INTERVARIETAL CHIMERA FORMATION BY GRAFTING IN CITRUS

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To myself
To the wonderful life

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LIST OF ABBREVIATIONS

BAP	6-Benzylaminopurine
DHS	Direction-Hormone-Slowlygrowing
FSS	Formamide: size standard
HLB	Huanglongbing
HPLC	High performance liquid chromatography
NAA	Naphthaleneacetic acid
PCR	Polymerase chain reaction
RAPD	Randomly amplified polymorphic DNA
UF-CREC	University of Florida Citrus Research and Education Center

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The Florida citrus industry plays a significant role in Florida's economy. Most of Florida citrus fruit (90%) is used for the juice or concentrate market, with the remainder (10%) sold as fresh fruit. Sweet orange and grapefruit are the major fruit grown in Florida. However, the rapid spread of citrus canker and Huanglongbing (HLB) throughout the citrus production regions of Florida has caused a serious problem to local citrus industries. The current methods for controlling citrus canker and HLB have been unable to stop the diseases' spread. Developing and releasing new citrus cultivars which are tolerant or resistant to citrus canker or HLB is highly desirable to maintain a healthy citrus industry in Florida. Also, developing new citrus cultivars with distinctive and novel traits, like easy peeling grapefruit or dark red pigmented mandarins, would better enable Florida growers to compete in national and international fresh fruit markets. Citrus species are highly heterozygous and have long juvenility; using traditional methods to develop new cultivars always takes a long time, and desirable cultivars are difficult to obtain. Therefore, finding new approaches for citrus scion cultivar breeding can be important in developing a viable method to combat citrus canker and HLB diseases, and developing new cultivars with novel traits. Graft chimera generation would lead to the development of unique phenotypes that could have significant potential value to the Florida industry. To have a better

understanding of how to synthesize chimera and what factors affect the efficiency of chimera formation, we: 1) evaluated the effect of two improved grafting methods (Improved Ohtsu's and improved Winkler's methods) on adventitious shoots and chimeric shoots formation; 2) investigated whether intervarietal citrus chimeras could be synthesized from a range of citrus cultivars by grafting. We found that seedling quality dictated the selection of graft methods in a combination, and that Ohtsu's grafting method is suitable for all of combinations.

We evaluated both improved grafting methods with ten different graft combinations using various cultivars of grapefruit, mandarin, kumquat, and blood orange, on adventitious and chimeric shoot formation. We used Simple Sequence Repeat (SSR) markers to detect chimerism of adventitious shoots. Eight putative chimeric shoots were regenerated from the combination of 'White' mandarin + 'Moro' blood orange using the improved Ohtsu's grafting method; no chimeric shoots were regenerated from any of the other combinations which revealed that graft partners affected the formation of chimera. Winkler's grafting method regenerated more adventitious shoots than the improved Ohtsu's grafting method in one combination, but no chimeric shoots were regenerated by improved Winkler's grafting method. More adventitious shoot regeneration does not necessarily result in a higher rate of chimeric shoot occurrence, only that adventitious shoot regenerating from the region of the graft union may lead to high rate of chimeric shoot formation. Our results have shown that only the improved Ohtsu's grafting method could successfully to regenerate chimeric shoots from one year old grafted seedlings.

CHAPTER 1
INTRODUCTION

Citrus is one of the world's most important fruit crops grown in tropical and subtropical climates in 140 countries (FAOSTAT, 2007). Citrus has the highest international trade value among all fruits (Norberg, 2008; UNCTAD, 2009) and citrus production in Florida accounted for 59 percent of total United States citrus production during the 2013-2014 season, far more than that of California (37%), Texas and Arizona (4% combined) (USDA-NASS, 2014). From an economic point of view, the citrus industry in Florida contributes to the bulk of Florida's financial revenue, and it directly and indirectly generated a total of 62,133 full or part time jobs in citrus fruit production, juice manufacturing, and fruit packing with total industry output impacts of $10.68 billion during the 2012-13 production season (FRED IFAS, 2014).

Compared to California where citrus fruit is mainly produced for fresh consumption (Geisseler and Horwath, 2014), Florida produces citrus fruit 90% of which is primarily destined for the juice or concentrate market, with the remainder 10% sold as fresh fruit (FASS, 2012). Sweet orange and grapefruit are the major fruits grown in Florida. Sweet oranges dominate Florida's citrus production (83.6%) with more than 95 percent of this crop being processed into orange juice, accounting for nearly all of the United States orange juice production. Florida is the largest US grapefruit producer which commands about two-thirds of the total US grapefruit production and about 30 percent of the world's grapefruit (USDA-NASS, 2010), and grapefruit ranks number 2 accounting for 12.6 percent of total Florida citrus production. Grapefruit is grown predominately for fresh market; however, a significant portion of this crop is processed for grapefruit juice as well due mainly to poor appearance, rind blemishes caused by citrus rust mites, scab, etc. Tangerines and tangelos are the specialty fresh fruit which account for a small portion of Florida citrus production (National Research Council, 2010).

Although Florida is the largest citrus producing state in the United States, Florida fresh citrus fruit production just makes a small portion of total Florida citrus production, only 5 percent of Florida orange crop and 50 percent of grapefruit crop go to the fresh market (USDA-NASS, 2010). Generally, very little grapefruit is produced with the intention of it being processed, but since approximately 35-40 percent of fruit fails to meet the fresh fruit quality standards (Mossler, 2011), these rejected fruit are usually processed. The production and consumption of fresh mandarin fruits have been continuously increasing in the United States and globally (Baldwin and Jones, 2013; Ladaniya, 2008). From 2012 to 2015, the annual US mandarin production and value of production increased from 682,000 tons and 426,101,000 dollars to 843,000 tons and 513, 932,000 dollars respectively, and fresh fruit utilization accounted for 84.7 % of production in 2014-2015 season; during the same time period production and value of oranges and grapefruit both decreased under threat of citrus greening. In 2014-2015 season, mandarin utilization of production in Florida and California, the two major areas, were 2,270,000 boxes and 18,200,000 boxes respectively (USDA-NASS, 2015). However, the growing area and production of mandarin fruit has been increasing in California in the past 10 years, but decreasing in Florida because of the lack of varieties suited to the environment that can meet consumer expectations (Yu, 2015). Unlike processing fruit, rind color and other characteristics such as easy to peel, seedlessness, and attractive appearance are more crucial to the fresh fruit market since perfect cosmetic appeal attracts consumers' attention more readily. To pass fresh fruit quality tests, greater pest management inputs are required for fresh citrus fruit production (Mossler, 2011). However, the rapid spread of citrus canker and Huanglongbing (HLB) (or citrus greening) throughout the citrus production regions of Florida has caused most

citrus to fail the fresh fruit quality test, and also caused a serious setback to local citrus processing industries.

Citrus canker, caused by the bacterium *Xanthomonas citri* subsp. *Citri*, is a blemishing disease that affects most citrus' fruits, attacking the plants' leaves and stems. Grapefruit are highly susceptible to canker and the fruit lesions occur frequently and with varying sizes due to the fruit having a longer growth phase with the end result of several infection cycles potentially being present on one individual grapefruit (Dewdney and Graham, 2016). Many management methods have been used to control canker, such as diseased tree removal, planting of windbreaks, leafminer control, and copper sprays (Graham and Leite, 2004; Leite and Mohan, 1990; Dewdney and Graham, 2016). However, each of the aforementioned methods have their own limitations in mitigating canker, tree removal is only effective when canker is localized and limited to a small number of trees. Windbreaks are effective for reducing the spread of canker but cannot stop the disease. Since weather and plant growth rate affect effectiveness of chemicals, and chemicals only reach the surfaces of leaves and fruits, several spray periods are needed to achieve the best effect (Dewdney and Graham, 2016; Leite and Mohan, 1990). Current methods also can be difficult to control canker on these susceptible citrus, like grapefruit and early season orange varieties.

HLB, caused by the bacterium *Candidatus* Liberibacter asiaticus (Las), is abacterial disease affecting all citrus species and varieties. HLB is spread by the Asian citrus psyllid (*Diaphorina citri* Kuwayama). When the psyllid feeds on bacteria-infected plants' new shoots and leaves, the psyllid ingests the bacteria, and transfers it to a healthy plant during the feeding process. Afflicted trees' fruits are often small, few in number, fail to color properly, and are prone to drop prematurely (Grafton-Cardwell and Daugherty, 2013). Currently, the methods for

controlling HLB are chemical control of the psyllid vector, removal of infected trees, use of disease-free nursery materials (Graça and Korsten, 2004; Bove, 2006) and use of nutrient therapy on existing infected trees (Giles, 2011; Spann et al., 2011); however, the aforementioned ways are not always effective.

Facing the rapid spread of canker and HLB in Florida, developing and releasing new citrus cultivars which can be tolerant or resistant to HLB or canker is certainly critical to maintain a healthy citrus industry in Florida. However, developing new citrus cultivars with distinctive and novel traits, like easy-peeling grapefruit or dark red pigmented mandarins, could also be important for Florida's industry, to attract consumers and compete in the national and international fresh fruit markets.

Conventional breeding method to make new citrus cultivars is challenging. First, citrus is highly heterozygous, with sexual hybridization creating a range of new genotypes with substantial variation of characters (Spiegel-Roy and Goldschmidt, 1996). Second, many citrus, like sweet oranges, are polyembryonic; crosses between closely related lines will produce very few, if any, zygotic seedlings, and these generally exhibit inbreeding depression. Most seedlings from such crosses arise from nucellar embryos and essentially resemble the maternal parent plant genetically and phenotypically. Last but not least, citrus has long juvenility; it takes 3 or more years for the first flowering to occur in citrus seedlings which makes citrus breeding not only a difficult but a costly program (Gmitter et al., 2009). Finding new approaches for citrus scion cultivar breeding can be important in developing a viable method to combat citrus canker and HLB diseases, and developing new cultivars with novel traits. Graft chimera generation would lead to the development of unique phenotypes that could have significant potential value to the Florida industry.

CHAPTER 2
LITERATURE REVIEW

What is Plant Chimera?

Schmidt (1924) proposed the 'tunica-corpus' theory to describe that distinct cell layers (or regions) exist in shoot apical meristem of plants and these cell layers finally develop into organs of the plants. Usually, most dicots have three distinct apical cell layers (LI, LII, and LIII), monocots have two or three layers in the shoot apex, and these layers remain independent from each other (Burge et al., 2002; Doring et al., 1999). In citrus fruit, the cells of the outermost layer (LI) produce the juice sac in fruit segments and the epidermis of the pericarp. The second apical layer (LII) forms the seed, segment wall, and the hypoderm and the mesocarp of pericarp, while the third layer (LIII) gives rise to the vascular bundle (Bartholomew and Reed, 1948; Frost and Krug, 1942; Ohtsu and Kuhara, 1994).

According to the theory of 'tunica-corpus', plant chimeras develop from these genetically different cells which exist in the layers of the shoot apical meristem that finally form organs of the plant. There are three types of chimeras based on position of the genetically distinct cells lying within these cell layers – sectorial, mericlinal and periclinal chimeras (Marcotrigiano, 1997). Burge et al. (2002) interpreted these three kinds of chimeras by using a figure below (Fig. 2-1) - sectorial chimeras have a sector of all cell layers that is genetically different (Fig. 2-1-A); periclinal chimeras have one or more entire cell layer(s) which is genetically distinct from another cell layer (Fig. 2-1-B); mericlinal chimeras have part of one or more layers that is genetically different (Fig. 2-1-C). Since periclinal chimeras develop from entire cell layer(s), they are the most stable form and can be multiplied by vegetative propagation. Some mericlinal and sectorial chimeras have a chance to give rise to stable periclinal chimeras eventually (Winkler, 1907; Burge et al., 2002).

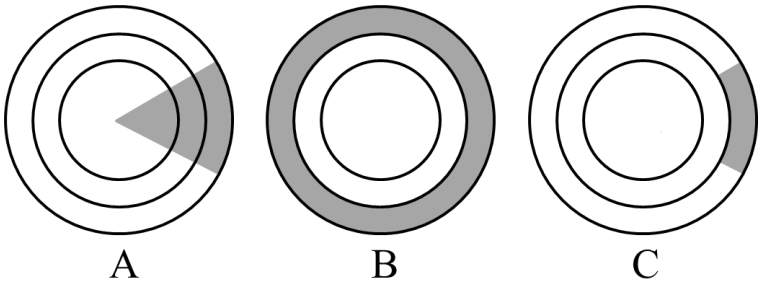

Figure 2-1. Cross sections through the shoot apical meristem showing the three cell layers (LI, LII, and LIII) for three types of chimeras. A) Sectorial chimera, B) Periclinal chimera, C) Mericlinal chimera (Burge et al., 2002).

Many commercially important cultivars are chimeras. Most of these chimeras have arisen spontaneously and have been reported previously, such as 'Hongjiangcheng' (*C. sinensis* + *C. reticulata*) (Shen et al., 1998), 'Gouheju' (*C. reticulata* Blanco + *C. aurantium* Linn.) (Shen et al., 1998), 'Zhihelu' [*C. reticulata* + *Poncirus trifoliata* (L.) Raf.] (Wu et al., 2004), 'Zaohong' navel orange [*Citrus sinensis* (L.) Osbeck + *C. unshiu* Marc.] (Zhang et al., 2007), Kobayashi Mikan (*C. unshiu* + *C. natsudaidai* Hayata) (Tanaka, 1980), Kinkoji Unshiu (*C. unshiu* + *C. obovoidea* hort. Ex Takahashi) (Tanaka, 1980), and 'Hongrou Taoye' [*Citrus sinensis* (L.) Osbeck + *Citrus unshiu* Marc.] (Zhang, et al., 2015). These chimeras all are naturally occurring graft chimeras due to accidentally being developed from the junction of scion and rootstock. Graft chimeras were also created artificially in lab. Japanese scientists synthesized periclinal chimera composed of 'Kawano Natsudaidai' (*C. natsudaidai* Hayata) and 'Fukuhara' sweet orange (*C. sinensis*) (Kuhara, 1989; Ohtsu, 1994). Another synthetic periclinal chimera of Satsuma mandarin and 'Hamlin' sweet orange (*C. sinensis*) was reported later by Sugawara et al. (2002). Some pink and red-fleshed grapefruits have arisen by somatic changes in white-fleshed varieties or within the colored group itself and were considered as periclinal chimeras (Cameron et al., 1964). Chimeras produce distinctive and valuable phenotypes for plant breeders, thus creation of periclinal chimeras is a potential breeding method for citrus.

The Successful Examples of Synthetic Chimeras

Many chimeras were artificially produced by grafting. Winkler (1907) produced interspecific chimeras from graft partners, nightshade (*Solanum nigrum*) and tomato (*Lycopersicon esculentum*). Burbank (1914) grafted a twig of a purple-leaved plum onto an old Kelsey plum tree, and he believed that the seedlings cultivated from Kelsey plum tree's seed were the result of graft hybridization between purple-leaved plum and Kelsey plum. Shinoto (1955) claimed to have obtained graft hybrids of eggplants by grafting Kantoao (blue fruits) and Sinkuro (black fruits). There are similar positive results involving eggplant graft hybridization obtained by Zu and Zhao (1957), Stroun et al. (1963) and Rajki and Pal (1966). Also, many other scientists claimed they obtained positive results from grafted plants in cabbage, pepper, tobacco, citrus and other plants (Noguchi et al., 1992; Zhu et al., 2007; Tian and Marcotrigiano, 1993; Ohtsu, 1994; Hirata and Yagishita, 1986; Hirata et al., 2003; Taller et al., 1998, 1999; Yagishita and Hirata, 1986, 1987; Yagishita et al., 1990). A wide range of plant characteristics has been introduced by chimeral breeding, such as flower color (Lindsay et al., 1995), flower size and morphology (Marcotrigiano, 1986), fragrance (Stewart et al., 1972), flowering (Binding et al., 1987), disease and pest resistance (Tingey and Laubengayer, 1981), and fertility of flowers (Binding et al., 1987).

New characters induced by grafting have been demonstrated as stable traits and can be used as novel genetic source material (Taller et al., 1999). Proof of concept results showed that a grafted pepper chimera had been stably inherited for at least 27 generations by seed propagation (Yagishita et al., 1990). Dole and Wilkins (1991) made auto and reciprocal grafts among different poinsettia cultivars, and his results showed that changes were retained after two generations of serial vegetative propagation and are considered permanent. All of the above

examples demonstrated that in certain cases, grafting is a powerful agent for causing the formation of new varieties (Neilson-Jones, 1969).

Methods of Artificial Synthesis of Chimera

Winkler (1907) grafted nightshade (*Solanum nigrum*) onto tomato (*Lycopersicon esculentum*) in an attempt to produce interspecific chimeras. After the graft union sealed, the junction was cut transversely. Many types of adventitious shoots were developed from the callus at the cut surface of both species, and some of these shoots which blended characters of both plants were interspecific chimeras. This method Winkler (1907) developed to create chimeras was used by many other scientists to produce chimeras (Jorgensen, 1927; Zu and Zhao, 1957; Goffreda et al., 1990; Hirata et al., 1990), with some producing graft chimeras and others failing to generate a viable chimera.

The formation of graft chimera requires the regeneration of adventitious shoots from the junction of two graft partners after it has been cut back; however, some species have a problem to produce adventitious shoots, such as potato (Jorgensen, 1927). Thus, improving shoot formation from graft unions may greatly increase the success of graft chimera development. Attempts have been made to solve this problem, such as improving grafting methods (Ohtsu, 1994), or using *in vitro* graft-culture techniques (Hirata et al., 1990; Noguchi et al., 1992).

Ohtsu (1994) used an improved method named Direction-Hormone-Slowlygrowing (DHS) method to develop a disease resistant citrus chimera. He used young nucellar seedlings (7-9 cm) which were grown in darkness in his experiments, and grafted them together at the hypocotyl. This graft method was used to graft two species together which was different from Winkler's (1907) method that grafted one partner (scion) onto another partner (rootstock). Ohtsu also made some change in how to cut back by cutting the hypocotyl of one species at an angle of 60° against the stem direction in order to increase the area of graft union, then treated the cut

surface of the hypocotyls with plant hormones. With this improved graft method, Ohtsu (1994) obtained one citrus periclinal chimera 'NF-5' (*Citrus natsudaidai* + *Citrus sinensis*) with desirable traits including resistance to citrus canker and citrus tristeza virus.

Noguchi et al. (1992) investigated an *in vitro* graft culture method which relies on the regeneration of adventitious shoots from cultured graft union tissues. Noguchi's lab sowed sterilized cabbage cultivars' seeds on MS basal medium, then grafted 7-day-old seedlings by a method named the approach-grafted hypocotyl culture method (AGSC). After the graft union, the graft site was cut into three parts (the fused apical part, a cross-cut section of the graft union, the united hypocotyl part), and subcultured in an improved MS medium containing cytokinins and auxins. Intervarietal chimeras were obtained by the subculture of cross-cut sections of the united part after the graft culture. Noguchi's lab concluded that the AGSC method they used was more effective than the usual Winkler's graft method and a simple mixed culture of heterogeneous cells or tissues. A similar conclusion was made by Hirata et al. (1990) who tried Winkler's graft method but failed, but succeeded using the *in vitro* graft culture method. Other approaches for producing synthetic chimeras, such as co-culture of pith slices (Carlson and Chaleff, 1974), mixed callus cultures (Marcotrigiano and Gouin, 1984) and co-culture of protoplasts (Binding et al., 1987), were used but these methods did not develop reliable synthetic chimera breeding techniques until now.

Efficiency of Synthetic Chimeras

Chimeral breeding currently is not a commonly used technique for horticultural crops due mainly to the inefficiency of chimera shoot formation. There are many publications about synthetic chimeras. Some scientists failed in their experiments, others claimed they obtained plant chimeras, but few researchers mentioned the efficiency of creating synthetic chimeras. Carlson and Chaleff (1974) used an *in vitro* mixed callus technique to generate chimeras and

obtained 28 chimeras (0.4%) from 7000 shoots. Ohta and Choung (1975a) grafted two red pepper cultivars together during their first experiment and only obtained few variants which accounted for 0.84% of total plants. In another set of experiments, Ohta and Choung (1975b) used the single-stranded RNA virus to infect stocks first, and found the rate of gene transfer increased dramatically from 2% for noninfected stocks to 16.5% for infected stocks. Marcotrigiano and Gouin (1984) produced three (1%) tobacco intraspecific chimeras from 294 adventitious shoots by using the mixed callus technique. Kaddoura and Mantell (1991) obtained five (3%) periclinal chimeras, 9 mericlinal and sectorial chimeras from 151 adventitious shoots by grafting *Nicotiana* and *Solanum* together. Tian and Marcotrigiano (1993) produced thirty-nine (9%) *Nicotiana tabacum* and *N. glauca* graft chimeras from 413 adventitious shoots. Lindsay et al. (1995) produced two (4%) interspecific chimeras from 52 adventitious shoots which regenerated from 10 graft unions between tomato and nightshade.

There are many factors that contribute to the rate of chimera formation. The grafting method is an important factor. Hirata et al. (1990) did not obtain stable intervarietal *Brassica oleracea* chimeras by using Winkler's graft method, but they produced chimeras subsequently by using *in vitro* graft culture methods. Ohtsu (1994) developed a much more efficient way (an improved DHS method) to produce synthetic periclinal chimeras of citrus; this allowed him to increase the rate of periclinal chimera formation from 1/205-1/600 to 1/7-1/21. Fuentes et al. (2014) showed that the formation of resistant calli from the graft sites was observed frequently (29 independent events selected from 13 grafts) by using transgenes plus improved *in vitro* graft culture methods. If desired results can be achieved without the use of transgenes, breeders can develop more graft chimeras reliably; however, the operator's skills must also be considered towards successful chimera production. For example, Zu and Zhao (1957) failed in their

experiment by using Winkler's graft method at first, but after improving their grafting skill, they obtained graft hybrids.

Graft species used in the process may influence the regeneration of shoots at a graft union. According to Lindsay et al. (1995), when nightshade was grafted onto tomato, only nightshade and chimera shoots were generated from the junction. Nightshade itself usually does not form regenerated shoots when a stem is cut back. Based on the information, reciprocal graft (A grafts onto B, or B graft onto A) may also influence the regeneration of shoots, with such questions as which should be the scion, which should be the rootstock that need to be addressed when using Winkler's graft methods. Graft chimeras are easier to produce when graft partners are between species, such as between *Brassica* species (Hirata et al., 1992; Noguchi et al., 1992) and between *Brassica oleracea* varieties (Hirata et al., 1990).

Seedling quality may be another one of the reasons that cause low percentage of chimera to form between graft partners. Michurin (1949) tried to introduce foreign materials' genes into a Russian fruit tree, but he failed due to using an adult tree. He finally figured out only seeds and young seedlings at the cotyledonary stage or at a younger stage are susceptible to changes. The younger the plant is, the more successful is the experiment. There are many other recorded negative results obtained by scientists on chimera formation. Sachs (1951) did not observe leaf-shape and fruit-color change in either the year of grafting or in the following generation after grafting different tomato cultivars. Stubbe (1954) did not produce tomato chimeras by grafting tomato mutants, and Kraevoi (1971) also claimed that he failed to obtain graft chimeras in his potato and tomato experiment. Thus, improving the efficiency of chimera shoot formation is essential for chimera breeding to become a standard breeding technique for horticultural crops.

Techniques to Identify Chimeras

A wide range of techniques, some of which are discussed in more detail below, have been used to identify synthetic chimera shoots, but their applicability mostly depends on the morphological and cytological differences between genotypes.

Phenotypic Evidence

Leaf size, shape and color can be used to differentiate between chimeras and donor plants (Neilson-Jones, 1969; Stewart et al., 1972; Binding et al., 1987; Hirata et al., 1990; Lindsay et al., 1995). Stewart et al. (1972) used stomatal size to provide evidence of the origin of the L1 layer in an interspecific *Camellia* chimera. Zhou et al. (2002) investigated stomatal morphology of periclinal chimera 'NFF' (layer constitution: LI-LII-LIII=NFF), 'FNN' (layer constitution: LI-LII-LIII=FNN) and their donor plants (Kawano natsudaidai 'NNN' and Fukuhara orange 'FFF'); they observed there were a significant difference in the length and width of the stomata between N layer and F layer. Hirata et al. (1990) used two *Brassica oleracea* varieties with different colored leaves to assist the identification of chimeras; chimeral shoots were easy to identify due to their green and purple stripes or segments. Fuentes et al. (2014) observed the differences in cell size between donor plants and chimeras.

Cytological Techniques

If nuclear transfer had taken place between grafting ancestors, the number of chromosomes in chimeras should be more than graft partners, and have different ploidy levels in the apical layers. Fuentes et al. (2014) revealed the allopolyploid status of chimeral plants by karyotype analysis, so observations of chromosome numbers can be used to determine the ploidy level of the layers in chimeras (Stewart et al., 1972; Dermen and Stewart, 1973).

Pollen and Embryos of L2 Origin

Marcotrigiano and Bernatzky (1995) indicated that gametes are usually produced by the L2 layer, which means pollen characteristics (pollen size, exine architecture) can be used to determine the L2 genotype. Stewart et al. (1972) showed differences in pollen types between the species of *Camellia* and the pollen from the chimera, which provided evidence that the L2 layer was not from the species that produced the epidermis; however, not all gametes are from the L2 layer (Burk et al., 1964; Stewart and Burk, 1970; Marcotriagiano and Bernatzky, 1995). Marcotriagiano and Bernatzky (1995) found that pollen and eggs were sometimes derived from L1 or L3 layers in an interspecific chimera between *Nicotiana tabacum* and *N. glauca*. These results suggest that histogen-derived tissue specificity is not absolute. Fuentes et al. (2014) found meiotic chromosome missegregation in pollen mother cells of autopolyploid tobacco chimeras.

Flow Cytometry

If nuclear transfer had taken place, the nuclear DNA content of chimera can be different from graft partners. Lindsay et al. (1995) compared *G*0/*G*1 and *G*2 channel numbers of nightshade and the chimera, with results showing that the relative number of nightshade nuclei was much lower in the chimera with only an L1 layer of nightshade (NTT) than in a chimera with L1 plus L2 layers of nightshade (NNT), which can be an effective method to provide evidence of an interspecific chimera. Fuentes et al. (2014) checked nuclear DNA content of the tetraploid chimeras by flow cytometry and found the genome size for tetraploid chimera equalled the sum of the genome sizes of graft partner A and graft partner B.

Isozyme Analysis

Isozyme analysis was used to investigate the histogenic structure of leaf tissues and the interactions between different genotypic tissues in citrus graft chimeras (Yamashita, 1983; Zhou et al., 2002). Zhou et al. (2002) reported results that revealed that each donor plant (*Citrus*

sinensis cv. Fukuhara orange (F) and *C. natsudaiddai cv*. Kawano natsudaidai (N)) had one unique band by peroxidase analysis, the N-peroxidase band was stably detected in both chimeras (citrus chimeras NFF (layer constitution: LI-LII-LIII=NFF) and FNN (layer constitution LI-LII-LIII=FNN)), and this band could thus be used as a reliable marker of N tissue in chimeric plants, but they didn't detect chimera specific isozyme band.

High Performance Liquid Chromatography (HPLC) Analyses

High performance liquid chromatography (HPLC) analysis of flavanone glycosides were used to investigate the histogenic structure of leaf, bark and/or fruit tissues in naturally occurring and/or artificially synthetic citrus periclinal chimeras (Ohtsu and Kuhara, 1994; Ohtsu, 1994; Zhang et al., 2007). The different layers' tissues from chimeras and donor plants had different amount of neohesperidin, naringin, hesperidin and narirutin which could be a useful way for identifying the variety of citrus tissues from layer I, II and III.

Molecular Markers

A wide range of markers have been developed by molecular biologists and some of them have been used in chimera breeding to help identify chimera shoots; for example, PCR genomic based markers were used to determine a genotype was present in a chimeral plant or genome transfer had taken place (Chen et al., 2006; Zhu et al., 2007; Fuentes et al., 2014). If using *in vitro* chimeral breeding techniques to produce intervarietal chimeras where there are few morphological differences between the genotypes, transgene markers can help identify chimeras easily; for example, if one genotype used to produce a chimera had the β-glucuronidase (GUS) or a green fluorescent protein (GFP) gene, allowing for fluorescent shoots to be rapidly screened in the leaf or stem tissue. The staining/fluorescence pattern would signify which layer was occupied by each genotype (Christou, 1990). Randomly amplified polymorphic DNA (RAPD) analysis were used to investigate the histogenic structure of leaf and/or fruit tissues in naturally occurring

and/or artificially synthetic citrus periclinal chimeras (Sugawara et al., 1995; Sugawara et al., 2002; Zhou et al., 2002). Primers that produced consistent and repeatable bands that differed between the parental cultivars were chosen to create discriminating band patterns (Sugawara et al., 1995). RAPD analysis showed that chimera has specific bands beside the donor plants' specific bands which suggests that interactions occurred between genotypically different cells (Zhou et al., 2002). Specific bands may exist in most of citrus chimeras could be an unique characteristic for using to identify citrus chimeras when using molecular markers. However, if the yield of DNA from each layer is low, RAPD primers may be failed to reveal bands derived from the tested plants in some case (Sugavara et al., 1995). RAPD analysis also has another limitation, RAPD band patterns cannot differentiate between a chimera and a hybrid when the template DNA is extracted from a mixture of cell layers (Sugavara et al., 1995). Other more robust DNA markers have been developed since these reports on citrus chimera characterization, such as citrus sequence derived EST-SSRs (Chen et al., 2006). However until now, such markers have not yet been utilized for this purpose.

Rationale and Objectives

Previous research revealed that periclinal chimeras have a great diversity of characteristics, and these new characteristics can be introduced from related species or cultivars. This suggests that synthetic chimera breeding could be an important method of developing new phenotypes and speed up the process of breeding. Naturally occurring chimeras have been produced with plants from a wide range of families; however, synthetic chimeras have only been developed between cultivars or species in a few families, such as Solanaceae, Cruciferae and Rutaceae, with more research required to improve our existing techniques.

As previously mentioned, various scientists used the same methods to generate chimeras and some of them obtained chimeras whereas others did not. This reveals that grafting methods

may affect the regeneration of adventitious shoots and chimeric shoots. Currently, two grafting methods were used most for synthesizing chimeras, one is Winkler's grafting method, another is Othsu's grafting method (also named Direction-Hormone-Slowlygrowing method); thus, our first objective was to evaluate the effect of two improved grafting methods on adventitious shoot and chimeric shoot formation.

Synthetic citrus periclinal chimeras were produced from combination of sweet orange and Natsudaidai (Ohtsu, 1994), but no reports were documented about citrus chimeras being synthesized between other citrus species, such as between grapefruit and kumquat. Therefore, our second objective was to investigate whether intervarietal citrus chimeras could be synthesized from a range of citrus cultivars by grafting.

CHAPTER 3
THE EFFECT OF GRAFTING METHODS AND DONOR PLANTS ON SHOOT REGENERATION AND CHIMERA FORMATION

Plant Materials

Eight donor cultivars, 'Ruby Red' grapefruit, 'Duncan' grapefruit, 'Hudson' grapefruit, 'Moro' blood orange, 'White' mandarin, '900' mandarin hybrid, 'Nagami' kumquat and 'Meiwa' kumquat, were used in this experiment. These cultivars were selected as experimental materials because they represent a diverse set of characteristics, including the easy peeling attribute as observed in 'White' mandarin, anthocyanin pigmentation characteristic seen in 'Moro' blood orange, and the canker resistance characteristic of kumquats. With the exception of the 'Moro' blood orange that was grown in the Florida Citrus Arboretum located at Winter Haven, all other citrus cultivars were grown in the University of Florida Citrus Research and Education Center (UF-CREC) groves with similar field soil, illumination, and irrigation and fertilization management.

Fruits with uniform size and color were randomly hand harvested in the middle of December 2014. Seeds were extracted from fruits and were sown in soilless potting mix after removing seed coats. All seeds were placed in growth chambers in darkness until seedlings reached 9-11 cm in height before grafting. Growth chambers were set to maintain 27/26 ℃ for 12h/12h day/night cycles. Seedlings were watered once or twice per week depending on the soil moisture.

Grafting Methods

Two grafting methods were used in our experiments. The first method was an improved Direction-Hormone-Slowlygrowing (DHS) method which was used by Ohtsu's (1994) experiments. In our experiment, some improvements were made, including increasing the length

of the graft part to 3-4 cm, treating with plant growth regulators on both cut surface of hypocotyls and stem. The method is described in the picture below:

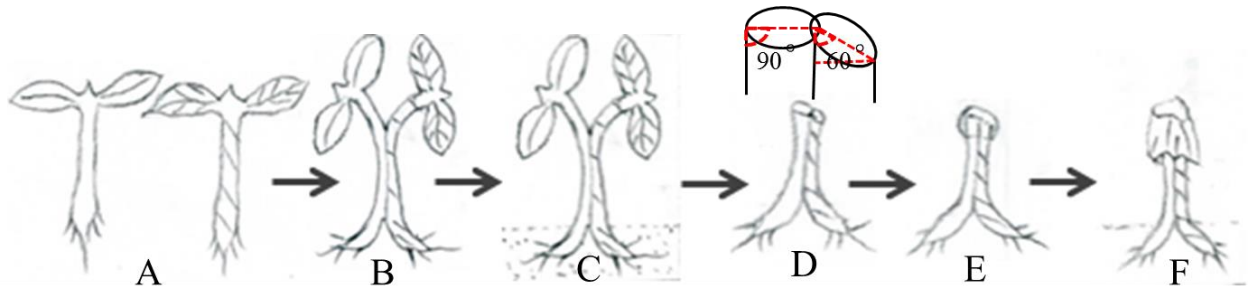

Figure 3-1. Procedure for chimera production by method 1- Improved Ohtsu's grafting method. A) Prepare 9-11cm tall seedlings (Sow seeds in soil, and grow in growth chamber at 27/26 ℃, 12h/12h day/night, in darkness for about one month), B) Graft donor plants at hypocotyls, then use parafilm to band stem segments together, C) Grow in light until callus formation, D) Cut hypocotyls at angles of 90 °and 60 °for each plant against the stem direction, E) Apply plant growth regulators on the cut side of hypocotyls and stem, F) Cover treated cut surface with parafilm.

Figure 3-2. Photos demonstrating chimera production by method 1- Improved Ohtsu's grafting method. A) Grafted donor plants at hypocotyls, then used parafilm to band stem segments together, B) Grew in light until callus formation, C) Cut hypocotyls at angles of 90 °and 60 °for each plant against the stem direction, then applied plant growth regulators on the cut side of hypocotyls and stem, D) Covered treated cut surface with parafilm, E) Adventitious shoots regenerated from cutting surface, removed parafilm from stem segments, F) Candidate chimeric shoot regenerated from conjunction part.

The second method is an improved Winkler's grafting method based on the method Winkler (1907) developed for producing interspecific chimeras between nightshade (*Solanum*

nigrum) and tomato (*Lycopersicon esculentum*). The improvement was made in this method including increasing the length of scion grafted onto rootstock.

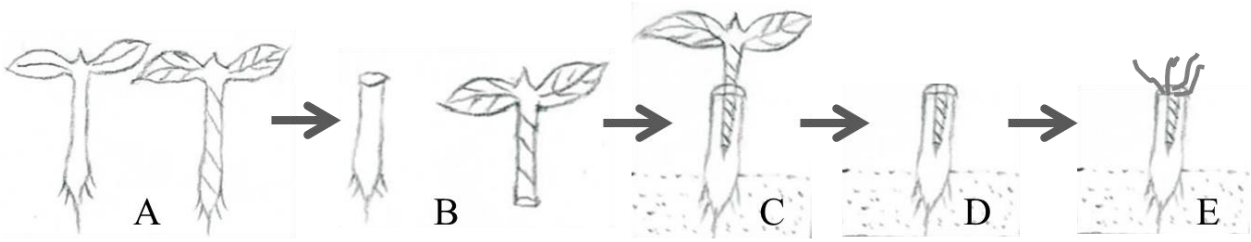

Figure 3-3. Procedure for chimera production by method 2- Improved Winkler's grafting method. A) Prepare seedlings (sow seeds in soil, and grow them in growth chamber at 27/26 ℃, 12h/12h, day/night, in darkness for about one month), B) Cut seedlings like preparing scion and rootstock, C) One graft partner is grafted onto another vertically, then use parafilm to band stem segments together, D) Cut junction transversely, apply plant growth regulators on the cut side of stem, cover treated cut surface with parafilm, E) Adventitious shoots regenerate from the cutting surface.

Figure 3-4. Photos demonstrating chimera production by method 2- Improved Winkler's grafting method. A) One graft partner was grafted onto another vertically, then used parafilm to band stem segments together, B) Grew in light until callus formation, C) Cut junction transversely, applied plant growth regulators on the cut side of stem, then covered treated cut surface with parafilm, D) Adventitious shoots regenerated from the cutting surface, E) Removed parafilm from stem segments.

All the seedlings were grafted by method 1 and method 2, were cut back 1.5 months after grafting. The cut surface of the hypocotyl was treated with 3mg/L 6-Benzylaminopurine (BAP) and 0.5mg/L 1-Naphthaleneacetic acid (NAA) based on the recipe Yu et al. (2002) used for citrus shoot regeneration. After 20 minutes, each treated hypocotyl was covered with parafilm

(Fig. 3-1-F, 3-3-D) and grown in the laboratory during the first month, then moved to the greenhouse.

Plant Graft Combinations

Ten graft combinations were set in our experiments as follows:

Graft combination one. Develop easy peeled, or canker resistant grapefruit cultivars:

‘Ruby Red’ grapefruit + ‘White’ mandarin (method 1:100 graft union; method 2:100 graft union)
‘Ruby Red’ grapefruit + ‘Meiwa’ kumquat (method 1:50 graft union; method 2:50 graft union)
‘Duncan’ grapefruit + ‘White’ mandarin (method 1:50 graft union; method 2:50 graft union)
‘Duncan’ grapefruit + ‘Meiwa’ kumquat (method 1:50 graft union; method 2:50 graft union)
‘Hudson’ grapefruit + ‘White’ mandarin (method 1:100 graft union; method 2:100 graft union)
‘Hudson’ grapefruit + ‘Meiwa’ kumquat (method 1:50 graft union; method 2:50 graft union)
‘Hudson’ grapefruit + ‘Nagami’ kumquat (method 1:30 graft union; method 2:50 graft union)

Graft combination two. Develop red pigmented or canker resistant mandarin cultivars:

‘White’ mandarin + ‘Moro’ blood orange (method 1:300 graft union; method 2:200 graft union)
‘900’ mandarin hybrid + ‘Moro’ blood orange (method 1:90 graft union; method 2:50 graft union)
‘900’ mandarin hybrid + ‘Meiwa’ kumquat (method 1:50 graft union; method 2:50 graft union)

‘Moro’ blood orange is the deepest pigmented blood orange, and the combinations with ‘Moro’ blood orange were all targeted for possible red pigmentation in those selections. ‘Meiwa’ and ‘Nagami’ kumquat both are canker resistant kumquats, anything with the kumquats was for canker resistance possibilities. ‘White’ mandarin has the easy peeling attribute, so the combinations with ‘White’ were designed to get easy peeling cultivars for the fresh fruit market.

Selection of Candidate Chimeras

Adventitious shoots that were regenerated on the cut surface of one donor plant and very near to the border of the two cultivars were selected as candidate chimera shoots. The donor plant mentioned above is the one which was designed to introduce new traits from the other graft

partner, such as the combination of ‘White’ mandarin + ‘Moro’ blood orange, we expected to introduce deep anthocyanin pigmentation trait to ‘White’ mandarin, and obtain red pigmentation mandarin chimeras.

DNA Analysis

Genomic DNA was extracted from fresh leaf tissues of candidate chimeras and donor plants using the CTAB-based method according to Aldrich and Cullis (1993). Applied Biosystems (ABI) 3130xl Genetic Analyzer and GeneMaker software were used for genotyping DNA samples. PCR and genotyping were repeated one to three times depending on whether the genotype fingerprints were clear or not.

The ABI 3130xl is a 16 capillary electrophoresis system that uses fluorescently labeled dyes for detection of DNA. There are four main steps required to genotype samples using the ABI 3130xl: 1) Polymerase Chain Reaction (PCR), 2) ABI plate setup, 3) run on ABI machine, and 4) DNA analysis using GeneMarker software (Chen et al., 2006). Currently, we use GeneMarker 2.4.0.

The first step: PCR setup for ABI using M13 Labeled Primers

	1 reaction	100 reactions
10x buffer	2.0 ul	200 ul
10xd NTP	1.0 ul	100 ul
25mM $MgCl_2$	0.8 ul	80 ul
Tag Polymerase (5 units/ul)	0.1 ul	10 ul
Primers (mixed F&R)	0.8 ul	80 ul
Dye	1.0 ul	100 ul
H_20	2.3 ul	230 ul
DNA template	2.0 ul	----
Total	10 ul	800 ul (dispense 8 ul per well)

We use M13 dye labeled primers (GTT GTA AAA CGA CGG CCA GT, designated M13, was added as a common tail to the 5' end of all SSR forward primers) in concentration of 1 uM. Eight primers, selected based on results presented by Chen et al. (2006), were used to reveal donor specific and/or chimera specific DNA markers and are listed in Table 1. All primers were ordered from Operon Technologies (Huntsville, AL, USA). Four fluorenscently labeled universal M13 primers, using 6FAM, VIC, NED, and PET, were synthesized by ABI (Applied Biosystems Inc., Foster City, CA, USA) and used for ABI G5 high throughput genotyping analysis on an ABI 3100 Genetic Analyzer (Chen et al., 2006). PCR conditions consisted of an initial denaturation at 94 ℃ for 3 min, followed by 10 cycles of denaturation at 94 °C for 30 s, annealing at 66 °C for 30 s with a 1 ℃ decrement each cycle, and extension at 72 °C for 45 s; this was followed by 30 additional cycles with a constant annealing temperature of 56 ℃ (other parameters were the same), plus a final extension at 72 ℃ for 30 min (Chen et al., 2006).

Table 3-1. Primers used to genotype donor plants and putative chimeras

Primer name	Primer sequence (5' to 3')
CX0010	F: GTTGTAAAACGACGGCCAGTAACCGAAGATGGAGGGAACT
	R: ACATTCATGGCCACATCTCA
CX0035	F: GTTGTAAAACGACGGCCAGTCCATTAACGAGAAAACCAAACA
	R: CAAAAAGGGGTTGCAAAGAA
CX2007	F: GTTGTAAAACGACGGCCAGTAAATCGGCTAGTTGCAAACG
	R: CCTTGACATTGTCGATGGTG
CX2021	F: GTTGTAAAACGACGGCCAGTAAGGTCATGTCTTTAGCACTTTGA
	R: CAAGTTGCCAATTCAGGAGG
CX5F57	F: GTTGTAAAACGACGGCCAGTCCTCGCCAATGACCTTTGTATTTA
	R: CAATACGTTTGGGTTCTAGTTCCG
CX6F04	F: GTTGTAAAACGACGGCCAGTAGTGAACTGTCCATTGGATTTTCG
	R: GTGTTGAATCCCGACCTTCTACC
CX6F06	F: GTTGTAAAACGACGGCCAGTTTCATTTGGAACAAAACCCAATTC
	R: GCTGCTAATCACAGCATCAAGAGA
CX6F07	F: GTTGTAAAACGACGGCCAGTCTGTTACCGTTGAGGAAACCAAAG
	R: CTCTTCAGCTGGTTTCTCTTCCTG
CX6F18	F: GTTGTAAAACGACGGCCAGTGTCTTCAACGAAGTTGCAGGCT
	R: TACTATTTCGAGAGAGCAGCAGCA

The second step: ABI plate setup

	1 reaction	100 reactions
PCR reaction*	4×0.8 ul *	----
Liz-500 Size Standard	0.25 ul	25 ul
Hi-Di Formamide	5.75 ul	575
Total	9.2 ul	600 ul (dispense 10 ul per well)

A size standard must be added to each PCR sample. The standard is labeled with an orange dye (LIZ) for dye set 'G5'. Prepare the formamide: size standard (FSS) mix on ice. Pipette 6.0 ul of the FSS mix into each well of new, empty ABI plate. Pipette 0.8 ul of each PCR sample from the PCR plate to the FSS mix in the ABI plate. Vortex and centrifuge all of samples. Place a rubber ABI septa on the plate, vortex, centrifuge and then denature at 95°C for 5 minutes. After denaturing, place on ice for at least 5 minutes in darkness and assemble plate into ABI plate sandwich. While the plate is cooling, create and import the plate record on the ABI computer.

The third step: Run ABI machine

Place the ABI plate sandwich on the autosampler carefully and check that all plates and reservoirs are fully seated and that all septa are in place, after which link the plates and run them.

The fourth step: Software analysis

Copy the run data to a portable USB key drive, and add the information into an analysis software package - GeneMarker software (SoftGenetics LLC., State College, PA, USA). Amplification from all four dyes will be shown on the computer screen.

Data Collection and Analysis

The diameter of seedlings' stem was measured using an electronic digital caliper before grafting (when seedlings reached 9-11cm in length) in each donor plant. The number of survived

seedlings after grafting, the number of survived graft seedlings after cutting back, the number of adventitious shoots from each graft plant, and the number of chimeral shoots from each combination were investigated. Data were analyzed using analysis of variance (ANOVA) and means were separated by Duncan Multiple Range Test at 95% confidence level (SAS version 9.1, SAS Institute Inc., NC, 2009).

Results

Effect of Seedling Quality of Cultivars on the Selection of Grafting Method

Statistical analysis revealed a significant difference in the stem diameter of grapefruit and other cultivars, with the stem diameter of grapefruit significantly larger than other donor plants (Table 3-2, 3-3). Grafting method 1- Improved Ohtsu's grafting method, could be used for any combination with grapefruit; however, Grafting method 2- Improved Winkler's grafting method, which requires a rootstock having a larger stem than scion part alone, or requires rootstock and scion to have similar size stem. Due to this condition, anything with grapefruit would require the rootstock would be grapefruit. The combination of 'Moro' blood orange and 'White' mandarin, since their stem diameters were not significantly different (Table 3-2) could be made in either combination.

Stem morphology of seedlings and statistical analysis both revealed the stem diameter of kumquats is significantly smaller than other cultivars (Table 3-2 and Fig. 3-5), kumquats would undoubtedly be the scion part in any graft combination using Improved Winkler's grafting method; however, due to the kumquat seedlings slower growth, kumquat seedlings face the problem that they grew up too slow compared to other partners when using Improved Ohtsu's grafting method in our experiments, as well.

Table 3-2. Seedling quality of donor plants – mean stem diameter

	'Ruby Red'	'Duncan'	'Hudson'	'Moro'	'White'	'900'	'Meiwa'	'Nagami'
Mean of seedling stem diameter* (mm)	1.35a	1.32a	1.33a	1.12b	1.10b	0.90c	0.77d	0.75d

Note: *Mean generated from 50 seedlings of each cultivar. Data was analyzed using analysis of variance (ANOVA) and means were separated by Duncan Multiple Range Test at 95% confidence level (SAS version 9.1, SAS Institute INc., NC, 2009).

Table 3-3. Results of analysis of variance (ANOVA) from SAS for data on seedling diameter of eight genotypes of citrus tested in a completely randomized design.

Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Model	56	22.98338100	0.41041753	29.63	<.001
Error	343	4.75058875	0.01385011		
Corrected Total	399	27.73396975			

Figure 3-5. The morphology of seedlings for grafting. A) 'Ruby Red' grapefruit, B) 'Duncan' grapefruit, C) 'Hudson' grapefruit, D) 'White' mandarin, E) 'Moro' blood orange, F) '900' mandarin hybrid, G) 'Meiwa' kumquat, H) 'Nagami' kumquat.

The Effect of Grafting Methods and Donor Plants on Adventitious Shoot and Chimera Formation

Grafting methods affected the formation of adventitious shoot and chimera. Donor plants grafted by improved Winkler's grafting method regenerated more adventitious shoots per graft union than improved Ohtsu's grafting method (Table 3-4). However, chimeric shoots were only regenerated from the combination of 'Moro' blood orange and 'White' mandarin by Improved Ohtsu's grafting method (Table 3-4).

The donor plants in each combination also affected the regeneration of adventitious shoot and chimeric shoot. For example, combinations with ‘Ruby Red’/ ‘Duncan’ grapefruit regenerated more adventitious shoots than combinations with ‘Hudson’ grapefruit. For combinations with ‘Ruby Red’ grapefruit, the combination of ‘Ruby Red’ grapefruit + ‘White’ mandarin regenerated more adventitious shoots than the combination of ‘Ruby Red’ grapefruit + ‘Meiwa’ kumquat (Table 3-4). Most of the combinations did not regenerate chimeric shoots except the combination of ‘Moro’ blood orange and ‘White’ mandarin (Table 3-4).

Chimera shoots were regenerated mainly from the cutting surface of the graft union near to the border of the two donor plants (Fig. 3-6-A), partially from the united hypocotyl part (Fig. 3-6-B, C). Most of chimera shoots grew slowly compared to other adventitious shoots.

Grafted seedlings were cut back to induce adventitious shoots from the cut surface. However, many grafted seedlings died at different growth stages (Fig. 3-7-A, B, C) after cutting back and treating with plant hormone that affected the percentage of survived graft union. As shown in Table 4, the lowest percentage of graft union formation (0%) was obtained from combinations with ‘Hudson’ grapefruit and combinations with ‘900’ mandarin hybrid, and the relatively high percentage of graft union formation was obtained from combinations with ‘Ruby Red’ grapefruit, combinations with ‘Duncan’ grapefruit, and combination of ‘Moro’ blood orange + ‘White’ mandarin.

Table 3-4. Effect of grafting methods and donor plants on adventitious shoots and chimera formation.

Combination	Grafting method	Total number of graft seedlings[a]	Number of survived graft unions[b] (%b/a)	Mean of adventitious shoot per graft union	Total number of adventitious shoots	Total number of shoots selected for SSR analysis	Total number of chimeral shoots
'Ruby Red'	a	100	73 (73)	1.95	142	36	0
grapefruit + 'White'	b	100	75 (75)	2.51	188	36	0
mandarin							
'Duncan' grapefruit	a	50	30 (60)	1.5	45	16	0
+ 'White' mandarin	b	50	26 (52)	3.81	99	17	0
'Hudson' grapefruit	a	100	7 (7)	1.43	10	0	0
+ 'White' mandarin	b	100	37 (37)	2.19	81	16	0
'Ruby Red'	a	50	18 (36)	1.39	25	0	0
grapefruit + 'Meiwa'	b	50	39 (78)	1.72	67	16	0
kumquat							
'Duncan' grapefruit	a	50	15 (30)	1.33	20	0	0
+ 'Meiwa' kumquat	b	50	40 (80)	2.68	107	16	0
'Hudson' grapefruit	a	50	0 (0)	0	0	0	0
+ 'Meiwa' kumquat	b	50	11 (22)	2.36	26	2	0
'Hudson' grapefruit	a	30	8 (26.7)	2	16	0	0
+ 'Nagami' kumquat	b	50	0 (0)	0	0	0	0
'White' mandarin +	a	200	117 (58.5)	1.74	204	24	8
'Moro' blood orange	b	200	29 (14.5)	2.28	66	6	0
'900' mandarin +	a	90	36 (40)	1.64	59	5	0
'Moro' blood orange	b	50	0 (0)	0	0	0	0
'900' mandarin +	a	50	0 (0)	0	0	0	0
'Meiwa' kumquat	b	50	0 (0)	0	0	0	0

Figure 3-6. Chimeric shoots from the combination of ‘White’ mandarin and ‘Moro’ blood orange. A) Putative chimeric shoot was regenerated from the cutting surface of the graft union near to the border of the two donor plants, B) and C) Chimeric shoot was regenerated from the united hypocotyl part.

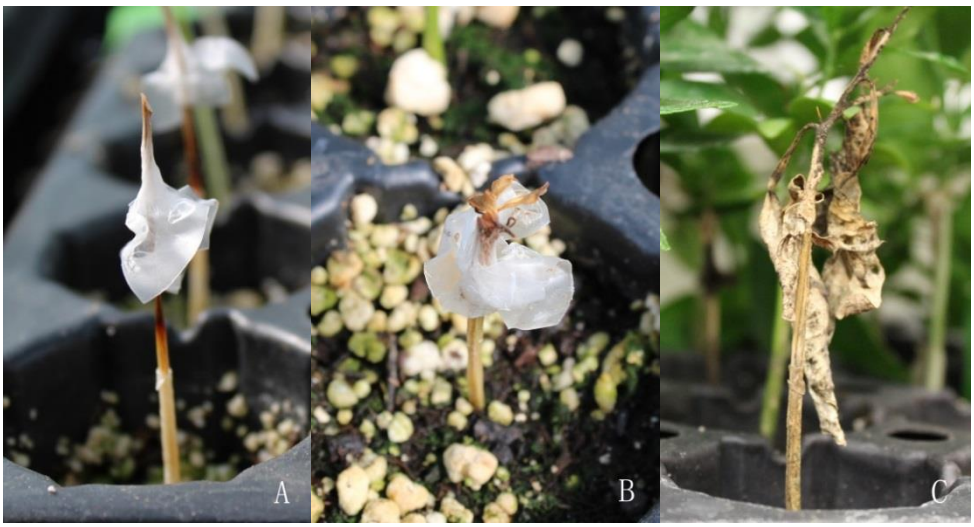

Figure 3-7. Graft seedlings died at different growth stages.

Primer Selection for Different Combinations

Nine primers were used to reveal single donor plant specific genotyping (Table 3-5). Primers selected for a specific combination to distinguish chimeras from donor plants are those that reveal specific alleles for both donor plants and chimeras. For example, with the primer CX0035, the specific alleles of sizes, 171.2 and 180.5, for ‘Moro’ blood orange and that of 157.7 and 171.3 (Table 3-5), unique for ‘White’ mandarin, were detected. If chimeras were generated

from the combination of ‘Moro’ blood orange and ‘White’ mandarin, the specific allele sizes of the chimera amplified with primer CX0035 should be 157.7, 171.2/171.3 and 180.5. However, taking the primer CX6F07 as a counter example, with the specific allele sizes for ‘Moro’ blood orange being 103.5 and 115.5, and for ‘White’ mandarin is 103.5 (Table 3-5); thus, the specific allele sizes for their chimeras should be 103.5 and 115.5 and therefore this primer fails to separate chimeras from ‘Moro’ blood orange with the same specific allele sizes. For the combination of ‘Ruby Red’ grapefruit and ‘White’ mandarin; however, primer CX6F07 could be used to reveal donor plants and chimeras.

For the combination of ‘White’ mandarin and ‘Moro’ blood orange, primers CX0035, CX2007, CX2021, CX6F04 and CX6F18 can be used to reveal chimeras (Table 3-6). For the combinations of ‘Ruby Red’/‘Duncan’ / ‘Hudson’ grapefruit and ‘White’ mandarin, primers CX0010, CX0035, CX6F07, CX5F57 and CX2021 can reveal chimeras effectively (Table 3-6). Primers CX0010, CX2021, CX5F57, CX6F06 and CX6F07 can be used to characterize donor plants- ‘Ruby Red’/‘Duncan’ / ‘Hudson’ grapefruit and ‘Meiwa’/‘Nagami’ kumquat, and reveal their chimeras (Table 3-6).

The alleles amplified using fluorescence labeled primers CX6F04, CX0035, CX2007, and CX6F07 revealed the difference between ‘Moro’ blood orange, ‘White’ mandarin and their chimeras (Table 3-7, Fig. 3-8).

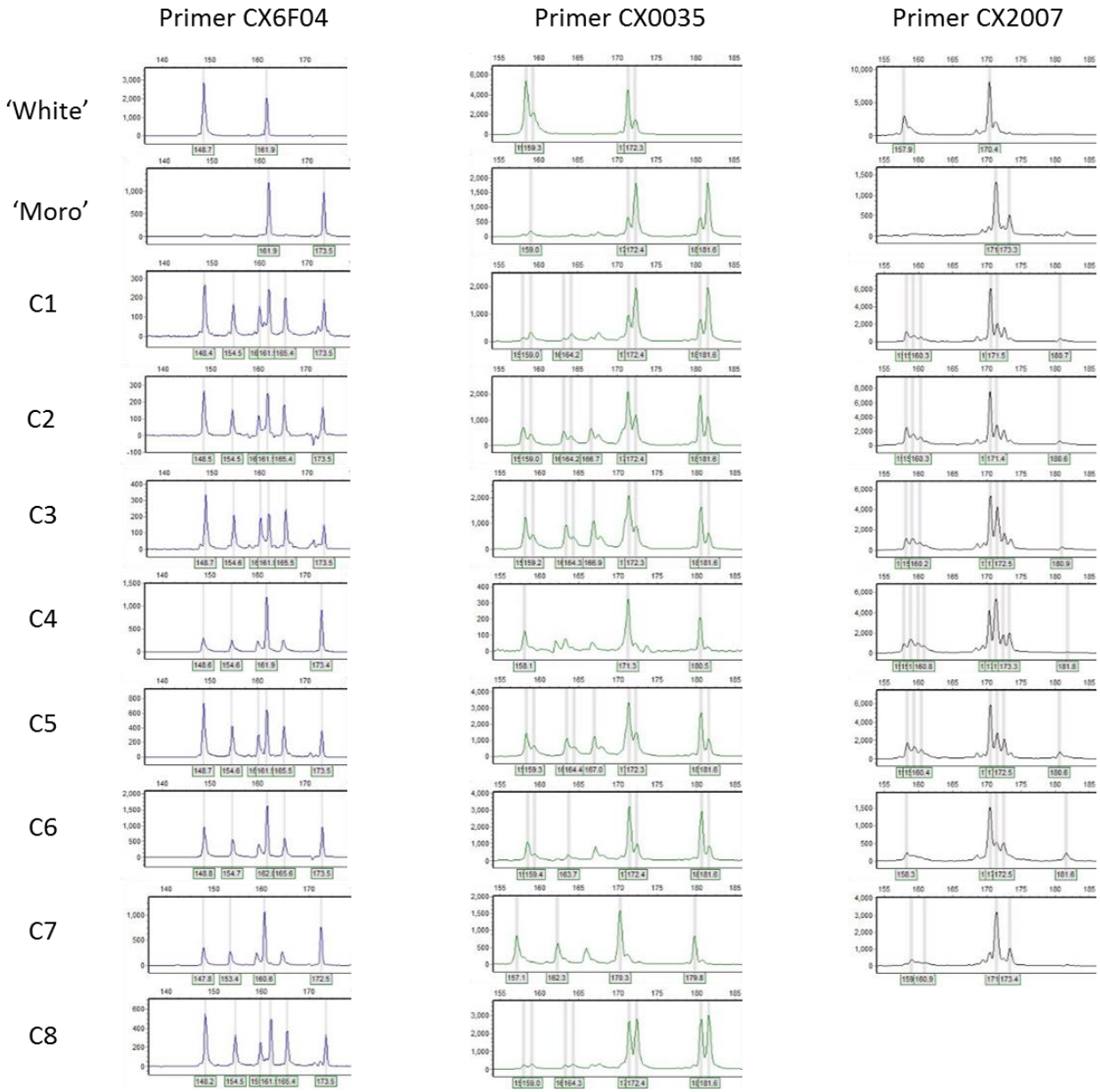

Figure 3-8. The alleles of donor plants and chimera observed after amplification with fluorescently labeled primers.

Table 3-5. Specific alleles at different loci of donor plants revealed by different primers

Primer name	Specific allele size															
	‘Ruby Red’		‘Duncan’		‘Hudson’		‘Moro’		‘White’		‘900’		‘Meiwa’		‘Nagami’	
CX0010	216.9	229.0	217.4	229.4	216.9	229.0	219.5	229.0	219.3	229.0	228.9	-	219.0	225.9	219.0	225.9
CX0035	180.7	181.7	180.7	181.7	180.7	181.7	171.2	180.5	157.7	171.3	171.4	172.5	180.7	181.7	180.7	181.7
CX2007	172.6	173.5	172.6	173.5	172.6	173.5	170.5	180.7	158.4	170.5	172.5	-	171.5	172.4	171.5	172.4
CX2021	149.5	152	149.5	152	149.5	152	147.8	149.7	147.8	152.0	147.3	152	149.6	-	149.6	-
CX5F57	151.0	166.8	150.9	166.7	151.0	166.8	154.3	166.4	155.9	166.3	150.6	166.3	156.0	-	156.0	-
CX6F04	149.0	162.1	149.0	162.1	149.0	162.1	161.8	173.3	148.4	161.8	153.9	162.1	155.9	162.1	155.9	162.1
CX6F06	168.7	171.7	168.7	171.7	168.7	171.7	168.7	171.6	168.7	-	168.7	-	168.7	-	168.7	-
CX6F07	115.8	-	115.5	-	115.8	-	103.5	115.5	103.5	-	103.5	115.5	103.5	115.5	103.5	115.5
CX6F18	154.0	159.4	154.0	159.4	154.0	159.4	154.0	159.4	142.3	153.8	154.1	-	160.5	-	160.5	-

Table 3-6. Primers that could be used to characterize donor plants and reveal chimeras

Combinations	Primer name
‘Ruby Red’/ ‘Duncan’ / ‘Hudson’ grapefruit + ‘White’ mandarin	CX0010, CX0035, CX2021, CX5F57, CX6F07
‘Ruby Red’/ ‘Duncan’ / ‘Hudson’ grapefruit + ‘Meiwa’/ ‘Nagami’ kumquat	CX0010, CX2021, CX5F57, CX6F06, CX6F07
‘White’ mandarin + ‘Moro’ blood orange	CX0035, CX2007, CX2021, CX6F04, CX6F18
‘900’ mandarin hybrid + ‘Moro’ blood orange	CX2007, CX2021, CX5F57, CX6F04
‘900’ mandarin hybrid + ‘Meiwa’ kumquat	CX0010, CX0035, CX5F57, CX6F06, CX6F07, CX6F18

Table 3-7. Allele size of donor plants and chimeras

Donor plant/Chimera	Allele size		
	Primer CX6F04	Primer CX0035	Primer CX2007
'White' mandarin	148.7/161.9	158.9/171.4	157.9/170.4
'Moro' orange	161.9/173.5	172.4/181.6	170.4/180.5
Chimera 1	148.4/154.5/161.9/165.4/173.5	159.0/164.2/172.4/181.6	157.9/170.4/180.7
Chimera 2	148.5/154.5/161.9/165.4/173.5	159.0/164.2/166.7/172.4/181.6	157.9/170.4/180.6
Chimera 3	148.7/154.6/161.9/165.5/173.5	159.2/164.3/166.9/172.3/181.6	157.9/170.4/180.9
Chimera 4	148.6/154.6/161.9/173.4	158.1/171.3/180.5	158.3/170.4/173.3/181.8
Chimera 5	148.7/154.6/161.9/165.5/173.5	159.3/164.4/167.0/172.3/180.7	157.8/170.4/180.6
Chimera 6	148.8/154.7/162.0/165.8/173.5	158.7/163.7/171.5/180.7	158.3/170.4/181.6
Chimera 7	147.8/153.4/160.6/172.5	157.1/162.3/170.3/179.8	159.0/171.2/173.4
Chimera 8	148.2/154.5/161.9//165.4/173.5	159.0/164.3/172.4/181.6	-

Discussion

In eight donor cultivars, grapefruit has the largest stem diameter (1.35 mm), kumquat has the smallest stem diameter (0.75 mm), mandarin and blood orange are in the middle of the aforementioned diameters. Improved Ohtsu's grafting method is suitable for all of combinations. For a combination, if the stem diameters of donor plants are significantly different, the larger one should be 'rootstock' and the smaller one should be the 'scion' when using improved Winkler's grafting method. However, for the combination of grapefruit and kumquat, no matter what method was used, kumquat seedlings faced the problem that they grew up too slowly compared to its partner. We observed that donor plants grafted with similar stem diameters grew better than donor plants grafted with significantly different stem diameters, thus we suggest that selecting seedlings of donor plants having similar stem diameter for grafting is preferred.

Most of reported naturally occurring citrus graft chimeras were regenerated from graft partners of sour/sweet/bitter orange and mandarin/Natsudaidai, such as 'Hongjiangcheng' (*C. sinensis* + *C. reticulata*) (Shen et al., 1998), 'Gouheju' (*C. reticulata* Blanco + *C. aurantium* Linn.) (Shen et al., 1998), 'Zaohong' navel orange [*Citrus sinensis* (L.) Osbeck + *C. unshiu* Marc.] (Zhang et al., 2007), Kobayashi Mikan (*C. unshiu* + *C. natsudaidai* Hayata) (Tanaka, 1980), and Kinkoji Unshiu (*C. unshiu* + *C. obovoidea* hort. Ex Takahashi) (Tanaka, 1980), 'Zhihelu' [*C. reticulata* + *Poncirus trifoliata* (L.) Raf.] (Wu et al., 2004), 'Hongrou Taoye' [*Citrus sinensis* (L.) Osbeck + *Citrus unshiu* Marc.] (Zhang, et al., 2015). Very few of naturally occurring graft chimeras were reported from other citrus graft partners. Synthetic periclinal chimeras were only developed from sweet orange and Natsudaidai. Japanese scientists synthesized periclinal chimera composed of 'Kawano Natsudaidai' (*C. natsudaidai* Hayata) and 'Fukuhara' sweet orange (*C. sinensis*) (Kuhara, 1989; Ohtsu, 1994). We only obtained chimeric shoots from the combination of 'Moro' blood orange + 'White' mandarin. It appears that donor

plants selected for grafting affects the regeneration of chimeric shoots. Previous research showed that it is easier to produce interspecific chimeras than intergeneric chimeras, and there are many successful interspecific chimera examples. Noguchi et al. (1989) were not able to obtain complete intergeneric chimera between tomato and eggplant combinations, for example. In our experiment, we did not obtain chimeras from any of the combinations with kumquat. This suggests chimera formation is related to the compatibility of coherent cells of two graft partners, and the compatibility of donor plants affects the efficiency of chimera formation.

Hirata et al. (1990) reported they were unable to obtain stable plant chimeras at high frequency using Winkler's graft method (1907), especially between distantly related species. Noguchi et al. (1992) pointed out that graft of the apical part (AGSC) is more effective than that of hypocotyls (AGHC: Noguchi et al., 1989). Their results show that grafting methods affected chimeric shoot regeneration. In our experiment, adventitious shoots were regenerated in each graft union by the improved Winkler's grafting method, more than improved Ohtsu's grafting method, but no chimeric shoots were regenerated by the former method. The rate of periclinal chimera formation in Ohtsu's experiment was 1/7 to 1/21. In our experiment, we obtained 204 adventitious shoots from 117 graft unions, but only 24 shoots regenerated from the conjunction part, and 8 of them were chimeric shoots (Table 3-4). The chimera formation rate was 8/204 which is much lower than Ohtsu's, though Ohtsu did not report how many adventitious shoots he obtained. Thus, more adventitious shoots regeneration does not mean more chimeric shoots developed, because inducing adventitious shoot regeneration from the junction is the key point affecting the efficiency of chimera formation. For one year old grafted citrus seedlings, our results showed that the improved Ohtsu's grafting method is better than improved Winkler's grafting method, but only for one combination, 'Moro' blood orange + 'White' mandarin.

However, there is no research on how grafting methods affect several years old grafted citrus seedlings. All of the naturally occurring graft citrus chimeras have arisen from the junction of scion and rootstock, suggesting that seedlings grafted by the improved Winkler's grafting method may regenerate chimeric shoots after cutting back and treating with hormones on the cut surface of the hypocotyl, no matter how old the graft seedlings are. Furthermore, if graft preparation is done in such a way that the two materials are symmetrically fit together, the graft union can be cut back every year until chimera formation.

Most of seedlings survived after grafting; however, after cutting back, some seedlings died at different growth stages. Many factors maybe contribute to this phenomenon. First, graft failures of unknown causes can occur between donor plants (Fig. 3-7-A), such as the combination of 'Hudson' grapefruit + 'White' mandarin. Second, greenhouse environment can affect young adventitious shoot development in their early stage (Fig. 3-7-B), parafilm should be removed immediately after grafted seedlings are moved to the greenhouse. Last but not least, fungi were associated with dead tissues (Fig. 3-7-C), and so grafting tools should be surface sterilized between seedlings to prevent any chances of transmission of fungi. Raising the grafted seedlings in a more clean growth chamber environment, for example, might overcome some of the challenges of growing these tender young plants in a normal greenhouse environment.

RAPD analysis showed the presence of citrus chimera specific bands besides the donor specific bands (Zhou et al., 2002). In our experiment, we observed similar phenomenon, there are some specific loci in chimeras besides the donor specific loci. With the primer CX6F04 for example, the allele loci 154.5 and 165.4, unique for chimera 1, 2, 3, 5, 6, 8 (Table 3-7, Fig. 3-8), were detected. This suggested that the interaction between different genotypes maybe led to genetic variation at the DNA level; however, nearly all of these fragment peaks are very small. It

is possible that some primer combinations generated these weak alleles when these chimeras' DNA were used in our PCR. Another possibility for the small peaks is the relatively low yield of DNA from chimeric leaves, because it was limited to only a very few of young leaves when we started to extract DNA from small chimeric shoots. To summarize, further investigations are required to confirm these contentious results.

Molecular analysis is a rapid and sensitive technique based on the polymerase chain reaction (PCR). To evaluate the ability of selected primers to detect cells derived from both parents in various tissues of a chimera, Sugawara et al. (1995) mixed DNA from the two parental genotypes in ratios of 1:9, 5:5, and 9:1, holding total template DNA concentration constant, their RAPD analysis revealed the distinct polymorphisms were detected even when the ratio of template DNA from one cultivars was 10% and 90% from the second one. In our experiments, all the primers we selected for identifying chimeras were from Chen et al. (2006), and our experiments showed some clear and positive results, thus SSR analysis could be a useful method to identify citrus chimeras.

CHAPTER 4
CONCLUSION

Seedling quality affected the selection of graft methods in a combination. Grapefruit has the largest stem diameter (1.35 mm), kumquat has the smallest stem diameter (0.75 mm), mandarin and blood orange are in the middle of the aforementioned diameters. Improved Ohtsu's grafting method is suitable for all of combinations. For a combination, if the stem diameters of the donor plants are significantly different, the larger one should be 'rootstock' and the smaller one should be the 'scion' when using improved Winkler's grafting method.

Ten combinations, 'Ruby Red' grapefruit + 'White' mandarin, 'Ruby Red' grapefruit + 'Meiwa' kumquat, 'Duncan' grapefruit + 'White' mandarin, 'Duncan' grapefruit + 'Meiwa' kumquat, 'Hudson' grapefruit + 'White' mandarin, 'Hudson' grapefruit + 'Meiwa' kumquat, 'Hudson' grapefruit + 'Nagami' kumquat, 'White' mandarin + 'Moro' blood orange, '900' mandarin hybrid + 'Moro' blood orange, '900' mandarin hybrid + 'Meiwa' kumquat, were used to investigate whether citrus chimeras could be synthesized from a range of citrus cultivars. Two grafting methods, improved Ohtsu's grafting method and improved Winkler's grafting method, were used to evaluate the effect of grafting methods on adventitious shoots and chimeric shoots formation.

Eight putative chimeric shoots were regenerated from the combination of 'White' mandarin + 'Moro' blood orange by improved Ohtsu's grafting method, but no chimeric shoots were regenerated from other combinations which revealed that graft partners affected the formation of chimera. Winkler's grafting method regenerated more adventitious shoots than improved Ohtsu's grafting method in one combination, but no chimeric shoots were regenerated by improved Winkler's grafting method. More adventitious shoots regeneration does not mean higher rate of chimeric shoot occurrence, only that adventitious shoots regenerating from the

conjunction part may lead to high rate of chimeric shoot formation. Our results have shown that improved Ohtsu's grafting method is better to regenerate chimeric shoots than improved Winkler's grafting method for one year grafted seedlings.

Simple Sequence Repeats (SSR) markers were used to detect chimerism of adventitious shoots. Primers that revealed specific loci that differed between the donor plants were chosen to discriminate citrus chimeric shoots. Primers CX0035, CX2007, CX2021, CX6F04, and CX6F18 can be useful for identifying the chimeric shoots regenerated from combination of 'White' mandarin + 'Moro' blood orange using SSR technology.

This is the first study to generate putative chimeras between 'Moro' blood orange and 'White' mandarin. However, these chimeric materials need to be validated further. Specific research questions to investigate could be: 1) the nature of the chimeras (sectorial, periclinal or mericlinal); 2) which layer(s) are derived from donor plant 'Moro' blood orange, and which layer(s) are derived from 'White' mandarin; 3) the nature of some potentially specific and unique alleles in citrus chimeras revealed at some SSR loci; and 4) whether there are interactions between different genotypic tissues, and whether any changes occurred at both nuclear and chloroplast genomes in these graft chimeras. Other research should also be done in the future to propagate these citrus chimeras, and determine whether they are stable. If they are stable and with desirable traits, these chimeras could be valuable resources for the citrus breeding program with potential to be developed into commercial cultivars. For example, a 'White' mandarin with anthocyanin pigmentation would represent a unique product, with attractive appearance and potentially improved health benefits. Alternatively, a 'Moro' orange with a peel that could be very easily removed likewise would represent something unique in the fresh citrus market. Demonstration of any such commercially important characteristics in fruit of these chimeras

would encourage further utilization of these techniques for citrus scion cultivars improvement in the future.

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BIOGRAPHICAL SKETCH

Yiling Zhou was born in China. She graduated from Beijing Forestry University with a bachelor's degree majoring in ornamental horticulture. During her undergraduate study, she worked in the project 'Plant Selection for Beijing's Green Walls' and finished her thesis titled in 'Preliminary Studies on Selection and Application of Plants for Green Walls in Beijing'. With strong interest in ornamental plants, she entered the graduate program of College of Landscape Architecture in Beijing Forestry University, majoring in ornamental horticulture. During her graduate study, she was involved in the program of 'National Key Technology R&D Program in the 11th Five Year Plan of China', concentrating on greenhouse floriculture- herbaceous peony forcing culture. In parallel, she completed her master's thesis research 'Floral Development and Chilling Requirements of *Paeonia lactiflora* Cultivars' and obtained the master's degree. In the spring of 2013, she was admitted to the graduate program at Department of Horticultural Sciences in University of Florida, she did the master's research on 'Intervarietal Chimera Formation by Grafting in Citrus'.