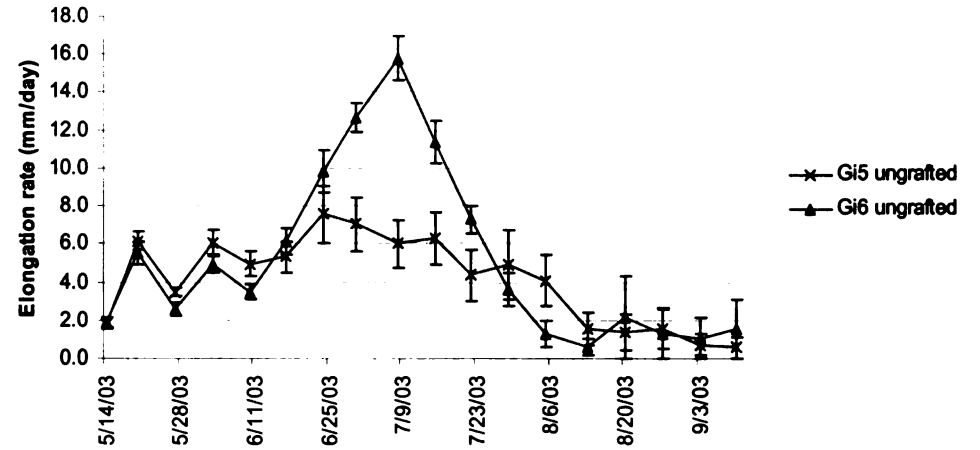
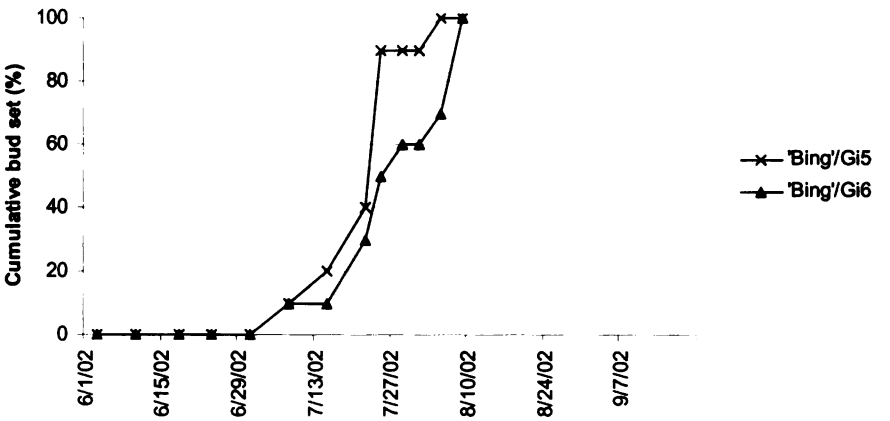


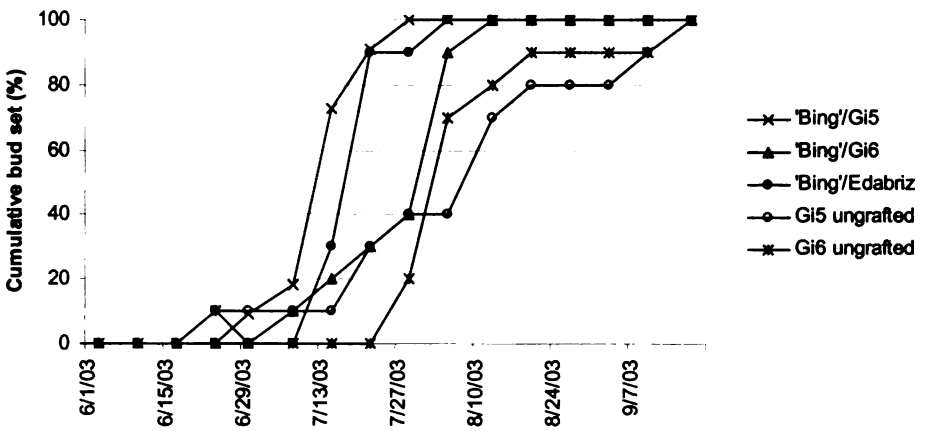
Figure 1.6: Shoot growth characteristics of ungrafted rootstocks Gi5 and Gi6 in the growing season of 2003. (A) Shoot length from bud break to bud set and (B) shoot elongation rate expressed as the ratio of weekly shoot growth divided by the number of days between measurements. Error bars indicate standard error.

Figure 1.7: Cumulative bud set in 'Bing'/Gi5 and 'Bing'/Gi6 trees for the growing seasons of 2002 (A), 2003 (B) and 2005 (C). Bud set was recorded for 'Bing'/Edabriz, Gi5 and Gi6 ungrafted rootstocks in the growing season of 2003 (B). Cumulative bud set is the percentage of trees for which the main shoot has set bud. The week when shoot growth cessation occurred was considered as the time of bud set (n=9-11). In 2002 and 2005 measurements were taken from 2-year old trees, while in 2003 measurements were taken from 3-year old trees

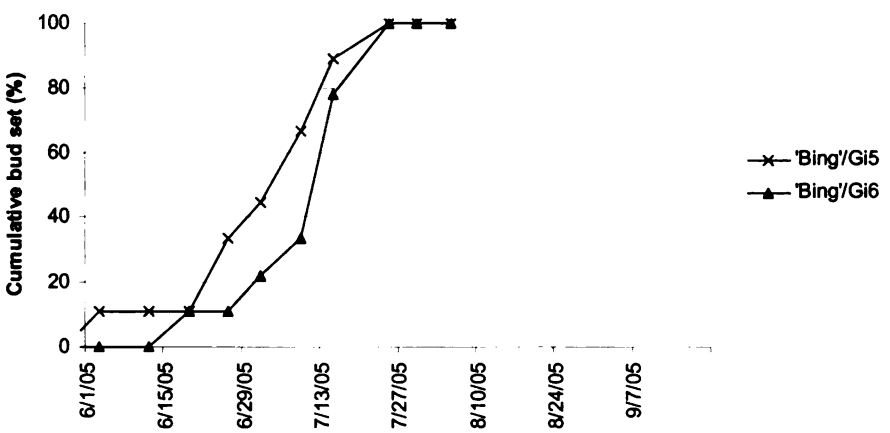
A


Cumulative bud set (%)
100
80
60
40
20
0
6/1/02
6/15/02
6/29/02
7/13/02
7/27/02
8/10/02
8/24/02
9/7/02
'Bing'/Gi5
'Bing'/Gi6

B


Cumulative bud set (%)
100
80
60
40
20
0
6/1/03
6/15/03
6/29/03
7/13/03
7/27/03
8/10/03
8/24/03
9/7/03
'Bing'/Gi5
'Bing'/Gi6
'Bing'/Edabriz
Gi5 ungrafted
Gi6 ungrafted

C


Cumulative bud set (%)
100
80
60
40
20
0
6/1/05
6/15/05
6/29/05
7/13/05
7/27/05
8/10/05
8/24/05
9/7/05
'Bing'/Gi5
'Bing'/Gi6

Measurements of internode number and metamer length

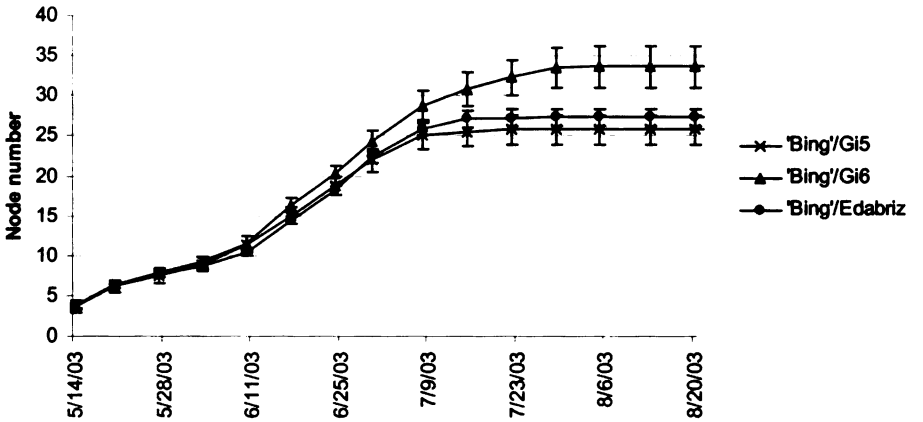
Shoot elongation measurements showed that tree height is controlled by shoot elongation and more specifically shoot growth cessation. The difference in shoot length was constant through the years, but it wasn't clear if growth cessation was the only determinant of this difference. The various plant hormones have been shown to affect shoot elongation, most notably gibberellin, which affects internode length and cell size (Fleet and Sun, 2005). Node number was measured in the same trees as for shoot elongation and trunk diameter, to detect any discrepancies in the number of nodes and length of internodes. In 2002 nodes were measured only after shoots ceased growing, but in 2003 and 2005 counting was performed in parallel to shoot elongation. In 2002, 50 trees were measured for each graft combination and gave an average node number of 30 in 'Bing'/GI5 and 39 in 'Bing'/GI6. In 2003 these numbers were 25 and 36 respectively, while for 2005 they were 23 and 29. The curve for node number increase was similar to the shoot elongation curves. When shoots were growing with the same rate, nodes were also added equally between dwarf and semi-vigorous trees (Figure 1.8). When shoot growth diverged between dwarf and semi-vigorous trees, node number followed this change (Figure 1.8). Metamer length at each time of the growing season was calculated as follows:

Mean Metamer length = shoot length/node number

Mean metamer length was not constant throughout the growing season (Figure 1.9). In the first 3 weeks of shoot growth metamers were short, since they are few in number and

are all elongating. During active shoot elongation, mean metamer length was maximal and it stabilized to a smaller length during shoot growth cessation (Figure 1.9). This decrease in metamer length at the end of the season does not indicate shrinking of the metamers, but rather inability of the last metamers to elongate fully, reducing thus the mean value. The length in the three growing seasons ranged between 1.7-2.0cm, but except from 2005 there was no significant difference in internode length between 'Bing'/Gi5 and 'Bing'/Gi6 trees (Table 1.2). In 2005 metamer length contributed to 29% of the difference in shoot length between 'Bing'/Gi5 and 'Bing'/Gi6 trees, with the rest of the difference (71%) being due to timing of shoot growth cessation.

A



B

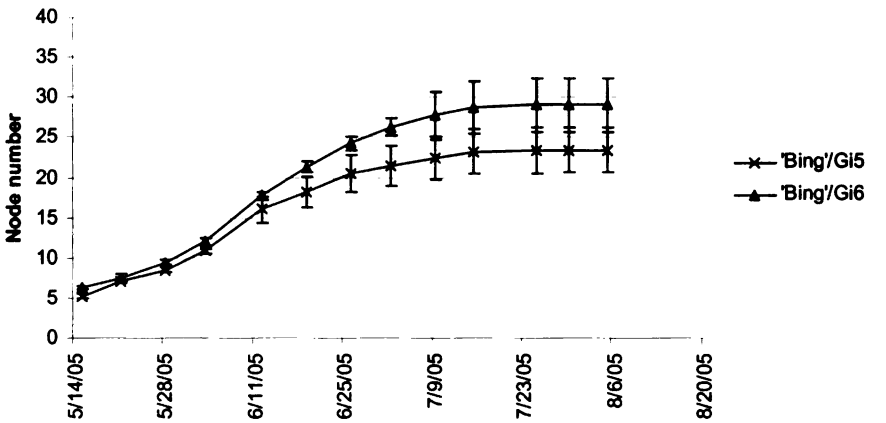
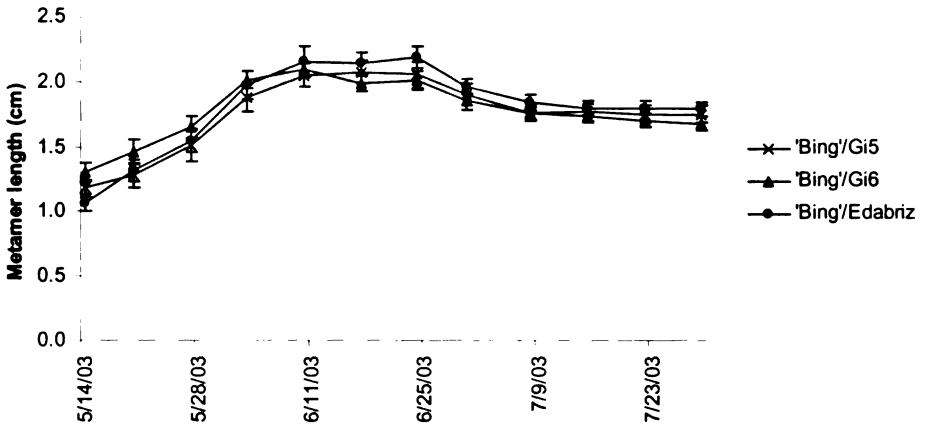


Figure 1.8: The number of nodes was measured in the main shoot of 'Bing'/Gi5 and 'Bing'/Gi6 in the growing season of 2003 (A) and 2005 (B). In 2005 measurements were taken from 2-year old trees, while in 2003 measurements were taken from 3-year old trees. Node number was also measured in 2-year old 'Bing'/Edabriz trees in the growing season of 2003 (A). Error bars indicate standard error.

A



B

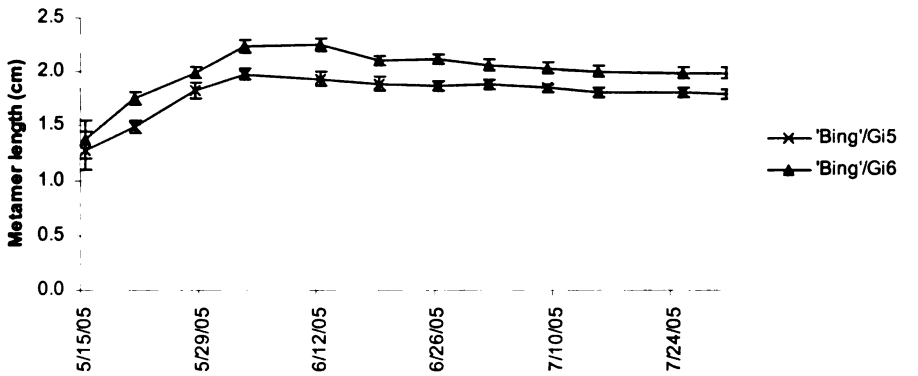


Figure 1.9: Metamer length of 'Bing'/Gi5 and 'Bing'/Gi6 trees in the growing season of 2003 (A) and 2005 (B). In 2005 measurements were taken from 2-year old trees, while in 2003 measurements were taken from 3-year old trees. Metamer length was also calculated for 2-year old 'Bing'/Edabriz trees in the growing season of 2003 (A). Metamer length was derived by the division of shoot length to node number for each measurement point. Higher metamer lengths were observed in June due to higher elongation rates. Reduction of metamer length reflects shorter internodes as shoot growth rates dropped near the time of shoot growth cessation.

Counting of cell size and number in metamers

The average metamer length was not significantly different between dwarfing ('Bing'/Gi5) and non-dwarfing trees ('Bing'/Gi6), with an average length of 1.7-2.0 cm in two of the three growing seasons as shown before. Even though the genetic background of the scions was the same, it was necessary to test whether there was a difference in growth at the cellular level within an internode. The size and number of cells that constitute the pith and the epidermis of the sixth internode were measured in shoots collected on 3 and 29 July 2002. On the 3rd of July, the sixth internode corresponded to approximately the first internode produced during the initiation of differential growth between dwarfing and non-dwarfing trees (Figure 1.4). Also, at this stage cells had stopped dividing and reached their final size. No significant differences were identified for cell number or size for the pith and epidermis from both graft combinations (Figure 1.10). This indicates that between the two rootstocks, main shoot internodes have equivalent cell numbers and cell sizes; however, in vigorous trees, the initial rate of shoot growth is maintained for a longer duration.

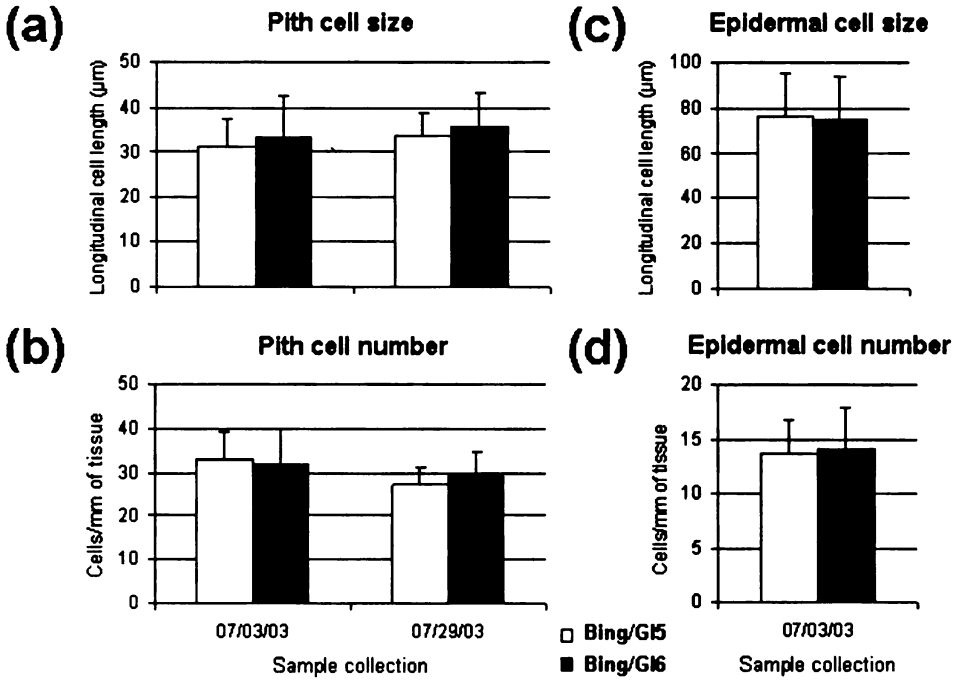


Figure 1.10: Cell size and number in the pith and epidermis of the 6th internode collected on the 3rd of July and the 10th internode collected on the 29th of July of 'Bing' sweet cherry trees on Gisela 5 (Gi5) and Gisela 6 (Gi6) rootstocks. (A) Pith cells were counted in radial sections of the internode. (B) Cell files in radial sections of the internode were used to measure the cell number per millimeter of pith. (C) Epidermal cell files were counted in tangential sections of the bark. (D) Cell number per millimeter of epidermis was counted in cell files of the 6th internode. Measurements were taken on 2-year old trees. Error bars indicate standard error.

DISCUSSION

Understanding the morphological changes that lead to dwarfism in cherry is crucial for the identification of the genetic factors that contribute to this phenomenon. Previous studies or rootstock trials have placed priority on the size of the trees at a reproductive age, which for most rootstocks is achieved after the 4th year of growth. Vigor is expressed through the trunk cross sectional area (TCSA) or the total tree height. But these are static measures that do not provide any insight in the progression of tree size. In the extended rootstock breeding program that produced the Gisela rootstocks, data on size were collected throughout the growing season to demonstrate the changes in size between the new rootstocks. In that trial, shoot elongation measurements of ungrafted and grafted rootstocks indicated a differentiation in the time of shoot growth cessation, as dwarf rootstocks stopped growing earlier (Seif and Gruppe, 1985; Franken-Bembenek, 1996). The same was observed in this study in grafted rootstocks.

The beginning of the growing season found all graft combinations to break bud at the same time. Shoot elongation was parallel for all the graft combinations. When shoot elongation reached the highest rate, there was difference between dwarf and semi-vigorous rootstocks. Dwarf rootstocks elongated more slowly and ceased growing earlier than the semi-vigorous rootstocks leading to shorter shoots and tree height. The initial parallel growth of all trees indicates the potential for growth exerted by the physiological status and genetic background of the scion. This also indicates that reserves and nutrient supply are not limiting at this stage of shoot growth. The onset of differential growth rate signifies a change in the biology of the various graft combinations. The signal for such a differentiation is not known, neither is its source. The driving force of this change should

relate to the environment, either directly or indirectly. Direct signals from the environment can be a change in photoperiod, a change in day to night temperature or water supply to the roots. Any effect of temperature or soil moisture can be excluded, since the response of the trees is consistent each year. In 2002 trees were not irrigated, while in 2003 they were, but the pattern of shoot growth did not change. Furthermore, the summer of 2002 was warmer than 2003 in mid-June, but shoot growth differentiated with a few days difference between the two years. When shoots of 'Bing'/Gi5 and 'Bing'/Edabriz trees started growing slower than 'Bing'/Gi6 shoots, photoperiod was reaching the summer solstice (June 22-23) at the Clarksville Horticulture Experiment Station. When 'Bing'/Gi6 shoots started growing slower the summer solstice was already past, thus downgrading the importance of photoperiod in the control of this phenomenon. In an extensive cataloguing of tree species according to their photoperiod response, Nitsch (1957) had placed cherry in the non-photoperiod responsive species, which is in agreement with the data presented in this chapter. Previous research on the effects of grafting has revealed a differential hyrdraulic resistance exerted by the difference in vessel diameter at the scion (Olmstead *et al.* 2006). Lower hydraulic conductance could have an impact on the support of growth especially at times of maximum growth rates. As shown in Figure 1.5B and C, shoot elongation rates differentiated between dwarfing and semi-vigorous trees when they exceeded 10mm/day. This was not evident in 2002 though, when shoot elongation rate differentiated as the rate of growth was dropping. Even though an effect of hydraulic conductance cannot be excluded, the consistency on the time of shoot growth cessation suggests a larger impact of another factor.

In contrast to the grafted trees, the ungrafted rootstocks continued growing until September, independent of their dwarfing ability. This observation increases the importance of the scion in the control of the dwarfing phenomenon and furthermore its interaction with the rootstocks. The same difference in shoot growth cessation between grafted and ungrafted rootstocks was identified by Seif and Gruppe (1985). Rootstocks grafted with 'Hedelfingen' had significantly earlier cessation of shoot growth compared to the respective ungrafted rootstocks that varied between 7 to 41 days. The genetic background of the rootstock is the indisputable factor that controls tree vigor, but based on the previous observation, the signal originates in the scion. It is then perceived differently by the various rootstocks and the process of shoot growth cessation is initiated. As indicated by the shoot elongation and node number curves, differential perception of the signal may be triggered when a threshold is achieved that is different between rootstocks. The nature of the response by the rootstock is not known. The absence of significant difference in the length of the internodes, the number and size of epidermal and pith cells, indicates that the difference in shoot length is due to control of the shoot apical meristem rather than cell division and expansion outside the meristem. Cells at the shoot apical meristem are produced in slower rates in the dwarf trees rather than the semi-vigorous. Cell division frequency thus should be lower in the first rather than the latter. As a result fewer nodes are produced by the end of the growing season in the dwarf trees (Figure 1.8).

In contrast to cherry, apples and peaches show a difference in shoot elongation between graft combinations of varying vigor, right from the beginning of the growing season (Weibel and DeJong, 2003; Webster, 1995). In the same systems, shoot growth

differences are a result of internode length and to a lesser extent of node number. This suggests an alteration in hormone perception or metabolism. Mutations in genes involved in gibberellin signaling lead to alterations in internode length and cell size (Peng *et al.* 1999; Boss and Thomas, 2002; Fleet and Sun, 2005). Loss of function mutants show reduced internode lengths, while gain of function mutations or application of gibberellins increases internode length (Fleet and Sun, 2005). Thus, in apples and peaches gibberellins may play a significant role in shoot elongation by affecting internode length. In cherries gibberellins do not seem to affect shoot growth, since internode length does not differ between grafts of different vigor.

Trunk diameter did not prove a good indicator of tree vigor within a growing season. Different graft combinations had parallel growth rates and any difference in vigor was not observed. Nevertheless, the diameter change is additive through the years and as the trees grow older the difference in vigor is made obvious. It should be noted though that trunk expansion rate was higher after shoot growth cessation, which is probably attributed to the change of sinks. Shoots are not allocating any more carbohydrates for their growth, but rather transfer it to the trunk and roots for storage. Thus, the trunk expands and stores more carbohydrates in the wood tissues. At the time of shoot growth cessation, new flower meristems start forming. Thus, dwarf trees that cease growing earlier than vigorous rootstocks can allocate more resources towards floral bud formation. This hypothesis can explain the increased productivity of dwarf rootstocks in contrast to vigorous ones, which allocate more photoassimilates to support shoot growth.

Similar growth patterns in cherry trees have been observed after the application of paclobutrazol (PBZ) through the soil. This growth regulator has been proposed as an

alternative to dwarfing rootstocks for cherry varieties planted on their own roots (Quinlan, 1985). Significant shoot length reduction has been reported in cherry trees after soil or foliar treatment with PBZ (Facteau and Chestnut, 1991; Snir, 1988; Asamoah and Atkinson, 1985; Looney and McKellar, 1987; Walser and Davis, 1989). Soil application of PBZ is comparable to the effect of the rootstock. The message for shoot growth cessation in dwarfing rootstocks originates in the root, while PBZ acts through the root. PBZ blocks the oxidation of *ent*-kaurene into *ent*-kaurenoic acid in the early steps in the biosynthesis of gibberellins (Rademacher, 2000). As a result of this blockage shoot internodes expand less compared to untreated shoots and the final shoot length is reduced (Webster, 1998).

Shoot elongation proved the most informative measure to depict the discrepancies in growth between dwarf and semi-vigorous graft combinations. Although the rootstock is controlling the final shoot length and as a result tree height, it is the scion that activates the initiation of shoot growth cessation. The nature of the signal originating in the scion is not yet determined. Study of the changes that occur between rootstocks at the time of initiation of shoot growth cessation can yield significant information on the nature of the agents and genes involved in this process.

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CHAPTER 2

IDENTIFICATION OF GENES EXPRESSED AT THE CRITICAL POINTS IN GROWTH BETWEEN DWARF AND SEMI-VIGOROUS GRAFT COMBINATIONS

INTRODUCTION

A significant amount of research has been performed in cherries and other fruit trees for the identification of the leading causes of rootstock induced dwarfing. Most of the research has focused on the physiological changes between dwarf and vigorous trees, as described in the Literature Review. Existing hypotheses involve changes in the concentration of chemical signals, such as hormones and phenolics, changes in the anatomy of the graft union or changes in the physiology of the trees (Literature Review). The leading cause for the ability of some rootstocks to dwarf scions lies in on the genetic makeup of those trees in comparison to vigorous rootstocks. Since allelic variation occurs in more than one genetic locus, it is expected that the varying degrees of vigor are due to many loci. This is supported by a map based cloning approach in apple aiming to identify dwarfing loci (Rusholme *et al.* 2004). Mapping resulted in the identification of a single dominant locus, though this could not explain 100% of the variation occurring in the segregating population. Many of the new cherry rootstocks are crosses between diploid and tetraploid *Prunus* species and they show a wide variety of vigor even within the same cross (Gruppe, 1985). This is a result of the numerous possible combinations of alleles originating from the parent species. Identification of this genetic variation would advance our knowledge of the interaction between rootstock and scion, and would lead to more efficient and rapid breeding or genetic engineering of new rootstocks. Recently, the importance of this genetic variation has been identified and a number of studies have emerged, attempting to identify genes involved in this phenomenon (Rusholme *et al.* 2004; Jensen *et al.* 2004). It is still early to conclude with certainty which signaling pathways or what enzymatic reactions control tree growth in a timely fashion.

The meristems responsible for the increase in plant size are the shoot apical meristem (SAM) and the vascular cambium. The activity of SAM determines aerial part architecture and plant size, whereas the cambium is involved in the increase of stem girth. Regulation of SAM has been extensively studied in plants, with *Arabidopsis* being the model for SAM development. Stem cell identity at the SAM is maintained by the *WUSCHEL* (*WUS*) - *CLAVATA* (*CLV*) interaction system (Williams and Fletcher, 2005). Expression of WUS is localized in the organizing center (OC), which is formed by the stem cells. Differentiation of stem cells occurs by the production of CLV3, a small peptide that binds to the CLV1-CLV2 receptor complex and signals the suppression of WUS outside the OC (Williams and Fletcher, 2005). *SHOOTMERISTMLESS* (*STM*) is another gene involved in the proliferation of the stem cells, acting in parallel to *WUS* in the maintenance of stem cells at the SAM (Veit, 2006). This is a very simplistic description of the regulation of stem cell maintenance that describes the major players in this process. New genes continue to be identified as suppressors or inducers of stem cell identity at the SAM. Cell divisions at the OC occur continuously during active growth to promote differentiation of new organs without depleting the OC of stem cells. Activation or deactivation of cell cycle related proteins occur at the post-translational level through phosphorylation or protein degradation (Horvath *et al.* 2003; Gegas and Doonan, 2006). During dormancy induction, cells at the apical meristem are arrested in the G1 phase of cell divisions, before replication of the DNA (Gegas and Doonan, 2006). The control of cell division during induction of dormancy is not well known. In poplars, shoot growth cessation is controlled by the CO/FT regulatory pathway (Bohlenius *et al.* 2005). *PtFT1* down-regulation through siRNA causes faster shoot growth cessation under short days

compared to wild type trees, indicating the importance of *PtFT1* in this process. The effect of *PtFT1* on cell cycle related genes has not been established. At the hormonal level, auxin and cytokinin are the most important players in the control of cell divisions (Shani *et al.* 2006; Veit, 2006). Cytokinin signaling positively regulates *STM*, but is negatively regulated by *WUS* to create a more precise control of SAM proliferation (Shani *et al.* 2006). Auxin has a role in organ development through suppression of *STM* and *CUP SHAPPED COTYLEDON1* (*CUC1*) (Shani *et al.* 2006; Veit, 2006). The seasonal regulation of SAM activity has not been studied extensively. At the molecular level, cessation of shoot growth in trees is an uncharted territory. More research is necessary to identify the mechanisms regulating cell cycle, hormone signaling and stem cell differentiation at the SAM on a seasonal basis.

Tree growth measurements described in Chapter 1 have identified the critical points that differentiate dwarf from vigorous trees in cherry grafts. More specifically, 'Bing'/Gi5 shoots consistently ceased growing earlier than 'Bing'/Gi6 trees across three growing seasons (Figure 1.3, Chapter 1). A similar response was identified for node number, as nodes in 'Bing'/Gi5 trees stopped being produced earlier than in 'Bing'/Gi6 trees, resulting in the cessation of shoot growth (Figure 1.8, Chapter 1). It was expected that changes in gene expression would precede shoot growth cessation at least for genes involved in the early stages of the response, while at the same time the same genes in 'Bing'/Gi6 trees would not be modified, but only later. During shoot growth cessation in 'Bing'/Gi5 trees, changes in gene expression also were expected in the graft union, which is the link between rootstock and scion. Crossing of the signal through the graft union

will occur at or before the time of shoot growth cessation. Thus, dwarfing rootstocks are expected to show earlier changes in gene expression.

A genomics approach was taken for the identification of genes differentially expressed between the main shoot and the graft union of dwarf 'Bing'/Gi5 and semi-vigorous 'Bing'/Gi6 trees. The main shoots of both graft combinations are of the same genetic background, but the rootstocks are the siblings of the interspecific cross between *P. cerasus* cv. 'Schattenmorelle' and *P. canescens*. As siblings, the two rootstocks are expected to share a high genetic homology, but also allelic variation (due to self-incompatibility driven heterozygosity). Complementary DNA Amplified Fragment Length Polymorphism (cDNA-AFLP) was used to screen main shoot and graft union samples and reveal a number of differentially expressed genes. Microarrays were constructed to confirm the differential expression of those genes. Ninety-nine genes were confirmed as differentially expressed, of which 43 were in the main shoot and 56 in the graft union.

MATERIALS AND METHODS

Plant material

Trees used in this experiment were purchased from commercial nurseries. The graft combinations were 'Bing'/Gi5 and 'Bing'/Gi6 in which the scion was 1-year-old and the rootstock 2-years-old when purchased. 'Bing' is a commercial sweet cherry cultivar while Gi5 and Gi6 rootstocks are triploid F1 progeny from an interspecific cross between the tetraploid sour cherry (*Prunus cerasus* L. cv. *Schattenmorelle*) and the

diploid greyleaf cherry (*Prunus canescens* Bois.) (Franken-Bembenek, 1996). The trees for each graft combination were planted in the spring of 2001 in two rows of 50 trees each with 6 meter row spacing and a 2 meter tree spacing with a North to South orientation at the MSU Clarksville Horticultural Experiment Station, Clarksville, Michigan. Pruning was performed every spring prior to bud break so that only the main stem was retained above ground. Flowers were removed before fruit set.

Sampling

In 2002, four trees were sampled per graft combination, 'Bing'/Gi5 and 'Bing'/Gi6, on 3 June, 20 June, 3 July. The 'Bing' shoots were sampled by removing the main shoot, defoliating it and freezing it on dry ice. The trees were then removed from the soil and the following tissue samples were obtained: rootstock (a 10 cm region directly below the graft union), graft union (including the swollen tissues of the rootstock and the scion) and scion (a 10 cm region directly above the graft union). All tissue samples were directly frozen in dry ice within separately labeled bags. For long term storage, the samples were kept in a -80^0 C ultralow freezer.

RNA extraction

For total RNA extraction, samples from each tree were ground separately in a mortar and pestle for shoots, or stainless steel blender (Waring, Connecticut) and subsequently stainless steel coffee grinder (BCG100, Kitchenaid, Michigan) for woody tissues (rootstock, graft union, scion). During grinding, tissues were kept frozen with liquid nitrogen. From the main shoot samples, the part containing the upper 10 buds was

used for grinding. Of that sample, the upper 3 nodes including the apical meristem (called "shoot apex") were ground separately from the "remaining shoot". Equal amounts of ground sample from three of four trees were mixed in a centrifuge tube totaling 2 ml of ground tissue. For the main shoot, 500 μl of the "shoot apex" and 1500 μl of the "remaining shoot" from three trees were mixed to produce 2 ml of tissue. This action was taken to enrich the extracted RNA with shoot apex mRNAs. For RNA extraction the protocol of Wang *et al.,* (2000) was used. This protocol was designed for RNA extraction from plant tissue samples with high content in polyphenols and polysaccharides. Briefly, the protocol is as follows:

Five volumes of homogenization buffer (0.3M LiCl, 200 mM Tris-HCl pH 8.5, 10 mM EDTA, 1.5% Sodium dodecyl sulfate, 1% (w/v) sodium deoxyholate, 1% (v/v) NP-40, 1 mM aurintricarboxylic acid, 10 mM DTT, 5 mM thiourea, 2% (w/v) PVPP) were mixed with one volume of ground tissue. The mixture was incubated at -80^0 C for 2 hours and then heated to 37^0 C until just thawed. Plant debris was removed by centrifugation at 5,000xg for 20 min. The supernatant was mixed with 1/30th of the volume 3M sodium acetate pH 5.2 and 100% ethanol to a final volume of 10%. The mixture was placed on ice for 10 min and then centrifuged at 5,000xg for 20 min. This step removes carbohydrates that are found in large concentration in cherry stems. The supernatant was mixed with 1/9th of the volume 3M sodium acetate pH 5.2 and 30% of the final volume iso-propanol. The mixture was placed at -20^0 C for 2 hours, followed by centrifugation at 5,000xg for 30 min. The pellet was diluted in 3 ml of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) and placed on ice for 30 min. The samples were then centrifuged at 10,000xg for 30 min and the supernatant was transferred to a fresh

tube, where it was mixed with ¼ of the supernatant volume 10M lithium chloride. After mixing well, the samples were placed at 4^0 C overnight. This step precipitates exclusively the RNA without transfer of double stranded DNA. The next day they were centrifuged at 10,000xg for 30 min. The pellet was resuspended in 1.5 ml of TE buffer pH 8.0 and mixed with 1.5 volumes 5M potassium acetate (pH not adjusted) and placed on ice for 3 hours. This step precipitates the RNA. Next, the samples were centrifuged for 30 min at 10,000xg. The pellet was resuspended in 1 ml of TE buffer pH 8.0 and placed on ice for 30 min, followed by centrifugation at 10,000xg for 30 min. The supernatant was mixed with $1/10^{th}$ of the volume 3M sodium acetate pH 5.2 and 2 volumes of 100% ethanol. It was then placed at -20^0 C for 2 hours, followed by centrifugation at 10,000xg for 30 min. The pellet was washed in 75% ethanol and centrifuged at 10,000xg for 10 min. The pellet was air dried and then resuspended in 30 μl of diethyl pyrocarbonate-treated (DEPC) water. RNA quantity was measured on a SmartSpec3000 (BIO-Rad, California) Spectrophotometer at OD_{260} with $OD_{260/280}$ serving as the quality control.

cDNA-AFLP analysis

The protocol was an adaptation of the protocol described by Bachem *et al.* (1996).

i) Preparation of double stranded cDNA

One hundred micrograms of total RNA were used for purification of polyA RNA. The Dynabeads® (#610.06, Dynal, New York) or poly(A)Purist™ Mag (#1922, Ambion, Texas) kits were used for the purification. Two hundred and fifty nanograms of polyA RNA were used for the preparation of double stranded cDNA. For synthesis of the first strand, polyA RNA was mixed with 1 ng of oligodT_{25} primer and water up to 12 μl and

incubated at 70^0 C for 10min. After denaturation, 4 μl of 1st strand buffer, 2 μl of 0.1M DTT, 0.5 μl of 20mM dNTPs and 0.5 μl of RNAse inhibitor (Promega, Wisconsin) were added. The mixture was heated to 42^0 C for 2 min, then 1 μl of SuperscriptII (200U/μl, Invitrogen, California) was added and the reaction was incubated at 42^0 C for 50 min followed by heat inactivation at 70^0 C for 15 min. For synthesis of the second strand, 8 μl of 10x 2nd strand DNA polymerase buffer, 2 μl 20 mM dNTPs, 1 μl RNaseH (2U/μl, Invitrogen, California), 4 μl DNA Polymerase I (9U/μl, Promega, Wisconsin) and 45 μl water were added to the first strand reaction and incubated at 16^0 C for 2 hours and 30 min, followed by deactivation at 68^0 C for 10 min. At this stage, 5 μl of the reaction were analyzed on a 1% agarose gel to examine if the concentration between samples remained equal.

ii) Preparation of the primary template

The cDNA was precipitated with 10% 3M sodium acetate pH 5.2 and 2.5 volumes of ethanol at -80^0 C for 2 hours or at -20^0 C for 8 hours. Samples were then centrifuged at 10,000xg for 30 min. The pellet was washed with 70% ethanol and centrifuged at 10,000xg for 10 min. The pellet was air dried and resuspended in 10 μl of HPLC grade water. The cDNA was digested with the MseI and ApoI restriction enzymes. Since the two enzymes have different restriction temperatures, the digestions occur sequentially and not at once. For the MseI digestion, 10 μl of cDNA were mixed with 4 μl 10x NEB4 buffer, 0.4 μl 100xBSA, 1μl of MseI (10U/μl, NEB, MA) and 25 μl of HPLC grade water. The reaction was incubated at 37^0 C for 2 hours and heat inactivated at 65^0 C for 30 min. For the ApoI reaction, the following components were added to the MseI

reaction: 1 μl 10x NEB4 buffer, 0.1 μl 100xBSA, 2 μl ApoI (4U/μl, NEB, MA) and 7 μl HPLC grade water, This was incubated at 50^0 C for 2 hours and heat inactivated at 80^0 C for 20 min. Following the digestion, ApoI and MseI specific adaptors are added to the digestion reaction. The adaptor sequences are:

Apo-adap-top : 5′- CTC GTA GAC TGC GTA CC - 3′

Apo-adap-bot : 5′- AAT TGG TAC GCA GTC TAC - 3′

Mse-adap-top : 5′ - GAC GAT GAG TCC TGA G - 3′

Mse-adap-bot : 5′ - TAC TCA GGA CTC AT - 3′

Prior to ligation, both strands of the adaptor were heat denatured at 65^0 C for 10 min and allowed to cool to room temperature.

For ligation of the adaptors to the digested cDNA, the following components were added: 0.5 μl 10x NEB4 buffer, 1 μl ApoI adaptor (5 pmoles), 1 μl MseI adaptor (50 pmoles), 0.6 μl 1M DTT, 1.6 μl 1M Tris-HCl pH 7.5 and 0.3 μl T4 DNA ligase (3U/μl, Promega, Wisconsin). The mixture was incubated at 16^0 C overnight. Four microliters of the reaction were loaded on a 1% agarose gel to inspect the quality of digestion. Bands should appear at sizes 100-1,000bp.

iii) Preparation of the secondary template

Secondary template is the PCR amplified primary template. The amplification primers are designed to anneal on the adaptor sequences. Primer sequences are:

Apo-pre : 5′ - CTC GTA GAC TGC GTA CCA ATT - 3′

Mse-pre : 5′ - GAC GAT GAG TCC TGA GTA A - 3′

The PCR reaction was as follows: 10 μl of Primary template, 5 μl of 10x Taq Polymerase buffer, 5 μl $MgCl_2$ (25 mM), 5 μl dNTPs (2 mM), 1 μl Apo-pre (10 pmoles/μl), 1 μl Mse-pre (10 pmoles/μl), 0.5 μl Taq polymerase (5 U/μl, Promega, Wisconsin) and 22.5 μl of HPLC grade water. The amplification program was as follows: 94^0 C for 30 sec, 52^0 C for 30 sec, 72^0 C for 1 min in 15 cycles. A 5 μl sample of the reaction was loaded on a 1% agarose gel to inspect the quality and quantity of DNA. At this point, it was very critical to have equal amounts of DNA for all the samples that were to be used in the cDNA-AFLP analysis. Quantification was performed on a 1% agarose gel using the gel image analysis software ImageQuant (Molecular Dynamics, California).

iv) Selective amplification of the secondary template

At this point, the secondary template was subjected to selective amplification to reduce the number of cDNA fragments present in each sample. For that reason, primers similar to the pre-amplification primers, but with a 3' extension into the sequence of the unknown gene, promoted selective amplification. The primers used were:

Apo-sel-CG : 5′ - GAC TGC GTA CCA ATT CG - 3′

Apo-sel-CA : 5′ - GAC TGC GTA CCA ATT CA - 3′

Apo-sel-CC : 5′ - GAC TGC GTA CCA ATT CC - 3′

Apo-sel-CT : 5′ - GAC TGC GTA CCA ATT CT - 3′

Apo-sel-TG : 5′ - GAC TGC GTA CCA ATT TG - 3′

Apo-sel-TA : 5′ - GAC TGC GTA CCA ATT TA - 3′

Apo-sel-TC : 5′ - GAC TGC GTA CCA ATT TC - 3′

Apo-sel-TT : 5′ - GAC TGC GTA CCA ATT TT - 3′

The Apo-sel primers had only two variable nucleotides in position 16 because the restriction site includes either G or A.

Mse-sel-GG : 5′ - GAT GAG TCC TGA GTA AGG - 3′

Mse-sel-GA : 5′ - GAT GAG TCC TGA GTA AGA - 3′

Mse-sel-GC : 5′ - GAT GAG TCC TGA GTA AGC - 3′

Mse-sel-GT : 5′ - GAT GAG TCC TGA GTA AGT - 3′

Mse-sel-AG : 5′ - GAT GAG TCC TGA GTA AAG - 3′

Mse-sel-AA : 5′ - GAT GAG TCC TGA GTA AAA - 3′

Mse-sel-AC : 5′ - GAT GAG TCC TGA GTA AAC - 3′

Mse-sel-AT : 5′ - GAT GAG TCC TGA GTA AAT - 3′

Mse-sel-CG : 5′ - GAT GAG TCC TGA GTA ACG - 3′

Mse-sel-CA : 5′ - GAT GAG TCC TGA GTA ACA - 3′

Mse-sel-CC : 5′ - GAT GAG TCC TGA GTA ACC - 3′

Mse-sel-CT : 5′ - GAT GAG TCC TGA GTA ACT - 3′

Mse-sel-TG : 5′ - GAT GAG TCC TGA GTA ATG - 3′

Mse-sel-TA : 5′ - GAT GAG TCC TGA GTA ATA - 3′

Mse-sel-TC : 5′ - GAT GAG TCC TGA GTA ATC - 3′

Mse-sel-TT : 5′ - GAT GAG TCC TGA GTA ATT - 3′

Visualization of the PCR product was accomplished by radioisotope labelling of the 5' nucleotide of the Apo-sel primers. Seven pmoles of labeled primer were used for each reaction. Depending on the number of wells for which each selective primer was going to be used its concentration was adjusted adequately for labeling. For example, if the primer was going to be used in 100 re-amplifications, we needed 700 pmoles. The labeling reaction was: 80 μl (700 pmoles) of Apo-selective primer, 10 μl 10x PNK buffer (NEB, Massachusetts), 4 μl [γ-^{33}P] ATP (NEG602H , NEN, Massachusetts), 3 μl PNK (NEB, Massachusetts) and 3 μl HPLC grade water. The reaction was placed at 37^0 C for 1 hour.

The selective PCR reaction was: 1 μl template (1/50 dilution of secondary template in water), 1 μl 10xTaq polymerase buffer, 1 μl $MgCl_2$ (25 mM), 1 μl dNTPs (2 mM), 1 μl labeled Apo-Sel (7 pmol/μl), 1 μl Mse-Sel (7 pmol/μl), 0.5 μl Taq polymerase (Promega, Wisconsin) and 3.5 μl HPLC grade water. The program used for the amplification was: 94^0 C for 30 sec, 65^0 C for 30 sec, 72^0 C for 1 min repeated in 10 cycles and followed by 94^0 C for 30 sec, 56^0 C for 30 sec, 72^0 C for 1 min repeated 25 times. After the PCR reaction finished, 2.5 μl of gel loading dye were added and the reaction was denatured at 99^0 C for 5 min.

A 5% polyacrylamide gel was previously prepared. The PCR reaction was heat denatured at 100^{0}C for 5 min and then cooled on ice. For loading, 2.5 μl of the reaction were used. A 50 bp step ladder (Promega, Wisconsin) was labeled with [γ-^{33}P] ATP in a reaction similar to that for the Apo-sel primers and 2.5 μl were loaded on the side lanes of

the gel. The gel was pre-run for 1 hour at 80 Watt and after loading it ran for 3.5 hours. The gel was then attached to a Whattman 3MM paper, covered on the other side with Saran wrap and dried on a 583 Gel dryer (BioRad, California) for 2 hrs at 80^0C. The dry gel was placed in a cassette with Glogos® II Autorad Markers (Stratagene, California) attached to its corners for alignment during excision. An X-ray film (Kodak, New York) was placed on top of the gel and exposed for 24 hours at -80^0 C. The film was developed by soaking for 2 min in developing solution, washed briefly in water, fixed for 2 min and then air dried.

Excision, cloning and sequencing of differentially expressed bands

The autoradiographic film was aligned to the dried gel and selected fragments were excised by cutting both film and gel using a scalpel (No 11). These are called Transcript Derived Fragments (TDFs). The excised TDFs were soaked in 50 μl of distilled water overnight at 37^0 C to elute the DNA. 5 μl were used for re-amplification of the TDFs in a 20 μl PCR reaction using the same primers as for pre-amplification (Apo-pre 5'-CTCGTAGACTGCGTACCAATT-3'; Mse-pre, 5'-GACGATGAGTCCTGAGTAA-3'). The following program was used: 30 sec at 94^0 C, 30 sec at 60^0 C and 1 min at 72^0 C for 30 cycles. The products were separated on a 1% agarose gel and the double or smeared bands were excluded from further processing. The remaining TDFs were purified by precipitation in 2 volumes of ethanol and 10% 3M sodium acetate pH 5.2, overnight at -20^0 C, washed in 100% ethanol, air dried and re-suspended in distilled water.

TDFs selected for sequencing were cloned into the pGEM-T Easy vector (Promega, Wisconsin) and inserted into Subcloning efficiency E.coli DH5a cells (#18265-017, Invitrogen, California) according to Dilks *et al.,* (2003). Four colonies were selected for each TDF which were then screened using a colonies PCR reaction with the following primers: M13F 5'-GTTTTCCCAGTCACGACGTTG-3' and M13R 5'-GAGCGGATAACAATTTCACACAG-3'. The colonies were diluted in the reaction mixture in a 96-well format and then streaked on an LB agar plate containing 100 μg/ml ampicillin, divided in a 96-well format and incubated at 37^0 C overnight. The reaction conditions were the same as for the re-amplification reaction. Positive colonies were then transferred from the agar plate to a 96-deep well plate containing 350 μl of LB with 100 μg/ml ampicillin and incubated at 37^0 C overnight with constant agitation. 150 μl of 50% sterile glycerol were then added for storage at -80^0 C. 1 μl of the culture was used for further confirmation of the clones with the Apo-pre/Mse-pre primers.

The M13F primer was used for sequencing the cloned TDFs in an ABI Prism 3700 DNA analyzer. Sequencing was performed at the Genomics Technology Support Facility, Michigan State University.

Microarray construction

The previously selected TDF clones were PCR amplified using 0.5 μl of the glycerol stock cells or $1/100^{th}$ dilution of PCR product for not cloned TDFs, as template. The reaction was set to 50μl final volume in 96-well plates. Apo-pre and Mse-pre were used as the amplification primers in the following reaction: template 0.5 μl, 1x Taq polymerase buffer (Promega, Wisconsin), 200 μM dNTP mix, 10 pmole Apo-pre, 10

pmole Mse-pre, 1 Unit Taq polymerase (Promega, Wisconsin) and HPLC grade water up to 50 μl. The reaction conditions were: 94^0 C for 30 sec, 60^0 C for 30 sec, 72^0 C for 1 min for 40 cycles. Four microliters of the product were analyzed on a 1% Agarose gel and the remaining sample was precipitated with 10% 3M sodium acetate pH 5.2 and 2 volumes of 95% ethanol overnight at -20^0 C. The next day the plates were centrifuged at 3,000 rpm for 40-50 min on a bench top centrifuge (HN-SI, Damon/IEC) and then washed in 75% Ethanol, followed by centrifugation at 3,000 rpm for 10 min. The pellet was air dried until no water was visible, re-suspended in 15 μl of 3xSSC solution to approximately 100 ng/μl and stored at 40^0 C for 24 hours and then transferred to -20^0 C.

DNA was transferred to 384 well plates to facilitate robotic printing. The Arrayit SuperAmine glass slides (SMM, Telechem, California) were used as substrate on a GeneMachines OmniGrid 100 robot (Genomic Solutions, Michigan) with Telechem Chipmaker pins. Each slide contained 1040 DNA samples printed in triplicate on distant locations of the slide to avoid position specific bias. The microarray was formed by 24 grids (each grid formed by one pin), each of which contained 132 spots or less.

Microarray hybridization

Probe was labeled with the amino-allyl method described by Hegde *et al.* (2000). The RNA used for probe preparation was the same as that used for the cDNA-AFLP analysis, in addition to RNA from the fourth tree that served as the biological replication. Arrays were hybridized at 42^0 C for 16 hr and washed once in 1xSSC, 0.2% SDS at 42^0 C for 5 min, 0.1xSSC, 0.2% SDS at room temperature for 5 min, and 0.1xSSC at room temperature for 5 min.

The experimental design was as follows:

Upper shoot			Graft union		
B5 6/3	↔ B5 6/20	↔ B5 7/3	B5 R	↔ B5 GU	↔ B5 S
↕	↕	↕	↕	↕	↕
B6 6/3	↔ B6 6/20	↔ B6 7/3	B6 R	↔ B6 GU	↔ B6 S

B5: 'Bing'/GI5 tree, B6: 'Bing'/GI6 tree, 6/3: mm/dd/2002, R: rootstock, GU: Graft union, S: Scion, two-headed arrows denote dye reversal. Dye reversal served as the technical replication and two RNA samples from independent trees served as the biological replication.

Microarray analysis

The slides were scanned in an Affymetrix 428 microarray scanner (Affynetrix, CA) and data were analyzed with the GenePix Pro v3.0 software (Molecular Devices, CA). Lowess normalization and ANOVA were performed using the R/maanova package (Wu *et al.* 2002; Churchill, 2004; http://www.jax.org/staff/churchill/labsite/index.html). The models used for the two experiments were:

a) Main shoot

y = Array+Spot+Dye+Date+Rootstock+Date:Rootstock+Sample

We were interested in the Date, Rootstock and Date:Rootstock effect

b) Graft union

y = Array+Spot+Dye+Position+Rootstock+Position:Rootstock+Sample

We were interested in the Position, Rootstock and Position:Rootstock effect.

Expression ratios at the log2 scale between 'Bing'/GI5 and 'Bing'/GI6 samples within Date or Position were obtained with the SMA package (http://www.stat.berkeley.edu/users/terry/Group/software.html) in an R language environment. Significantly differentially expressed genes were those that showed a P-value lower than 0.05, more than 1.5 fold-difference within Date or Position, and had more than 3 spots not flagged as bad per clone per sample. Grouping of the significant genes was performed manually due to the small number of genes. Genes were sorted based on their fold-difference and then grouped based on the similarity of their expression pattern.

Northern hybridization

Total RNA was extracted as above. A 5 μg sample of total RNA was analyzed on a denaturing 1% agarose gel containing formaldehyde. RNA was then transferred on a nylon membrane (Hybond N+, Amersham-Biosciences, New Jersey) according to Sambrook *et al.* (1989). The probe was prepared from the cDNA-AFLP re-amplified band using primers Apo-pre/Mse-pre as before. The probe was gel purified with the QIAEX II gel purification system (QIAGEN, California). For probe labeling, 25 ng of purified PCR product were incorporated in the one tube reaction RedyPrime system (Amersham-Biosciences, New Jersey) with the addition of [α-^{32}P]ATP (NEN-Perkin Elmer, Massachusetts). The hybridization reaction was performed at 42°C for 16 h and

the membrane was then washed (according to the manufacturer) and exposed to an X-ray film (Kodak, Connecticut).

RESULTS

Complementary DNA amplified fragment length polymorphism (cDNA-AFLP) analysis of scion main shoots in grafts showing differential cessation of growth

Comparison of growth between dwarfing and semi-vigorous trees revealed that 'Bing'/Gi5 shoots cease growing earlier than 'Bing'/Gi6 shoots (Figure 1.4, Chapter 1). Differentiation in shoot growth between these two graft combinations occurred approximately on the 17th of June 2002. To understand the genetic changes leading to this differential growth, a differential display cDNA-AFLP screen was performed on main shoot samples of 'Bing'/Gi5 and 'Bing'/Gi6 trees, collected in 2002. Using the shoot elongation curve as a reference, samples were collected on three different dates from each graft combination. The first sample was collected on 3 June, when shoots in both combinations were elongating at the same rate. The second sample was collected on 20 June at the point of initiation of differential shoot elongation. Finally, the third sample was collected on 3 July when both combinations had reduced elongation rates and approached cessation of terminal meristem growth. The restriction enzymes ApoI and MseI were used in the cDNA-AFLP analysis. ApoI can recognize 4 consensus sequences (RAATTY, R=A or G, Y=C or T), including that of EcoRI (GAATTC), thus reducing its hypothetical restriction band size to 1,024 bp, instead of 4,096 bp for the regular six cutter enzymes. Such an enzyme combination can increase the representation of cDNAs

screened by four-fold compared to the standard cDNA-AFLP protocol. Selective primers for MseI had two random nucleotides in the 3' end giving rise to 16 primers, while ApoI selective primers had a C or T in the second to last 3' end and a random nucleotide in the last position giving rise to 8 primers. Thus the number of all possible primer combinations consisted of 128 primer pairs. At a rate of 100 bands per primer set after PCR amplification and 6 samples per primer set, the total number of transcript derived fragments (TDFs) was 76,800 (128 x100 x 6).

Overall gene expression was similar between the two graft combinations for samples collected at the same day. Only 111 Transcript Derived Fragments (TDFs) showed differences in their expression level between 'Bing'/Gi5 and 'Bing'/Gi6 trees (Figure 2.1). The change in expression occurred earlier in 'Bing'/Gi5 samples, as it is clear in Figure 2.1. Differences in expression were apparent as early as the 3rd of June when shoots of 'Bing'/Gi5 and 'Bing'/Gi6 were still growing at the same rate. Some of the most common expression patterns are presented in Figure 2.1B. Patterns 1-4 show genes that were down-regulated first in 'Bing'/Gi5 shoots, while patterns 5-8 show genes that were up-regulated first in 'Bing'/Gi5 shoots (Figure 2.1B). Patterns 9 and 10 represent genes whose expression changes at the same time for 'Bing'/Gi5 and'Bing'/Gi6 shoots, in response to a non-defined signal (Figure 2.1B). Pattern 1 shows a gene which is down-regulated in 'Bing'/Gi6 shoots while it is not already transcribed at the time of sampling in 'Bing'/Gi5. Pattern 2 shows a gene with an expression change that seems to follow the change in pattern 1, but is consistently down-regulated first in 'Bing'/Gi5 trees. In patterns 3 and 4 gene expression is not completely blocked, but it is down-regulated first in 'Bing'/Gi5 on June 20. Such genes are expected to be downstream of

genes exhibiting patterns 1 and 2 and act in the late stages of shoot growth cessation. Since samples represent only points in time the exact date of change in expression of those genes is not known. In contrast to patterns 1-4, pattern 5 shows a gene that is up-regulated in 'Bing'/Gi5 on the 3rd of June, while in 'Bing'/Gi6 up-regulation occurs later on the 20th of June. These genes are expected to be positive regulators of shoot growth cessation. Pattern 6 shows a gene which is up-regulated first in 'Bing'/Gi5 shoots on the 20th of June and later on the 3rd of July in 'Bing'/Gi6. Patterns 7 and 8 represent genes that show a late up-regulation in 'Bing'/Gi5 on the 3rd of July. The same genes have very low expression or they are not expressed in 'Bing'/Gi6. Such genes may not be involved in the process of shoot growth cessation, but rather in other processes, such as formation of floral primordia or induction of dormancy. All 111 TDFs were cloned in a plasmid vector to obtain pure fragments. Cloning produced 277 clones and each TDF was represented by one or more clones.

cDNA-AFLP analysis of the graft union area during shoot growth cessation

The graft union presents another point of influence in the dwarfing phenomenon. It was expected that analysis of gene expression would reveal genes that are either differentially expressed in the rootstock, graft union and scion or that are differentially transported through the graft union. The second hypothesis is the most interesting, but also more difficult to prove. cDNA-AFLP analysis of the main shoot revealed that the majority of the genes differentially expressed between 'Bing'/Gi5 and 'Bing'/Gi6 trees occur in 20 June sample. Rootstock tissue, graft union and scion trunk above the graft

union were used in a second cDNA-AFLP analysis to screen the graft union region for possible differential gene expression.

Overall, there was a high level of similarity in the gene expression profiles between rootstock and scion (Figure 2.2), even though they represent different species. The genotype of the scion is *P. avium* and the rootstock is a hybrid of *P. cerasus* and *P. canescens*. The genetic similarity revealed by the cDNA-AFLP analysis can be explained by the close phylogeny of *P.avium* to *P.cerasus* (Bortiri *et al.* 2001). The most variable pattern is shown in Figure 2.2 (pattern 5), in which the TDF is only present in the graft union of the 'Bing'/Gi6 trees. Sequencing of TDFs exhibiting this cDNA-AFLP profile revealed genes with high similarity to peach, apricot or almond ESTs, which excludes the possibility of contamination of the sample by other organisms. Also, alignment of TDFs with those ESTs excluded the presence of genomic DNA that might have contaminated the RNA. Northern hybridization and RT-PCR analysis with independent RNA samples for both graft combinations did not show the expected difference in expression, but rather equal level of expression (data not shown). This further complicates the reason for this pattern, because it excludes the possibility that the 'Bing'/Gi6 sample was contaminated. If 'Bing'/Gi6 samples were contaminated, we should not expect to see expression in 'Bing'/Gi5 samples. Any further step to characterize this pattern was not taken, as the cDNA-AFLP data could not be replicated.

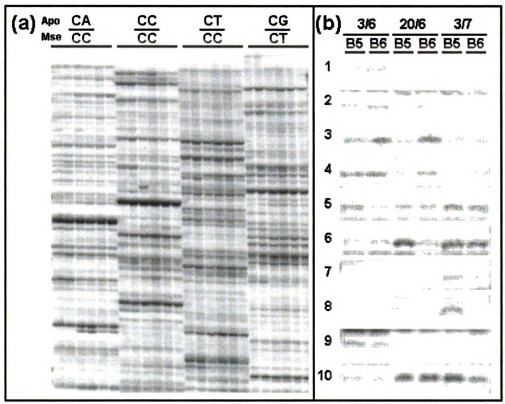

Figure 2.1: cDNA-AFLP analysis at the main shoot. (A) Portion of a cDNA-AFLP gel produced by four primer pairs and showing high degree of co-regulation between samples. (B) Detail of gene expression patterns putatively related to shoot growth cessation, identified in 8 cDNA-AFLP gels as the one shown in (A). 3/6: 3 June, 20/6: 20 June, 3/7: 3 July, B5: 'Bing'/Gi5, B6: 'Bing'/Gi6.

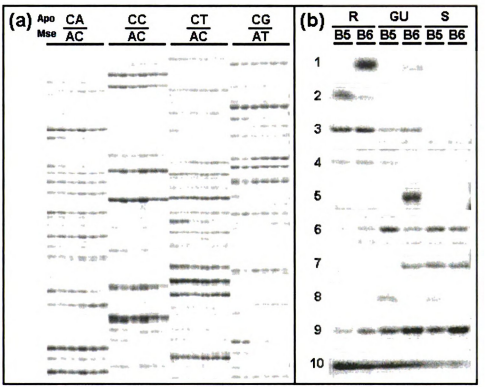

Figure 2.2: cDNA-AFLP analysis at the graft union. (A) Portion of a cDNA-AFLP gel produced by four primer pairs and showing high degree of co-regulation between samples. (B) Detail of interesting expression patterns identified in 8 cDNA-AFLP gels as the one shown in (A). R: Rootstock, GU: Graft Union, S: Scion, B5: 'Bing'/Gi5, B6: 'Bing'/Gi6.

Genes, expressed either in the rootstock or scion, also were present in the graft union, since this tissue combines cells from both genotypes (Figure 2.2B, patterns 1-4, 6-10). In some cases (Figure 2.2B, patterns 9 and 10), genes exhibited a gradual reduction in expression from the rootstock to the scion and *vice-versa*. This pattern may indicate RNA transport across the graft union through the vascular system. It is not known whether or not this pattern was due to RNA transport across the graft union through the vascular system, since it proved difficult to extract phloem sap from cherry trees.

The cDNA-AFLP analysis revealed 249 differentially expressed TDFs. Sixty-four of the TDFs were differentially expressed in the rootstock (Figure 2.2B, patterns 1-4, 10), 49 in the graft union (Figure 2.2B, pattern 5) and 136 in the scion (Figure 2.2B, patterns 6-9). Patterns 3, 4 and 6-8 were differentially expressed in the graft union, but the genes originate in the rootstock or the scion and are categorized as such. The 249 TDFs were cloned into a plasmid vector to obtain pure fragments. One or more clones were obtained for each TDF resulting in 646 clones for all the TDFs.

Confirmation of cDNA-AFLP with the use of microarrays

Microarrays were used to confirm the expression profiles of cloned TDFs. Sixty-nine constitutively expressed TDFs from the shoot and the graft union, and 48 ESTs from the shoot apical meristem, also were cloned and printed on the arrays. In total 1040 DNA samples (277 clones from shoot, 646 clones from graft union, 48 ESTs from the SAM region and 69 control sequences form the shoot and the graft union) were printed in triplicate on the arrays. Two experiments were conducted, which tested the gene expression changes in the main shoot and the graft union region, respectively. The

experimental design for both microarray experiments was in accordance to the cDNA-AFLP experiments (see Materials and Methods). All 3120 spots printed on the microarray were included in the data analysis for both experiments with the aim to maximize the output of differentially expressed genes and identify genes differentially expressed at both locations in the plant.

Of the 1040 clones printed on the microarray, only 99 showed statistically significant differences following microarray analysis: 43 in the shoot and 56 in the graft union experiments (Tables 2.1 and 2.2). Of those, 6 clones were only expressed in 'Bing'/Gi5 trees in both experiments and these belong to the Cherry Virus A (CVA) RNA genome (Table 2.1, Chapter 3). CVA is a capillovirus that has no defined symptoms in cherry trees (Jelkman, 1995). No other TDFs showed an expression pattern similar to that of the virus, indicating reduced or no effect on the trees by the presence of the virus. The other sequences exhibiting significant differences in microarray expression could serve as primary candidates for the promotion of dwarfing, since they show a differential response between the two rootstocks.

The differentially expressed genes were clustered according to their pattern of expression in 6 clusters. The majority of the genes differentially expressed in the main shoot fell into two clusters, Clusters 1 and 3 (Table 2.1). The genes in Cluster 1 were expressed higher in 'Bing'/Gi5 compared to 'Bing'/Gi6 trees on 3 July, when the rate of growth for the first graft combination was decreasing. These genes are similar to a phospholipaseD (RGUS1010), catalase1 (RGUS1271-72), MYB protein (RGUS1306), a subtilisin serine protease (RGUS1529-31), a touch induced protein (RGUS1636) and an unknown sequence (RGUS1342) (Table 2.1). In Cluster 3, genes are expressed higher in

'Bing'/GI6 shoots on 3 July than in 'Bing'/Gi5. Genes in Cluster 3 include an AP2 domain containing protein (RGUS1210), an Armadillo beta-catenin domain protein (RGUS1367), an AtVOZ1-like transcription factor (SM1045) and a zinc finger protein (SM1026) (Table 2.1). In the same cluster, two genes represented by four clones (RGUS1121, RGUS1124, SM1063, SM1064) are putatively involved in senescence. Cluster 2 includes four genes that were down-regulated in 'Bing'/Gi5 compared to 'Bing'/Gi6 shoots.

Table 2.1: Genes differentially expressed at the upper shoot microarray experiment. Positive fold change values refer to B5 up-regulation while negative values to B6 up-regulation. 3/6: 3 June, 20/6: 20 June, 3/7: 3 July, B5: 'Bing'/Gi5, B6: 'Bing'/Gi6, R: Rootstock, S: Scion, GU: Graft Union, Sh: Shoot, ns: non-singificant.

TDF ID(1)	TDF size(2)	TO(3)	Genebank accession number	Microarray fold change				Annotation		
				B5/B6 6/3	B5/B6 6/20	B5/B6 7/3	Clu-ster	Product	PubMed ID	E-value
RGUS1010	171	GU	DV175385	ns	ns	1.50	1	Phospholipase D	At3g15730	5E-18
RGUS1271	130	GU	DV175414	ns	ns	1.56	1	Catalase 1	At1g20630	1E-11
RGUS1272	129	GU	DV175415	ns	ns	1.65	1	Catalase 1	At1g20630	5E-12
RGUS1306	316	S	DV175422	ns	1.11	1.58	1	MYB transcription factor	At5g49330	3E-23
RGUS1342	200	S	DV175426	ns	1.29	1.60	1	No hit		
RGUS1636	296	R	DV175451	ns	1.33	1.52	1	Putative calcium binding protein	XP_550050	1E-09
RGUS1529	251	S	DV175439	ns	-1.17	1.50	1	Subtilisin-like protease	AAQ54525	2E-23
RGUS1530	285	S	DV175440	ns	-1.22	1.70	1	Subtilisin-like protease	AAQ54525	7E-22
RGUS1531	319	S	DV175441	ns	ns	1.58	1	Subtilisin-like protease	AAQ54525	7E-24
RGUS1511	213	S	DV175437	ns	ns	-1.59	2	Nucleic acid binding / pancreatic ribonuclease	At5g67210	0.001
RGUS1589	157	GU	DV175447	ns	ns	-1.57	2	UDP-D-glucuronate 4-epimerase GAE1	At4g30440	3E-17
RGUS1616	180	S	DV175449	ns	ns	-1.51	2	No hit		
RGUS1619	132	S	DV175450	ns	ns	-1.54	2	Delta 8-sphingolipid desaturase	AAG43277	6E-63
RGUS1084	155	R	DV175391	ns	-1.27	-1.63	3	No hit		
RGUS1117	202	R	DV175394	ns	-1.97	-1.79	3	Alcohol dehydrogenase (ADH)	At1g77120	3E-05
RGUS1121	251	GU	DV175395	ns	-2.58	-2.16	3	Putative replication factor C 36kDa subunit	XP_468050	2E-22
RGUS1124	305	GU	DV175396	ns	-2.03	-2.13	3	Putative senescence-associated protein	AAR25995	1E-44
RGUS1127	211	GU	DV175397	ns	-1.76	ns	3	Putative ATP-dependent RNA helicase A	At2g47680	1E-09
RGUS1210	140	GU	DV175401	ns	-1.71	-1.42	3	AP2 domain containing protein RAP2.12	AAC24587	2E-74
RGUS1230	202	S	DV175405	ns	-1.11	-1.69	3	Hemoglobin	CAA68405	3E-16
RGUS1231	202	S	DV175406	ns	-1.30	-1.55	3	Hemoglobin	CAA68405	3E-16
RGUS1321	170	R	DV175424	ns	-1.31	-1.51	3	No hit		
RGUS1367	177	GU	DV175429	ns	-1.53	ns	3	Armadillo/beta-catenin repeat U-box protein	At3g07360	5E-16
RGUS1478	234	S	DV175435	ns	-2.24	-1.80	3	Cytochrome P450 like_TBP	BAA10929	2E-28
RGUS1563	230	GU	DV175446	ns	-1.87	-1.57	3	Polyprotein -related	At1g21945	3E-20
SM1045	391	Sh	DV175457	ns	-1.34	-1.56	3	Transcription factor AtVOZ1	At1g28520	2E-21
SM1063	297	Sh	DV175459	ns	-1.45	-2.60	3	Putative senescence-associated protein	BAB33421	5E-19
SM1064	367	Sh	DV175460	ns	-1.79	-2.46	3	Putative senescence-associated protein	BAB33421	2E-19
SM1123	587	Sh	DV175463	ns	-1.97	ns	3	GDSL-motif lipase/hydrolase	AAM64368	1E-23
SM1228	386	Sh	DV175468	ns	-2.22	-1.88	3	DEAD/DEAH box helicase, putative RH15	At5g11170	4E-38
SM1026	337	Sh	DV175454	-1.23	-1.68	-1.49	3	CZF1/ZFAR1 zinc finger protein	At2g40140	1E-11
RGUS1068	255	S	DV175389	2.48	ns	ns		SPX domain-containing protein	At5g20150	1E-20
RGUS1303	252	S	DV175420	1.29	-1.49	-1.94	3	RNA polymerase alpha chain	ArthCp055	1E-08
SM1146	244	Sh	DV175466	53.12	32.49	3.63	4	RNA dependent RNA polymerase [CVA]	AAL60496	9E-29
SM1124	245	Sh	DV175464	50.34	35.62	5.23	4	RNA dependent RNA polymerase [CVA]	AAL60496	2E-28
SM1002	204	Sh	DV175453	27.90	28.82	2.46	4	RNA replicase; coat protein [CVA]	CAA57896	5E-17
RGUS1393	153	R	DV175430	13.33	22.69	2.99	4	RNA dependent RNA polymerase [CVA]		
RGUS1336	273	GU	DV175425	-2.40	ns	ns	5	Chloroplast omega-3 desaturase	AAM77643	5E-35
SM1155	213	Sh	DV175467	-1.80	ns	ns	5	Valencene synthase	AAX16077	3E-11
SM1089	275	Sh	DV175461	-1.40	ns	2.26	6	Sesquiterpene cyclase	CAA04773	3E-27
SM1090	275	Sh	DV175462	-1.36	ns	2.34	6	Sesquiterpene cyclase	CAA04773	3E-26

1.RGUS: gene obtained in the graft-union cDNA-AFLP experiment, SM: gene obtained in the upper shoot cDNA-AFLP experiment; 2.TDF size refers to the sequence length in base pairs; 3. Tissue of origin according to the cDNA-AFLP profile.

Table 2.2: Genes differentially expressed in the graft union microarray experiment. Positive fold change values refer to B5 up-regulation; negative values to B6 up-regulation. R: Rootstock, GU: Graft Union, S: Scion, Sh: Shoot, B5: 'Bing'/Gi5, B6: 'Bing'/Gi6, ns: non-significant.

TDF ID(1)	TDF size(2)	TO(3)	Genebank accession number	Microarray fold change				Annotation		
				B5/B6 R	B5/B6 GU	B5/B6 S	Cluster	Product	PubMed ID	E-value
RGUS1219	302	S	DV175402	ns	ns	1.65	1	transferase family protein	At1g31490	3E-30
RGUS1305	204	S	DV175421	ns	ns	1.75	1	pentatricopeptide (PPR) repeat-containing	At1g19720	3E-22
RGUS1590	302	S	DV175448	ns	ns	1.60	1	transferase family protein	At1g31490	3E-30
SM1057	174	Sh	DV175458	ns	ns	1.74	1	AP2 domain-containing transcription factor	At4g39780	7E-09
RGUS1001	254	S	DV175382	ns	ns	-1.51	3	CSLD2 cellulose synthase catalytic subunit-like	At5g16910	2E-29
RGUS1336	273	GU	DV175425	ns	ns	-1.68	3	chloroplast omega-3 desaturase	AAM77643	5E-35
RGUS1358	171	S	DV175427	ns	ns	-1.63	3	No hit		
RGUS1399	135	S	DV175431	ns	ns	-1.51	3	No hit		
RGUS1449	300	S	DV175434	ns	ns	-1.59	3	COBRA-like protein 7 precursor	At4g16120	8E-33
RGUS1526	210	S	DV175438	ns	ns	-1.53	3	calcium binding protein	CAC43238	2E-50
RGUS1542	96	GU	DV175443	ns	ns	-1.68	3	expressed protein	At5g54870	2E-58
RGUS1589	157	GU	DV175447	ns	ns	-1.52	3	UDP-D-glucuronate 4-epimerase GAE1	At4g30440	3E-17
RGUS1114	267	R	DV175393	ns	1.53	ns	5	terpene synthase/cyclase family	At3g29190	1E-12
RGUS1136	272	S	DV175398	ns	1.75	ns	5	sesquiterpene cyclase	CAA04773	9E-30
RGUS1143	223	S	DV175399	1.92	1.73	1.41	4	No hit		
RGUS1067	197	R	DV175388	1.98	1.56	1.78	4	BG3 beta-1,3-glucanase	At3g57240	5E-20
RGUS1080	164	R	DV175390	1.64	ns	1.43	4	glycosyl hydrolase family 17	At4g16260	7E-07
RGUS1278	270	S	DV175417	1.59	ns	1.44	4	calreticulin 3	AAQ19995	2E-32
RGUS1279	201	S	DV175418	1.62	ns	1.30	4	calreticulin 3	AAQ19995	2E-32
RGUS1636	296	R	DV175451	1.49	ns	1.81	4	putative calcium binding protein	XP_550050	1E-09
RGUS1534	142	R	DV175442	1.18	ns	1.69	4	CHIB1 Acidic endochitinase	At5g24090	0.0002
RGUS1234	327	S	DV175407	1.61	1.47	ns	4	alpha 1,4-glucan phosphorylase L isozyme	AAK15695	4E-41
RGUS1235	327	S	DV175408	1.61	1.46	ns	4	alpha 1,4-glucan phosphorylase L isozyme	AAK15695	4E-41
RGUS1007	172	R	DV175383	1.60	ns	ns	4	subtilisin-like serine protease	At3g14240	2E-07
RGUS1009	170	GU	DV175384	1.52	ns	ns	4	No hit		
RGUS1022	289	GU	DV175387	1.61	ns	ns	4	zinc finger protein 291	NP_065894	2E-21
RGUS1108	302	GU	DV175392	1.50	ns	ns	4	putative serine threonine kinase CIPK9	AAL85889	7E-29
RGUS1166	436	S	DV175400	1.54	ns	ns	4	BRI1-associated receptor kinase 1 (BAK1)	AAK68074	6E-26
RGUS1223	140	GU	DV175403	1.52	ns	ns	4	C3HC4-type zinc finger protein (RING finger)	At1g69330	6E-15
RGUS1226	141	GU	DV175404	1.51	ns	ns	4	RING zinc finger protein, putative	ABF94597	6E-14
RGUS1239	449	S	DV175409	1.72	ns	ns	4	DNA-binding bromodomain-containing protein	BAA97526	2E-32
RGUS1252	273	GU	DV175410	1.92	ns	ns	4	heat shock protein hsc70-1 (hsp70-1)	At5g02500	3E-40
RGUS1276	426	S	DV175416	1.62	ns	ns	4	putative nodulin-like protein	BAD34364	3E-43
RGUS1544	94	GU	DV175444	1.60	ns	ns	4	expressed protein	At5g54870	5E-06
SM1039	369	Sh	DV175455	1.68	ns	ns	4	putative heat shock protein 90	At5g56000	9E-51
SM1040	369	Sh	DV175456	1.59	ns	ns	4	putative heat shock protein 90	At5g56000	9E-51
SM1126	287	Sh	DV175465	1.57	ns	ns	4	t-complex polypeptide 1	BAC22124	2E-37
RGUS1362	381	R	DV175428	1.81	ns	-1.44	4	centromere protein	At3g22790	2E-24
RGUS1018	256	S	DV175386	-1.42	ns	-1.71	6	putative regulator of gene silencing	XP_463755	3E-15
RGUS1418	329	S	DV175432	-1.51	ns	-1.57	6	chorismate mutase, cytosolic (CM2)	At5g10870	4E-31
RGUS1419	363	S	DV175433	-1.59	ns	-1.62	6	putative chorismate mutase CM2	At5g10870	3E-31
RGUS1282	191	R	DV175419	-1.61	1.07	ns	6	cinnamate 4-hydroxylase CYP73	AAF66065	1E-12
RGUS1506	207	R	DV175436	-1.62	ns	ns	6	cinnamate 4-hydroxylase CYP73	AAF66065	1E-12
RGUS1679	138	R	DV175452	-1.52	ns	ns	6	Ttrans-cinnamate 4-monooxygenase (C4H)	At2g30490	0.009
RGUS1342	200	S	DV175426	-1.66	-1.19	ns	6	No hit		
SM1312	322	Sh	DV175469	-1.36	-1.52	ns	6	xyloglucan endotransglycosylase, putative	At4g03210	9E-11
SM1348	386	Sh	DV175470	-1.44	-1.64	ns	6	No hit		
RGUS1316	365	GU	DV175423	-1.57	ns	1.58	6	weak similarity to CTD phosphatase-like 3	At5g58000	7E-12
RGUS1545	187	S	DV175445	-1.22	ns	1.55	6	inwardly rectifying potassium channel subunit	CAG27094	3E-05
RGUS1264	401	R	DV175411	-1.98	-2.37	-1.90	7	No hit		
RGUS1265	401	R	DV175412	-1.72	-2.21	-1.71	7	No hit		
RGUS1266	402	R	DV175413	-1.88	-2.28	-1.76	7	No hit		
RGUS1530	285	S	DV175440	-1.31	-1.56	ns	7	subtilisin-like protease	AAQ54525	7E-22

1. RGUS: gene obtained in the graft-union cDNA-AFLP experiment, SM: gene obtained in the upper shoot cDNA-AFLP experiment; 2. TDF size refers to the sequence length in base pairs; 3. Tissue of origin according to the cDNA-AFLP profile

The differentially expressed genes in the graft union experiment fell into 7 clusters (Table 2.2). Cluster 1 includes 3 genes up-regulated in the scion of 'Bing'/Gi5 trees compared to 'Bing'/Gi6, which encode for a transferase (RGUS1219, RGUS1590) and the other two for a lectin (RGUS1305) and an AP2-domain protein (SM1057) (Table 2.2). In contrast, Cluster 3 is formed by 10 genes that are up-regulated in the scion of 'Bing'/Gi6 compared to that of 'Bing'/Gi5. A COBRA-like 7 protein and a calcium binding protein are among these genes. Cluster 4 contains the largest number of clones (24), which represent genes expressed higher in 'Bing'/Gi5 than 'Bing'/Gi6 rootstocks. Many of the genes are involved in post-translational modification such as kinases, molecular chaperones and proteases (Table 2.2). The most interesting of the kinases is a BRI1-associated receptor kinase1 (BAK1,RGUS1166) involved in the perception of brassinosteroids (BRs) through interaction with the BRI1 receptor (Li *et al.* 2002; Nam and Li, 2002; Russinova *et al.* 2004). RGUS1252 is also interesting since it encodes for HSC70-1 a molecular chaperone with the ability to move non-cell autonomously within the phloem sieve elements and may act in long distance signaling (Aoki *et al.* 2002). Cluster 6 is comprised of 12 clones and represents 'Bing'/Gi6 genes expressed at higher levels in the rootstock than in 'Bing'/Gi5. The majority of the genes are homologous to cell wall formation related genes, while one gene is similar to a putative regulator of gene silencing from rice (RGUS1018).

Four transcription factors were identified in the shoot microarray experiment: a putative homolog to an AtVOZ1 protein from *Arabidopsis* (SM1045), an AP2-domain containing protein (RGUS1210), a MYB-domain protein (RGUS1306) and a zinc-finger

containing protein (SM1026). AtVOZ1 is a transcription factor in *Arabidopsis* with only one homolog called AtVOZ2 (Mitsuda *et al.* 2004). AtVOZ1 is expressed specifically in the phloem (Mitsuda *et al.* 2004), but its function remains unknown. The AP2-domain containing protein is similar to an ethylene responsive transcription factor (ERF/AP2) with unknown function that belongs to the B2 family of ERF/AP2 factors (Nakano *et al.* 2006). The MYB-domain homolog of *Arabidopsis* is called MYB111 and belongs to the R2R3-MYB subfamily, but its function remains unknown (Stracke *et al.* 2001). A phylogenetically close homolog of MYB111, MYB12 is involved in the transcriptional control of flavonoid biosynthesis genes (Mehrtens *et al.* 2005). Interestingly, most of the flavonoid biosynthesis related genes were identified in the graft union region and not in the shoot (Tables 2.1 and 2.2). Except for RGUS1306, the other three genes are expressed higher in 'Bing'/Gi6 on 20 June and 3 July, due to the reduction in 'Bing'/Gi5 expression. In contrast to the shoot, only one transcription factor was differentially expressed between 'Bing'/Gi5 and 'Bing'/Gi6, in the graft union. It is similar to an AP2-domain protein (SM1057), but different from that identified in the scion (Tables 2.1 and 2.2). This AP2-domain protein also belongs to the ERF/AP2 family of transcription factors and falls into subfamily A6. It is not known if these two proteins function in the same process.

Finally, five TDFs were expressed differentially both at the shoot and the graft union region. The most interesting of those genes is a touch-induced protein with a calmodulin calcium-binding domain (RGUS1636), because it may function in the brassinosteroid signaling pathway. The other four genes are a subtilisin-like serine

protease (RGUS1530), a nucleotide sugar epimerase (RGUS1589), a chloroplast omega-3 desaturase (RGUS1336) and a sequence with no homology to known genes (RGUS1342).

DISCUSSION

Differences in phenotype between graft combinations are accompanied by genetic changes

Shoot elongation measurements revealed a mid-growing season differentiation in shoot elongation rate between dwarfing and semi-vigorous trees. Such changes in growth were expected to be the result of signaling events occurring in the tree system. These events involve an array of proteins whose levels or activity is modified to produce the observed changes. The level of some proteins is controlled transcriptionally or post-transcriptionally and can be screened with high-throughput methods such as cDNA-AFLP. The collection of samples before, during and after the divergence in shoot growth between 'Bing'/Gi5 and 'Bing'/Gi6 trees enabled the identification of genes that were differentially modified between the two grafts and possibly contributed to the changes in shoot growth. It was expected that cessation of growth is followed by other physiological processes, such as flower primordia formation, reserve storage and dormancy. Thus genes differentially expressed on 3 or 20 June, before or at the onset of shoot growth cessation, respectively, are expected to be involved in growth cessation. Genes conforming to this criterion are represented by patterns 1, 2, 3, 5 and 6 (Figure 2.1B).

cDNA-AFLP is a powerful molecular technique for high throughput screening of transcriptomes across organisms (Breyne and Zabeau, 2001). Its major advantage is the

ability to analyze any RNA sample without previous knowledge of the genome sequence for the respective organism. Due to its reliance on PCR amplification, even low abundance signals can be recovered and compared. The sensitivity of detection is high enough to detect temporary RNA molecules, such as miRNA precursors (Prassinos *et al.* 2005). The profiles produced in each run are highly reproducible making it a method of choice over differential display (Kuhn, 2001). Allelic variation and post-transcriptional modification, such as miRNA cleavage, can be detected with the use of adequate restriction enzyme combinations. One disadvantage of the technique is the dependence on the technical skills of the researcher that will allow reliable comparison between samples. The enzyme combination ApoI/MseI used in this study was selected due to its higher restriction frequency compared to other six/four cutter combinations. ApoI has the potential to identify four restriction sites [(A/G)AATT(T/C)] compared to the popular restriction enzymes such as EcoRI, HindIII, PstI, BamHI that recognize only one. As a result, the number of TDFs produced by this combination can reach up to 4 times more fragments than the enzymes mentioned before. Each sample used in this study returned approximately 100 TDFs. The primer combinations used were 128 and the samples used for each primer pair were 6. Thus the total number of TDFs produced per cDNA-AFLP experiment reached 76,800 (128 x 100 x 6).

Despite the large number of genes screened by cDNA-AFLP, comparison of gene expression at the main shoot, between dwarfing and semi-vigorous trees, revealed a high degree of co-regulation. Only 111 TDFs were selected as differentially expressed between the two grafts. Such a low number of differentially expressed genes can be explained by several observations such as: a) the genotype of the scion is the same

('Bing') in both graft combinations, b) the trees were growing under the same environmental conditions, c) apart from the difference in shoot elongation there was no other obvious phenotype and d) shoot length did not differ substantially between graft combinations indicating a very similar transcriptome. Thus, it is prudent to expect that these 111 TDFs are involved in shoot growth cessation. Nevertheless, evaluation of differential expression was performed visually and thus it was necessary to quantify the level of expression at each sample. Furthermore, cloning of some TDFs returned more than one gene sequences due to contamination from neighboring fragments. The microarray technology was selected to solve these problems and simultaneously serve as a second independent replication of the experiment. In contrast to cDNA-AFLP, microarrays use sequence homology to detect the level of gene expression. Thus, microarrays cannot differentiate effectively between alleles, reflecting the cumulative expression of a gene. Since the microarrays in this analysis were constructed using the cloned TDFs as template, there could be no selection over the quality of the sequence. Lack of sequence information did not allow filtering of the TDFs on the basis of sequence conservation. The average size of the TDFs was 200bp, thus highly conserved genetic regions could cover its complete sequence. Even under stringent hybridization conditions, cross-hybridization of conserved regions cannot be avoided. Statistical analysis of the microarray data allowed the identification of significant differences in gene expression. Genes with statistically significant differences in expression between rootstocks were subjected further to filtering for fold change in expression levels. A fold change of 2 proved to be very stringent, since as it is shown in Tables 3 and 4 only 17 TDFs could pass that filter. Thus genes with fold change higher than 1.5 were selected.

This is considered a small change in gene expression, but tagging as statistically significant indicates a consistent difference between samples. That difference in the concentration of transcripts may be enough to produce the observed phenotypic change. For these reasons only 43 gene clones were proven to be differentially expressed. Since the microarray included genes from the shoot and the graft union regions, some of the differentially expressed genes were originally identified in the graft union cDNA-AFLP experiment.

The graft union region is transcriptionaly diverse between graft combinations at the time of differentiation in shoot growth

The graft union is the bridge between the rootstock and the scion, where tissues from both genotypes combine to produce a functional tree. Swelling at the graft union denotes an interaction between rootstock and scion, a phenomenon inversely proportional to rootstock vigor (Wagner and Gruppe, 1985). Identification of the interaction effect at the genetic level was performed by cDNA-AFLP. Analysis at the shoot apex indicated the 20th of June as the most indicative time for differential gene expression between the two graft combinations. This date was selected as the most appropriate for the graft union analysis. Due to the common origin of the two rootstocks, it was expected that they share the same genes, but at the same time they exhibit allelic variation.

cDNA-AFLP analysis at the graft union revealed the expected variation in TDF profiles as shown in Figure 2.2. The majority of the differentially expressed TDFs were identified in the scion area bordering the graft union. This is unexpected since the scion tissue for both graft combinations used in the experiment have the same genetic

background ('Bing'). Only 64 TDFs were differentially expressed in the rootstock, which would have been expected to show a higher degree of differential expression than the scion. Furthermore, TDFs with patterns similar to pattern 1 (Figure 2.2) were the result of allelic variation and not due to a difference in the transcriptional levels as it was proven by the microarray analysis. Nevertheless, allelic variation can affect the function of the gene at the translational level. Enzymes or signaling proteins with altered sequence in one genotype may have different affinity for their substrate or other interacting proteins. This possibility was not tested however. Gene expression at the graft union proved to be even more complex. The graft union combines cells from both rootstock and scion that interact at the point of union formation allowing interaction between cells and concomitantly the rootstock and the scion. TDFs with patterns such as pattern 15 (Figure 2.2), were initially believed to be 'Bing'/Gi6 graft union specific. Microarray analysis proved that these genes were expressed in both graft combinations without any sign of differential expression. The reason for this anomaly in the cDNA-AFLP profile is not known. Except for 2 TDFs expressed in 'Bing'/GI5, all others were specifically expressed in 'Bing'/GI6 graft unions according to the cDNA-AFLP. This observation excludes the effect of allelic variation in restriction digestion. In that case, we would expect a similar number of differentially expressed bands between the two graft combinations. Furthermore, sequencing of some of these TDFs excluded contamination by other organisms or genomic DNA, due to high similarity to other *Rosaceae* cDNA sequences available in public databases and the methods used to isolate and purify the RNA. Nevertheless, the inability of these genes to pass the filters set for the microarray experiment did not allow any further examination of their behavior in the cDNA-AFLP experiment.

Parallel gene regulatory pathways between cherry and apple graft combinations

An interesting gene (RGUSS1166) encodes a protein similar to the *Arabidopsis* BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1 (BAK1/SERK3). This gene also has been found to be up-regulated in dwarfing apple grafts, but at the stem region (Jensen *et al.* 2003). The parallel differential expression of BAK1 in these two dwarfing systems implies an important role for brassinosteroids (BRs) in the control of tree growth. Other genes found in both studies include an AP2 domain-containing protein (RGUS1210), a GDSL-motif lipase hydrolase (SM1123), a touch-induced protein (RGUS1636) and a C3HC4 zinc-finger protein (RGUS1223), indicating an across-species response mechanism to the dwarfing phenomenon.

Genes implicated in brassinosteroid response

Three independent microarray studies on the effect of BRs in Arabidopsis gene expression revealed a significant number of affected genes (Müssig *et al.* 2002; Yin *et al.* 2002; Goda *et al.* 2004). Interestingly, genes with similar annotation are between those differentially expressed in the current study. The cherry orthologs of the Arabidopsis genes affected by BRs are a touch-induced protein (RGUS1636), the subtilisin serine protease (RGUS1529-1531) and a beta-1,3-glucanase (RGUS1067). Together with the homolog of BAK1 (RGUS1166), this makes a significant number of differentially expressed BR related genes and requires special attention. In the shoot, RGUS1636 and RGUS1529-31 are up-regulated in 'Bing'/Gi5 on the 3rd of July, compared to 'Bing/Gi6. RGUS1636 also is up-regulated in the rootstock and scion of 'Bing'/Gi5 trees, together with RGUS1067. RGUS1166, the homolog of BAK1, is up-regulated in the rootstock of

'Bing'/Gi5 trees. Only RGUS1530 shows up-regulation in the rootstock and graft union of 'Bing'/Gi6 trees. This is contradictory to the role of BRs in the control of growth since, in this study, genes responsive to BRs are up-regulated in the dwarf 'Bing'/Gi5 trees rather than the semi-vigorous 'Bing'/Gi6. It is in agreement, though, with the findings in dwarf apple trees (Jensen *et al.* 2003). Recently, it was shown that the epidermis plays a crucial role in the promotion of growth in Arabidopsis (Savaldi-Goldstein *et al.* 2007). This was shown in response to local activation of BR signaling, indicating the important role of BRs in the control of growth. A more in-depth analysis of the response of various rootstocks to brassinolide treatment or internal concentration of BRs is necessary to clarify their role in the dwarfing phenomenon.

Conclusions

The parallel analysis of gene expression at the shoot and the graft union region allowed a more complete coverage of the changes in the biology of the two graft combinations. We have shown that shoots of the common genotype 'Bing' respond differently when grafted on two rootstocks of different vigor. The dwarfing rootstock Gi5 caused earlier changes in gene expression of the 'Bing' shoot, compared to the non-dwarfing rootstock Gi6. The graft union region experiment revealed much more diverse gene expression in the rootstock (Table 2.2), which is expected to be the cause of differential shoot growth in the scion. Additionally, as shown in Figure 2.2B patterns 9 and 10, several TDFs exhibited a differential expression across the graft union, which implies transport of the signals between rootstock and scion. It is not known, however, whether these genes are transported through the vascular system or if they are expressed

in the trunk cells. These observations lead to the formulation of a fourth hypothesis that "rootstock-induced dwarfing is caused by rootstock encoded signals able to move through the graft union and affect scion growth". In support of this hypothesis, recent studies have shown that there are a significant number of mobile macromolecules that can move through the vascular system (Kim *et al.* 2001; Mallory *et al.* 2003; Ding *et al.* 2003; Lucas and Lee, 2004). Signaling proteins or RNA can move through the graft union and small differences in the receptors/targets of those signals can have negative or positive effects on growth (Kim *et al.* 2001; Mallory *et al.* 2003).

Rootstock-induced dwarfing remains a complex and poorly understood phenomenon. Physiological data have provided much information on the changes occurring in the grafted trees, especially at the vicinity of the graft union. Clues provided by these studies also point to the idea that all dwarfing systems do not function by the same mechanism. The genotypic differences of rootstocks that exert varying levels of vigor to the same scion variety prompted us to study the response of genes in both the scion and the rootstock. We have successfully focused on the transition stage during the growing season when gene expression differentiates between dwarfing and vigorous rootstocks and studied both the biology of the shoot and the graft union area. Further characterization of the identified genes should eventually lead to the confounding signals that are responsible for the determination of the transitions in shoot growth. Control in the production of these signals could eventually lead in the control of tree growth depending upon the desirable attributes of the exploited trees.

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CHAPTER 3

CHERRY VIRUS A HAS NO DIRECT EFFECT ON ROOTSTOCK-INDUCED DWARFING OF GRAFTED CHERRY TREES

INTRODUCTION

Plant viruses can cause significant losses in agricultural production. Their impact is usually in the quality of the product, which is usually unacceptable for the market, but also in the quantity of the product, which is reduced. Protection of crops from viruses is usually performed through the distribution of certified material that is virus-free, since there are no products in the market for virus eradication. Virus-free material can be produced by tissue culture or propagation of uninfected stocks. For the detection of viruses in crops many tests are commercially available and are usually based on antibody detection, such as the Enzyme-Linked ImmunoSorbent Assay (ELISA). However, tests are not available for all viruses. Development of a test depends on the importance and severity of infection by the virus on various crops. Fruit crops are prone to virus infections due to their longevity, clonal propagation and attraction of sap feeding insects that can transmit viruses through their proboscis. Previously reported viruses in cherry are the Prune Dwarf Virus (PDV), Prunus Necrotic Ringspot Virus (PNRSV), Little Cherry Virus-1 (LChV-1), Little Cherry Virus-2 (LChV-2), Cherry Necrotic Rusty Mottle Virus (CNRMV), Cherry Mottle Leaf Virus (CMLV), Cherry Rasp Leaf Virus (CRLV), Cherry Virus A (CVA), and Cherry Green Ring Mottle Virus (CGRMV) (Jelkmann, 1995; Lang and Howell, 2001; Isogai *et al.* 2005). As the name of PDV suggests, the virus reduces the size of the infected trees, but not in cherries (VIDE web site). Nevertheless, the ability to produce dwarf cherry trees may exist in other viruses.

Plants in contrast to mammals do not produce antibodies against viruses. They have developed a system of suppression of the viral genomic RNA called silencing. In this system, double stranded RNA molecules are recognized and cleaved by internal plant

enzymes that belong to the Dicer-like family (Wang and Metzlaff, 2005). Cleavage results in small double stranded RNAs of 21 and 24 nucleotides in size (Lecelier and Voinnet, 2004). These small dsRNA molecules are perceived by the 'RNA induced silencing complex' (RISC), which will then convert them into single stranded RNAs and use them as templates for the detection and cleavage of more viral RNAs (Lecelier and Voinnet, 2004). Plant RNA dependent RNA polymerases (RdRPs) are responsible for the amplification of the silencing signal by producing more dsRNAs (Lecelier and Voinnet, 2004). Small RNAs can also move systemically into the plant vascular system and confer virus resistance to the rest of the plant body (Yoo *et al.* 2004). Systemic silencing is necessary to protect plants from systemically spreading viruses. Some plant viruses can accomplish long distance trafficking with the use of a movement protein (Nelson and Citovsky, 2005; Lucas, 2006). Silencing though is not enough to protect plants from viral infection. Viruses have developed mechanisms to overcome host-specific resistance. An example is the ability of the HC-Pro protein to suppress the accumulation of virus induced siRNAs, thus promoting viral infection (Llave *et al.* 2000).

Cherry Virus A (CVA) was reported first in sweet cherry (*Prunus avium* L.) by Jelkmann (1995) in a study aimed at isolating Little Cherry Disease (LCD). The virus belongs to the genus *Capillovirus*, which includes the type member Apple Stem Grooving Virus (ASGV). CVA is a single stranded RNA virus, with a 3' attached poly-adenylated tail. Its genome has a size of 7,383bp and contains two open reading frames (ORFs, Figure 3.1). The first ORF (ORF1) covers almost the complete sequence of the virus and produces a sequence of 2,360 amino acids in frame 1. ORF1 contains a domain of unknown function (DUF1717), a RNA dependent RNA polymerase (RdRP), a viral

helicase 1 and a coat protein. The second ORF (ORF2) is located in the C-terminal region of the virus and has a sequence of 463 amino acids in frame 3. It contains the movement protein of the virus.

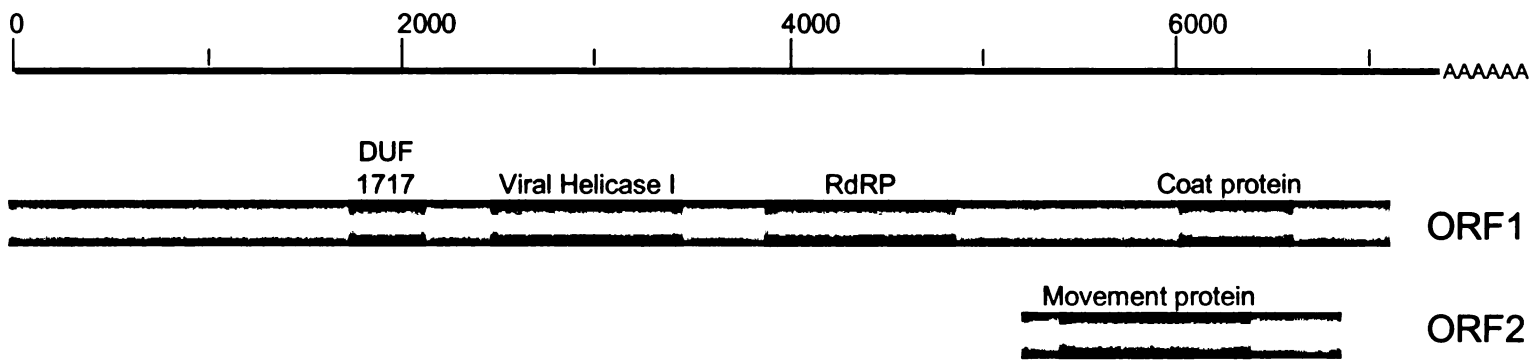


Figure 3.1: Genome organization of Cherry Virus A. Shaded areas denote the position of the proteins as described above these areas. ORF1: Open Reading Frame1, ORF2: Open Reading Frame2, DUF: Domain of Unknown Function, RdRP: RNA depended RNA Polymerase. ORF1 is 2,360aa and ORF2 is 463aa (Jelkmann, 1995)

Since 1995, the virus has been detected in Germany, Canada, the United Kingdom, France and Japan (Eastwell and Bernardy, 1998; Foissac *et al.* 2001; Isogai *et al.* 2004; James and Jelkmann, 1998; Jelkmann, 1995; Kirby *et al.* 2001). No symptoms on sweet cherry trees or fruits have been linked to the virus, which explains its relatively new and accidental discovery (Jelkmann, 1995; Eastwell and Bernardy, 1998). CVA does not exhibit a synergistic effect in the presence of LCD and thus does not amplify the symptoms associated with LCD (Eastwell and Bernardy, 1998).

Here we report the discovery of CVA in sweet cherry cultivars grafted on interspecific hybrid rootstocks in Michigan, United States. The virus was identified in a complementary DNA-Amplified Fragment Length Polymorphism (cDNA-AFLP) screen between dwarfing and non-dwarfing trees, aimed at identifying genes involved in the phenomenon of rootstock induced dwarfing. Genes showing differential expression between the two graft combinations were of primary interest. Nine cDNA-AFLP fragments that were present only in the dwarf trees aligned to various regions of the 7,383 bp CVA genome, confirming that they originated from CVA RNA. We tested whether the presence of CVA is linked to dwarfism induced by rootstocks of varying vigor.

MATERIALS AND METHODS

Plant material

Tissue samples were harvested for the cDNA-AFLP experiment from orchard-grown shoots and trunk of two-year-old 'Bing' sweet cherry on GiSelA5 and GiSelA6 (both clonal rootstocks derived from hybridization of *Prunus cerasus* L. x *Prunus*

canescens Bois.) (Horticulture Experiment Station, Clarksville, Michigan). Shoots were collected on 3 June, 20 June and 3 July 2002, while trunk samples of the rootstock and the scion of the same trees were collected on 20 June 2002. For the additional screening tests, shoot samples were collected in June 2003 from: 1) shoots of 'Hedelfingen' sweet cherry grafted on the clonal rootstocks GiSelA3 (*P. cerasus* x *P. canescens*), GiSelA5, GiSelA6, Edabriz (*P. cerasus*), Gi195/20 (*P. canescens* x *P. cerasus*), Weiroot10 and Weiroot158 (both *P. cerasus*), as well as on the seedling rootstocks Mazzard (*P. avium*), Mahaleb (*Prunus mahaleb* L.) and Erdi V (*P. mahaleb*) (Northwest Michigan Horticultural Experiment Station, Traverse City, Michigan); 2) shoots of 'Bing', 'Hudson', and 'Attika' sweet cherry grafted on GiSelA5 and GiSelA6 rootstocks (Horticulture Experiment Station, Clarksville, Michigan); and 3) shoots of 'Sam'/ GiSelA5, 'Brooks'/ GiSelA5 and 'Bing'/Edabriz trees (Horticulture Experiment Station, Clarksville, Michigan). Samples consisted of the upper 10 cm of the shoot without the leaves. One to three shoots were collected from each of three to four trees for every graft combination. Samples harvested in the orchard were placed in coolers filled with dry ice, transported to the laboratory, and ground to a fine powder in liquid nitrogen. Shoot samples were ground with a mortar and pestle while trunk samples from the rootstock and the scion were ground in a 1 liter stainless steel commercial blender (Waring, Torrington, Connecticut). All of the samples were maintained frozen in liquid nitrogen during grinding (samples were frozen in liquid nitrogen, but were dry when the blender was operated). Ground samples were stored at -80^0 C until RNA could be extracted.

RNA extraction

A common total RNA extraction protocol was used throughout these experiments (Wang *et al.* 2000). mRNA was isolated for the cDNA-AFLP experiment with Dynabeads paramagnetic particles (Dynal Biotech, Lake Success, New York) as described by the manufacturer.

cDNA-AFLP analysis

As described in Chapter 2.

Sequencing

The cDNA-AFLP fragments were PCR amplified using the Apo-pre and Mse-pre primers and directly sequenced using ApoI-pre (Molecular Structure Facility, Michigan State University) as the sequencing primer. Sequencing was performed on an ABI 7700 Sequencer (Genomics Technology Support Facility, Michigan State University). The sequences were introduced in the BlastN and BlastX search engines of the National Center for Biotechnology Information (NCBI) for alignment to known gene or protein sequences, respectively.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

RT-PCR primers were designed based on the initially reported CVA sequence (X82547) by Jelkmann (1995). The downstream primer was CVA4 (6372): 5' TCCTTTGAGAATTGCACTTATC 3', and the upstream primer CVA5 (4840): 5' CGTACAATAAAGGCGATCACC 3'. A brief description of the RT-PCR protocol is as

follows: Total RNA (1 μg) was reverse transcribed using an oligo(dT_{25})N primer and SuperscriptII reverse transcriptase, as described by the manufacturer (Life Technologies, Rockville, Maryland). The reaction was incubated at 42^0C for one hour followed by deactivation of the enzyme at 65^0C for 15 min. Ten percent (2 μl) of the reaction was used for PCR amplification in a 20 μl reaction using 10 pmol of CVA4 and CVA5 primers that produce a 1,532 bp fragment. The reaction conditions were as follows: 1) 94^0C for two min; 2) 40 cycles of 94^0C for 30 s, 52^0C for 30 s, and 72^0C for 70 s; 3) 72^0C for 7 min; and 4) storage at 4^0C. The product was analyzed on a 1% agarose gel.

Growth measurements

Shoot length measurements were taken from trees growing at the Northwest Michigan Horticultural Research Station at Traverse City, Michigan. All trees had 'Hedelfingen' as the scion and the rootstocks were Gisela5, Edabriz, Gi195/20, Gisela 6, Weiroot10, Weiroot158, Mahaleb, Mazzard and Erdi V. Eight trees were used for each graft combination and three shoots were measured per tree. The model and analysis of variance were for unequal number of replications and subsamples. The SAS statistical package was use in the analysis and for multiple comparisons using the proc glm function.

Northern hybridization analysis

Ten micrograms of total RNA were analyzed on a 1.2% denaturing agarose gel and then transferred overnight to a nylon membrane Hybond N+ (Amersham Biosciences, Piscataway, New Jersey) by capillary transfer (Sambrook *et al.* 1987). The probe consisted of three cDNA-AFLP fragments (F1, F6, F8) that represented the 5' and

the 3' region of the CVA genome and were labeled with ^{32}P using the Rediprime II DNA labeling kit (Amersham Biosciences, Piscataway, New Jersey). Hybridization was performed in UltraHyb hybridization buffer (Ambion, Austin, Texas) at 42^{0}C for 18 h. The membranes were washed twice in 42^{0}C 2xSSC, 0.1% SDS solution for 5 min and twice in 42^{0}C 0.1xSSC, 0.1% SDS solution for 15 min. After washing and drying, membranes were exposed to an X-Ray film (Kodak, X-OMAT) overnight.

RESULTS

Initial detection of the virus

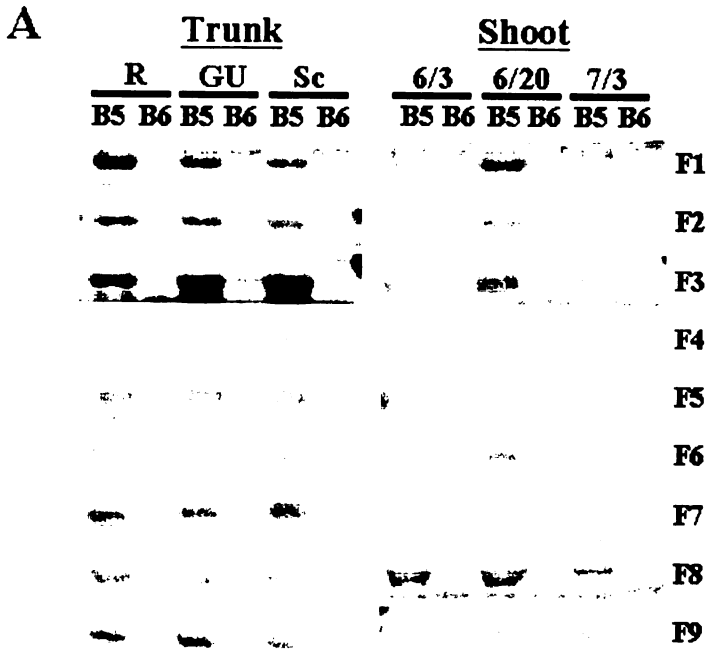
A study was conducted to identify gene expression changes between dwarfing and non-dwarfing scion-rootstock graft combinations in sweet cherry. 'Bing'/Gi5 and 'Bing'/Gi6 trees were used as the dwarfing and non-dwarfing plant materials, respectively. Screening of shoot and trunk samples by cDNA-AFLP revealed various types of gene expression differences between dwarfing and non-dwarfing trees (Chapter 2). Some of these expression patterns showed genes expressed only in 'Bing'/Gi5 trees (Figure 3.2A). Sequencing of the fragments representing these patterns revealed a high degree of similarity to Cherry Virus A. Of the 13 fragments showing this pattern, 9 were sequenced and aligned to the virus genome. The distribution was balanced across the viral genome, spanning from the 5' to the 3' region of the 7,383bp sequence (Figure 3.2B). The average size of the fragments was 157bp, with the smallest being 60bp and the largest 343bp. The same exact fragments were present in the shoot, the scion trunk, the graft union and the rootstock trunk (Figure 3.2B). Alignment of the translated sequences

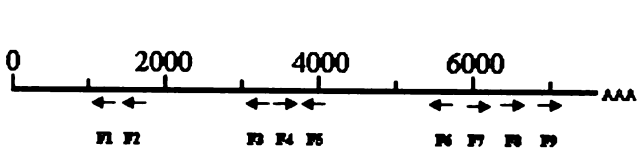
revealed a high degree of conservation between the virus identified in Germany and the United States, with few non-conserved amino-acid changes (Figure 3.2C). Fragments F6 and F7 align to the ORF2 that codes the movement protein, while the remaining fragments code for ORF1 which contains the other viral proteins. It should be noted that even though cDNA-AFLP was used as a gene expression screening tool, the identification of CVA was made possible by the polyadenylation and not the transcription of its genomic RNA. Thus the cDNA-AFLP patterns for CVA reflect the concentration of the viral genome and not viral gene expression.

CVA is not associated with rootstock control of scion vigor

The distinct presence of the virus in scion and rootstock tissues associated with the dwarfing Gi5 rootstock, yet absence in the same tissues associated with the semi-vigorous Gi6, led to the hypothesis that CVA may play a role in the ability of dwarfing cherry rootstocks to reduce sweet cherry scion vigor. To test this hypothesis, shoots from 'Hedelfingen' sweet cherry grafted on nine different rootstocks of varying vigor were screened for presence of the virus. The screening was performed using RT-PCR and Northern hybridization. The primers designed for the RT-PCR were based on the published sequence as another proof for the sequence conservation, producing a fragment of 1,532 bp. Northern hybridization was used as a confirmation method to avoid problems with miss-priming due to mutations on the virus sequence. The two methods returned consistent results, with CVA genomic RNA clearly present in the samples from 'Hedelfingen' on Gi5, but not on Gi6 (Figure 3.3). However, CVA RNA was

Figure 3.2: Cherry Virus A detection. (A) cDNA-AFLP profiles across tree sections and growing season dates for different parts of the CVA genome that was digested with *ApoI/MseI* restriction enzymes, R: rootstock, GU: graft union, Sc: scion, B5: 'Bing'/Gi5, B6: 'Bing'/Gi6. (B) The position of each cDNA-AFLP fragment on the 7,383bp CVA genome is indicated by arrows. Direction of the arrow is from the *ApoI* to *MseI* restriction site. (C) Alignment of the translated cDNA-AFLP fragments with the two open reading frames (ORF) of CVA. The alignment was obtained from the BlastX results. Amino acid conservation is indicated as follows; black: identical, gray: conserved, white: non-conserved. ORF1 and ORF2: open reading frames of the CVA genome with GenBank accession numbers CAA57896 and CAA57897, respectively. Amino acid numbering of the cDNA-AFLP fragments is based on the individual TDF amino acid sequence, while for CVA ORF1 and ORF2 it is based on the complete amino acid sequence. Sorting of the sequences is based on the CVA ORFs.





C

```
F1  : 1     NKRSPWSFLSDAKNYIDSWIIQSPFLRRIFPVGSRAITELIRDWIANAESLKIQTSSASLTF  62
ORF1: 375   NKRSPWSFLSDAKNYVDSWIIQSPFLRRIFPVGSRAITELIRDWIANAESLKIQTTCSSLTF  436

F2  : 1     TSKRFSGGSYSMVGRKGLLVDSMRQNTSSSSEVFVDLFPSTVRPA  45
ORF1: 475   TSKRFSGGSYSMVSRNGLLVDSMRQNTSFSCEVFVDLFPSTIRPA  519

F3  : 1     NEAQGSTFNSVILIVTRDFFSNPIESIIVAITRHQKNLLMDFPAAIQSEMDF  52
ORF1: 1056  NEAQGSTFNSVILIVTRDFFSNPIESIIVAITRHQKNLLIYFPAAIQGEMDF  1107

F4  : 1     KLQPIEENSTEVKENLKTHLPISYSGL  27
ORF1: 1170  KLQQIEENSIESKENLKTHLPISYSGL  1196

F5  : 1     PNTDQRMYDESVSEYEEQKISTNAPM  26
ORF1: 1298  PNFDQRMYDESVSEFEEKKISKNAAM  1323

F8  : 1     ISDMALSIVEHIYNEIRRGLGNYIWENMIDPRDLLHLTAKPAVEASEGVAATPAITLSENQRAVKNTIE  69
ORF1: 2110  ISSMALSIVEQNYNEIRRGLGNYIWENMIDPRDLLHLTAKPAVEASEGVAATPAITLSENQRAVKNTIE  2178

      70    NYYLRIMFGNLAVMGTSEQTDYPGEHLAIPRPVIENQELTAHLPAGMSLL  120
      2179  NYYLRIMFGNLAVMGTSEQTDYPGEHLAIPRPVIENQELTAHLPAGMSLL  2229

F9  : 1     NHGAVSFIYLKNPGAYFNCPAVVFDFNKGLPLTIIKIGKNANAISACNQRLFNREGKKAVFAAQGEVNL  69
ORF1: 2270  NHGAVSFIYLKNPGAYFNCPAVVFDFNKGLPLTIIKIGKNANAISACNQRLFNREGKKAVFAAQGEVNL  2338

F6  : 1     KKPINGRIVYFDPRFLDKNDACQAGFSFQLQTGSRYYLYRPNYPMSTHDPN  51
ORF2: 104   KKPINGRIVYFDPRFLDKNDACQAGFSFQLQTGSAYYLYRPNYPMSTHDPN  154

F7  : 1     FNGQYQLSIQPLQKRQRAADVGAPFQALFGSSGLPNPESFLEDEDIINPPTVALIDVSVDQSF  63
ORF2: 181   FGVMYQLSNQSTAEKTTAADVGAQFQALFGSSGLPNPESFLEDEDIVNPPTVALIDVSVDQSF  243
```

Table 3.1: cDNA-AFLP fragments with homology to CVA. Location of each fragment is given in nucleotides on the CVA genome. Size of each fragment is given in bases.

Fragment	Start	Finish	Size
F1	1180	1341	161
F2	1475	1598	123
F3	3223	3371	148
F4	3508	3588	80
F5	3977	4037	60
F6	5718	5861	143
F7	5975	6118	143
F8	6391	6734	343
F9	6862	7067	205

only faintly present in `Hedelfingen' on the dwarfing rootstock Edabriz, yet RNA concentration was quite strong in `Hedelfingen' on Weiroot158, which is similar in vigor to Gi6 (Table 3.2). The rootstock Gi3 is the most dwarfing rootstock among those tested, but there was no indication of the presence of CVA. These results do not support the hypothesis that CVA plays a role in the dwarfing ability of cherry rootstocks.

The absence of CVA is not linked to rootstock resistance

Based on the cDNA-AFLP, RT-PCR and Northern data, a second hypothesis was developed to determine whether the absence of the virus from Gi6 is due to resistance. To test this hypothesis, tissues were examined from four different sweet cherry cultivars grafted onto Gi5 and Gi6 rootstocks. RT-PCR and Northern hybridization were used as described above. The virus was present in all scions grafted on Gi5, while two scions ('Hudson' and 'Attika') grafted on Gi6 also were infected (Figure 3.4). 'Bing' on Edabriz which has the same vigor as 'Bing'/Gi5 was also found to be infected. These data indicate that the absence or presence of the virus in a particular rootstock or scion cultivar is probably by chance and not due to genetic resistance or susceptibility. Inoculation studies should be performed to clarify the level of resistance of each rootstock to CVA.

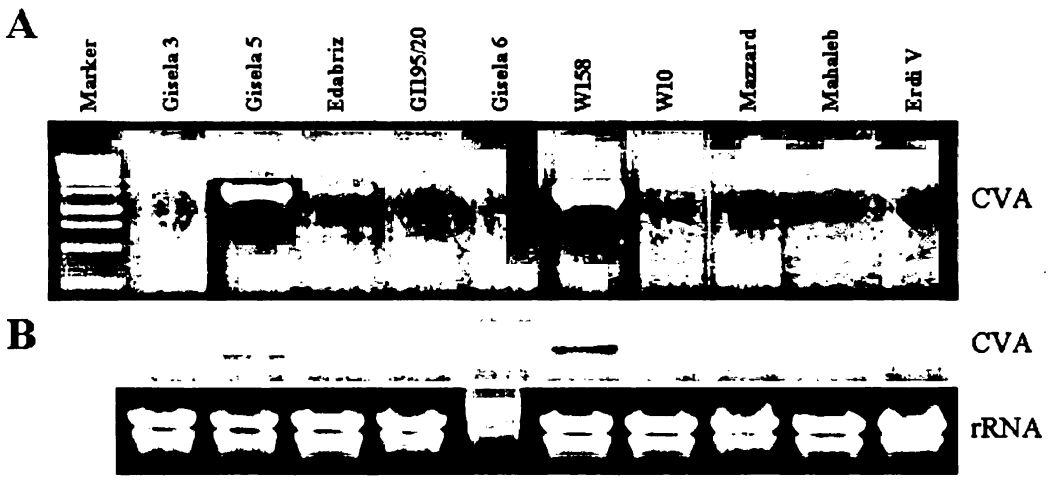


Figure 3.3: CVA detection in the sweet cherry variety 'Hedelfingen' grafted on 9 rootstocks that exert different degrees of vigor to the scion. (A) RT-PCR amplification of a 1532bp fragment of the CVA genome using primers CVA4-CVA5. W158: Weiroot158, W10: Weiroot10. Rootstocks are arranged from the most dwarfing (Gi3) to the most vigorous (Erdi V). (B) Northern blot hybridization was used to confirm the RT-PCR result. The image is aligned to the RT-PCR image in (A). rRNA denotes the RNA loading control.

GiSelA5	Edabriz	Gi195/20	GiSelA6	W.10	W.158	Mahaleb	Mazzard	Erdi V.
21.2a	21.7a	21.8a	27.0ab	31.7b	33.7bc	38.1c	38.5cd	44.9d

Table 3.2: End of season shoot length of 'Hedelfingen' scions grafted on 9 different rootstocks. Values present the average shoot length on July 29 and letters denote statistical differences at a=0.05.

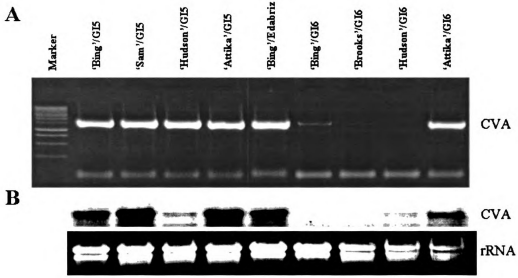


Figure 3.4: CVA detection in 5 sweet cherry varieties grafted on 3 rootstocks. (A) RT-PCR amplification of a 1532bp fragment of the CVA genome using primers CVA4-CVA5. GI5: GiSelA5, GI6: GiSelA6. (B) Northern blot hybridization was used to confirm the RT-PCR result. The image is aligned to the RT-PCR image in (A). rRNA denotes the RNA loading control.

DISCUSSION

Viruses of the genus *Capillovirus*, such as ASGV, Citrus Tatter Leaf Virus (CTLV) and Lilac Chlorotic Leaf Spot Virus (LCLV), have not been linked previously to tree height or vigor reduction (Büchen-Osmond, 2004). While our initial study revealed a coincidental association of CVA with the dwarfing cherry rootstock Gi5, but not the more vigorous Gi6, our subsequent screenings suggest that CVA is not responsible for the alteration of tree vigor in dwarfing cherry rootstocks. CVA was present in trees on relatively vigorous rootstocks (Weiroot158 and GiSelA6 with some scion varieties) that show the expected growth. Comparing previous records on the virus (Eastwell and Bernardy, 1998; James and Jelkmann, 1998; Jelkmann, 1995) and the experiments reported in this study, the absence of the virus from certain trees or graft combinations is circumstantial rather than due to a consistent association with rootstock vigor or genotypic tolerance.

There have been few reports on the occurrence of CVA since its discovery by Jelkmann (1995), likely because CVA has not been linked to any symptom that reduces cherry tree productivity or fruit quality (Eastwell and Bernardy, 1998; James and Jelkmann, 1998). As a result, no commercial detection assay has been developed to track virus abundance or distribution. Thus, it is possible that, in addition to its natural occurrence in mature trees, the virus has been spread via propagation of nursery stock as well. Indeed, that appears to be the case from the range of scions and rootstocks we screened as young trees in this study. Additionally, even though we found that mutations have been accumulating at the nucleotide level, they have been translated to only a few amino acid changes.

The cDNA-AFLP profiles during three different dates (Figure 3.2A) show that the concentration of the viral RNA is higher in mid-June, when the trees have reached the peak of growth activity. This observation is important for easier detection of the virus (when a genome detection method is used). Furthermore, total RNA was enough to detect the virus, since the CVA genome consists of a long RNA molecule with a poly-adenylated tail. The cDNA-AFLP data also provide another proof to Jelkmann's report (1995) that this virus is graft transmissible. This is important for the effective control of the virus, especially at the nursery level.

Future research on CVA should include further characterization of its possible symptomatology (possibly in synergy with other common viruses like Prunus Necrotic Ringspot and Prune Dwarf Virus, which are both pollen-borne and hence can infect an orchard at maturity); production of a fast detection assay (perhaps by immunoassay); and since it may be spread through grafting, determination of whether CVA-free certification should be a consideration for nursery mother blocks to prevent its continued spread.

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DISSERTATION SUMMARY, CONCLUSIONS AND IDEAS

SUMMARY CONCLUSIONS AND IDEAS

The aim of this study was the identification of genes involved in rootstock induced dwarfing. Several ideas have been put forth, some of which were attempted and some remained in the blueprint. In this section initial ideas will be presented and conclusions will be drawn based on the current results.

The idea for this project was formed by the need for genetic markers that will assist the efficient breeding of dwarfing rootstocks. Many of the current cherry rootstocks are products of interspecific crosses that are sterile, thus making genetic mapping impossible. Availability of genetic markers linked to RID would make breeding of dwarfing rootstocks an easier task. A fact in RID is that the genetic background of the rootstock is the driving force for the degree of vigor excerpted to the scion. Thus rootstocks with the same genetic background and different dwarfing capacity should be easier to compare for the identification of genetic loci involved in this phenomenon. Gisela5 and Gisela6, as discussed previously, are two of the most successful cherry rootstocks that confer different degrees of vigor to the cherry variety 'Bing'. The mechanism by which the rootstock dwarfs the scion is not clear. Several hypotheses were discussed in the Introduction, most of which relate to secondary effects of grafting. cDNA-AFLP was selected to test the hypothesis that assumes the existence of a mobile mRNA signal from the rootstock to the scion and its effect on gene expression. One concern was whether this approach is going to return any meaningful result, but the data in Chapter 2 proved that it did. It would have been a much more straight forward approach to extract phloem sap and study its transcriptome. Cherry, however, is a

difficult plant to obtain phloem sap from, and several attempts to do so proved unsuccessful. Another concern was that changes in the transcriptome may not be reflected in the proteome or the opposite. Such differences would not be detected by cDNA-AFLP. Two-dimentional (2-D) protein electrophoresis would be able to provide some clues on this question. Nevertheless, 2-D gels are very difficult to produce or reproduce, and low level signals cannot be detected. Thus, the method was excluded from further consideration as impractical and deviating from the main target, which was identification of RNA signals.

During the course of the project it was shown that dwarf trees respond faster to a signal that is consistent throughout the growing seasons. The signal is unknown, but the consistency of the data indicates that it is a periodic environmental signal, which may function through a signaling cascade or through changes in the physiology of the tree that trigger downstream signaling. The dominance of this signal is not known. Is it deprivation of some significant growth compound that is reduced or is it a repressor of growth that triggers dwarfing? To test that question two approaches were taken. In the first and probably the most direct, 'Bing'/Gi5 and 'Bing'/Gi6 trees were approach grafted to produce trees with two rootstocks. Grafts were performed at the 'Bing' scions to avoid any incompatibility issues. Measurements were taken on these trees to test their behavior in comparison to regular 'Bing'/Gi5 and 'Bing'/Gi6 growth rates. Unfortunately, the approach grafts created enormous wounds to the trees, which were not able to recover and eventually grafts were aborted. Information from this experiment was going to be very informative on the dominance of the dwarfing signal and will provide clues on the nature of the signal. Experienced grafters may be the solution to the problem of aborted

grafts. The second approach was reciprocal grafting, which would allow testing the effect of one rootstock on top of the other. Unfortunately, even in this case the grafting was unsuccessful due to the advanced growth of the ungrafted trees. The success rate was 2-5%, which is translated to 1-3 trees per combination. This number of trees is not enough for a well designed experiment.

One would expect that changes in shoot length are caused by the action of a certain hormone and especially the traditional hormones linked to stem elongation, such as auxin and gibberellin. Such an analysis seems reasonable, but the growth measurements showed that differences in shoot length between graft combinations are due to cessation of growth rather than metamer length. This was an initial indication that auxin or gibberellin are not involved in this phenomenon for these particular rootstocks. Indeed, cDNA-AFLP analysis did not return any of the known auxin or gibberellin regulated genes. Nevertheless, a group of genes was annotated as brassinosteroid regulated genes. Brassinosteroids are also linked to plant growth and development. It cannot be concluded though if these genes are responding to brassinosteroids, since no such experiment has been conducted in this project.

Abscisic acid (ABA) is another hormone that has been related to growth and more specifically with dormancy in seeds. It is reasonable to believe that cessation of shoot growth and bud set are part of the dormancy process. Nevertheless, cDNA-AFLP analysis did not return any ABA related genes. This may be explained by the absence of dormancy at the time of shoot growth cessation. Even though shoots have ceased growing and bud has set, the trunk is still active and expands until September. Leaf drop occurred later in October, thus explaining the absence of ABA regulated genes in June.

From these observations it seems that shoot growth cessation is related to the reduction in the rate of cell divisions and cell elongation. Such a process is difficult to explain since all mechanisms for SAM maintenance and cell differentiation from the SAM are functional, but they only occur in slower rates and eventually stop. This is supported by the absence of SAM regulatory or cell division related genes from the analysis.

The use of rootstocks from the same cross provided the opportunity to study gene expression at the graft union. If the rootstocks were not closely related then genetic differences would be enhanced and thus make the analysis of gene expression more difficult. Nevertheless, in the analysis of gene expression at the upper main shoot, the ideal comparison would be between graft combinations with significant difference in vigor. That selection would have made the differences in gene expression more obvious and may have also returned many more genes.

Genes identified in this project should be analyzed further for their involvement in the dwarfing phenomenon. To achieve this goal it is important to involve more graft combinations and test the correlation of gene expression to rootstock vigor. Consistency between gene expression and rootstock vigor should qualify these genes for markers in the screening of promising rootstock breeding trials. The regulation and the impact of polymorphisms (SNPs, indels) in these genes should be studied further to allow more accurate screening in rootstock trials.

The presence of the CVA in the 'Bing'/Gi5 combinations was initially a concern, since viruses can have a significant effect in tree physiology. Tests presented in Chapter 3 reduced this possibility since the virus is also present in vigorous trees. Nevertheless,

presence of the virus may have affected the expression of some genes. Expression patterns similar to the CVA profile were not observed in the cDNA-AFLP analysis or the microarray screening providing another proof that the virus is not affecting the physiology of the tree.


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