CONTROLLED HYBRIDIZATION WITH AN APPLE BREEDING SELECTION OF

REDUCED SCION VIGOR AND THE STUDY OF THE TREE ARCHITECTURE OF ITS

OFFSPRING

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

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May 2012

CONTROLLED HYBRIDIZATION WITH AN APPLE BREEDING SELECTION OF
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Cornell University 2012

Selection 1, an apple (*Malus ×domestica* Borkh.) progeny of 'Fuji' x Co-op 18, was characterized by reduced stature and repeated occurrence of forks (lateral branches of the same length). Crossing Selection 1 with parents with contrasting architectures generated progeny 805 and 806. Forked branching and reduced internode length were transmitted from Selection 1 to some of its progeny. Clear segregation of dwarf plants was observed in progeny 806 in the first growing season. These dwarfs resembled 'sturdy dwarf' suggested by Alston (1976) and were controlled by two recessive genes (d_1, d_3). Fewer columnar plants and more intermediate types were present in progeny 805 than expected, a manifestation of interaction of different architectural types.

Shoots were collected from apple cultivar 'Redcort' (on 'M.M.106' rootstock), as well as dwarf and standard plants (own rooted) from progeny 806. These shoot were treated with [^{14}C]GA$_{12}$. Twenty-one metabolites were identified and used as tracers for the purification of endogenous GAs. The existence of endogenous GA$_{12}$, GA$_{15}$, GA$_{53}$, GA$_{44}$, GA$_{19}$, GA$_{20}$ and GA$_3$ was demonstrated by GC-MS, an indication that [^{14}C]GA$_{12}$ was metabolized mainly through the 13-hydroxylation pathway. Dwarf and standard seedlings from progeny 806 produced similar metabolites from [^{14}C]GA$_{12}$, except for peak N, which was only detected in standard plants

between 3 and 6 h. The metabolic rate in standard plants was almost double that of dwarf plants, which may be due to the low vigor of the dwarf seedlings or to low bioactivity of certain enzymes in the pathway.

Exogenous GA_3 and GR24 (a strigolactone analog) were applied to plants in progeny 806. Branches treated with GA_3 had more new growth due to increased number of nodes as compared to control branches in dwarf plants. Decapitation was conducted before the application of GR24 to induce outgrowth of axillary buds. Different degrees of dominance were observed for shoots induced by decapitation in the greenhouse on population 28 (derived from open pollinated Selection 1), whereas for plants in progeny 806, shoots from bud 1 and bud 2 always became dominant. GR24 did not effectively inhibit branching in our study.

BIOGRAPHICAL SKETCH

Xiaohua Yang was born in Huhehaote, Inner Mongolia, China to Yang He-Li and Gao Ze-Hong. Growing up by the side of Yangzi River in Wuhan, Hubei, she went to No.1 High School affiliated with Central China Normal University and obtained her B.S. in Biotechnology in Huazhong University of Science and Technology (HUST). Upon graduation, she was exempted from the entrance exam to graduate school due to her outstanding academic performance in HUST and accepted to Wuhan University, where she worked in the National Key Lab of Genetics with Dr. Li Yang-Sheng with a research focus on the salt tolerance of rice.

In 2006, Xiaohua joined Dr. Susan Brown's research program at Cornell University to pursue her doctorate degree. Here, Xiaohua used a special apple selection to study the components and mechanisms of apple tree architecture. Xiaohua was also engaged in extension outreach activities. In September 2010, she accompanied her advisor Dr. Susan Brown and Dr. Kathryn Boor, Dean of the College of Agriculture and Life Science (CALS), to attend the NY Farm Day in Washington D.C., where she helped introducing new Cornell apple varieties and addressed audiences from different arrays.

Xiaohua was very active in extracurricular activities while pursuing her PhD at Cornell. She served as the treasurer of SoHo, a graduate student organization, for two years. She was also the Management Consulting Specialist of Cornell Graduate Consulting Club (CGCC), a professional club that serves graduate students who are interested in pursuing a career in consulting. A dancer for many years, Xiaohua performed frequently with Cornell's dance department and Ithaca's local community.

献给我的爸爸（杨和礼）和妈妈（高泽红）

ACKNOWLEDGMENTS

This dissertation would not have been possible without the help of my committee members, colleagues, families and friends.

I owe my deepest gratitude to my advisor Dr. Susan Brown. I want to thank her for accepting me into her program and giving me the opportunity to further my education. Pursuing one's PhD can be difficult at times and Susan is always there for me along the way, supportive and encouraging. As a mentor, not only does she give me guidance in my research, she also cares about me deeply at a personal level. This is a rare but such an important quality for professors who serve as advisors for PhD students, who are often at a critical moment both in their professional and personal life. I am especially grateful for her patience during the process of my dissertation writing.

My special thanks to Dr. Peter Davies. My collaboration with him constituted an important part of my research. He has shown me how to think logically and critically and why a scientist needs to be a good mechanic as well. I want to thank him for the many weekends that he came in to rescue me from disasters and the lovely Thanksgiving dinner he served with his wife Linda.

I am fortunate to have Dr. Lailiang Cheng on my committee. Being Chinese, he understands the difficulty of pursuing a career abroad thus has given me his generous support. My many discussions with him were enlightening. His knowledge about apple physiology saved Peter and me a lot of time and effort in our attempt to extract gibberellins from apple tissues.

I want to thank Kevin Maloney, our technician. It was a true pleasure to work with him and without him, nothing can be achieved. My thanks also extend to other crew at Geneva, whom I may have never met but tended my plants in the field.

Thanks to Dr. Kenong Xu for allowing me to use his lab for DNA extraction and to Dr. Aide Wang who helped me get settled in the lab. I am also grateful for all the professors, colleagues and staff at Cornell, who collectively make Cornell such a great working place.

I owe a big thank you to all my friends: Yan Jian, Fangfang, Leslie, Qi Ling and many more. Thank you for making the hard times bearable and happy times more cheerful.

Lastly, I would like to thank my mother and father for making me who I am today. I hope I have made you proud.

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LIST OF ABBREVIATIONS

6-BA	6-benzyladenine
ABA	abscisic acid
AFL	*apple floricaula*
AFLP	amplification fragment length polymorphism
ARGOS	auxin-regulated gene controlling organ size
ALSV	apple latent spherical virus
AM fungi	arbuscular mycorrhizal fungi
AN3	*ANGUSTIFOLIA3*
ANT	*AINTEGUMENTA*
AP1	*APRTALA1*
BAP	benzylaminopurine
BFT	*brother of FT*
BL	brassinolide
CCD	*CAROTENOID CLEAVAGE DIOXYGENASE*
CEN	*CENTRORADIALIS*
CK	cytokinin
COL	*CONSTANS* (*CO*)-*like* (*COL*)
CS	campesterol
FLO	*floricaula*
FT	*FLOWERING LOCUS T*
GA	gibberellins
GAI	*Gibberellin (GA) Insensitive*
GC-MS	gas chromatography-mass spectrometry

GEBV	genomic estimated breeding value
GRF	growth-regulating factor
GS	genomic selection
GSI	gametophytic self-incompatibility
GU	growth unit
HPLC	high performance liquid chromatography
IAA	indolyl-3-acetic
KODA	9, 10-octadecadienoic acid
KRI	Kovats Retention Index
le	a locus encodes GA 3β-hydroxylase in pea
LFY	*LEAFY*
LG	linkage group
MAS/MAB	marker-assisted selection (also known as "marker-assisted breeding")
MeTMSi	trimethylsilyl ether of methyl ether
MYB	myeloblastosis
NPA	an inhibitor of auxin transport
PAC	paclobutrazol
PIF	phtyochrome-interacting factors
RAPD	random amplification of polymorphic DNA
RFLP	restriction fragment length polymorphisms
SCAR	sequence characterized amplified region
SI	self-incompatibility
SIM	selected ion monitoring

SL	strigolactone
SOC1	*SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1*
SNP	single nucleotide polymorphism
S-RNase	S locus encoded ribonuclease
SSR	simple sequence repeats (microsatellite)
SVP	*Short Vegetative Phase*
TDZ	thidiazuron
TFL	*TERMINAL FLOWER 1*
TIBA	2,3,5-triiodobenzoic acid
VOZ	vascular plant one zinc finger protein
X-CK	xylem sap cytokinin

CHAPTER 1

INTRODUCTION

GENERAL BACKGROUND OF APPLE

Apple is in the *Rosaceae* family, *Maloideae* subfamily and *Malus* genus (Brown and Maloney, 2004). Within the genus, there are 30 primary species that can be readily hybridized (Korban, 1986; Way et al., 1991). Other *Malus* species and hybrids are grown as ornamentals for their flower display and attractive foliage (Fiala, 1994).

As one of the most important fruit crops worldwide, apples are widely planted throughout the temperate and sub-tropical climate (Brown, 2012). The world leading apple producers are China and USA (Brown, 2012), followed by European Union (Poland, Italy and France). Apples can be used for fresh eating (second most eaten fresh fruits, after bananas), or to make juice (second most popular juice, after orange juice) and sauce. They can also be fermented to cider, wine and vinegar (Brown and Maloney, 2004).

The largest overall apple market involves fresh fruits, which are sold based on appearance (size, color, shape and free of blemishes), quality (taste-with regional differences), and texture. Current leading varieties include 'Delicious', 'Golden Delicious', 'Granny Smith', 'Fuji' and 'Gala' (Brown and Maloney, 2004). Five of Cornell's best-known releases are 'Empire', 'Jonagold', 'Cortland', 'Macoun' and 'Liberty'. Some of the emerging varieties and their trademarks are listed in Table 1-1.

Table 1-1. Emerging apple varieties and their trademarks (Hancock et al., 2008).

Varieties	Trademarks
Cripps Pink	Pink Lady™
Honey Crisp	Honeycrunch™ (Europe)
Scifresh	Jazz™
Delblush	Tentation™
Civini	Ruben™
Corail	Pinova™ or Pinata™

APPLE TREE ARCHITECTURE

Tree architecture of apple is diverse in nature and important for orchard production efficiency, fruit quality and labor input (Kenis and Keulemans, 2004). It can be regulated genetically, environmentally or by cultural techniques. Genetic control of apple tree architecture can occur in the scion (our focus), or rootstocks/interstocks. Other common practices include pruning/training and application of growth regulators.

Factors Affect Apple Tree Architecture

Scions with Unconventional Architectural Types

Unconventional apple scion architectural types (dwarf, columnar, spur type, weeping type) are reviewed in Chapter 2. Among those, dwarf trees are especially desired by modern orchards that are designed to move quickly from juvenility to production (Miller and Tworkoski, 2003).

The small stature of dwarf trees is suitable for high density planting (Miller and Tworkoski, 2003). It facilitates better light penetration which leads to more efficient photosynthesis and better fruit quality; it aids even spraying and minimizes chemical drift. Dwarf trees also require less pruning and training and are easier to harvest (El-Sharkawy et al., 2012). They are now a normal practice in both North America and Europe.

Dwarfing Rootstocks

Due to the heterozygous nature of *Malus ×domestica* Borkh., most apple trees in commercial orchards are comprised of a genetically distinct fruiting part, the scion, and the rootstock. As a principle aid to apple scion propagation, rootstocks are also used to control "the intrinsic vigor of the scion, its habit, it precocity and efficiency of cropping and the quality of fruits produced" (Webster and Wertheim, 2003). Some common rootstocks and their features are listed in Table 1-2.

Two main effects of dwarfing rootstocks are early flowering and tree volume reduction (Costes and García-Villanueva, 2007). Dwarfing conferred by rootstocks is different from that of a compact scion cultivar (genetic dwarfs), which reduce tree size by shortening the internode length of extending shoots. When genetic dwarfs are caused by an overall reduction in bioactive GAs, they showed both a reduced internode length and number (Bulley et al., 2005). Dwarfing rootstocks, however, do not necessarily reduce internode length. They reduce the rate of shoot growth throughout the season (Webster and Wertheim, 2003), and often terminate the vegetative growth earlier in the late summer or early fall, resulting in fewer nodes (Seleznyova et al., 2003).

As cell division in apices is also influenced by GAs (Davies, 2010) the form could reflect an early cessation of GA production.

Table 1-2. Vigor and features of common apple rootstocks (Webster and Wertheim, 2003).

Rootstock	Vigor	Feature
'M.27[1]'	super dwarfing	Poor root systems; smaller fruits compared to 'M.9'; sensitive to cold injury
'M.9'	dwarfing	Sensitive to winter injury, fire blight, wooly apple aphid (big problem in southern hemisphere, severely damaging the root system); poorly anchored; tolerates hot and dry soils poorly
'M.26'	semi-dwarf	Better winter tolerance than 'M.9'; tendency to produce burr-knots on the shank providing entry site for pest and pathogen and also reduce growth of scion; less efficient in calcium uptake than 'M.9'; susceptible to fire blight, tomato ringspot virus
'M.M.106'	semi-vigorous	Resistance against wooly aphid; popular with juice and cider growers; susceptible to collar rot, tomato ringspot virus

The difference in growth of scions grafted on dwarfing rootstocks and on invigorating rootstocks only become obvious after they enter the reproductive period. Therefore, dwarfing could be a direct effect of the rootstocks or the results of precocity, which in turn alters the physiology of the tree, then limits growth. The answer could be both, as dwarfing rootstocks lead to more fruitfulness and smaller trees (Faust, 1989).

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[1] 'Malling 27'; 'Malling Merton 106'.

In addition to shoot length and node number, rootstocks were also shown to affect branching density, location and branch characteristics (Costes et al., 2006). However, the percentage of bud breaks of axillary buds along extension units was determined by the number of nodes developed in the previous season and not affected by the rootstocks. Hence, rootstock effects on scions are probably cumulative and superimposed year after year.

van Hooijdonk et al. (2005) grafted 'Royal Gala' apple scions to 1-year old rootstock liners of 'M.9' (dwarf), 'M.M. 106' (semi-dwarf), 'Merton 793' (invigorating) and 'Royal Gala' (very vigorous, own rooted scion). At the end of the first season, length and node number were similar on primary shoots, but 'M.9' seemed to limit the number of secondary shoots formed on primary shoots during the summer. Dwarfing apple rootstocks were suggested to limit root-produced GA_{19} supply to shoot apices of the scion. However, Bulley et al. (2005) demonstrated that when the level of bioactive GAs in the scion variety 'Greensleeves' was reduced, the dwarfing effect was not corrected by grafting the scion onto an invigorating rootstock ('M.M.106' and 'M.25').

Costes and García-Villanueva (2007) attempted to clarify the effects of dwarfing rootstocks on the vegetative and reproductive growth of the scion during tree development. Cultivars 'Ariane' and 'X3305' were chosen for their difference in growth and fruiting habit. Half of the plants were own-rooted while the rest were grafted onto 'Pajam 2[®] Cepiland' rootstock, a 'M.9' clone. Total number of axes was reduced by dwarfing rootstocks, both at the terminal and axillary positions, after an increase of flowering, and this reduction was achieved by having proportionally more medium and short axes than long ones. An increase in the number of floral axillaries and flowering regularity was also observed in grafted trees. The authors concluded that the root systems mainly affected the number of medium shoots developed in the scions, while the number of long shoots and flowering potential were more cultivar specific.

Seleznyova et al. (2008) studied second-year annual shoots from different rootstock/interstock/scion combinations ('Royal Gals' scion; 'M.9' and 'M.M.106' rootstocks) and concluded that rootstock and interstock affect annual shoots growth by regulating the type and number of extension growth units produced during the growth cycle but not the number of nodes per growth unit.

Given the effect of rootstocks on scion growth, it is not surprising that rootstocks will affect scion gene expression. When scion variety 'Gala' was grafted to seven different rootstocks that confer different vigor, distinct gene expression patterns were identified for each scion and rootstock combination using a DNA microarray based on available apple ESTs (Jensen et al., 2010). Among the 55,230 transcripts compared, 116 were found to have expression levels correlated with tree size. The transcript with the best correlation was predicted to encode SDH (sorbitol dehydrogenase), which converts sorbitol to fructose in sink tissues, suggesting that shoot tips on more vigorous trees were possibly stronger carbon sinks.

A cross between dwarfing rootstock 'M.9' and invigorating rootstock 'R.5' led to a population of 146 rootstocks seedlings that were able to confer a segregation of size on grafted scions from the cultivar 'Braeburn' (Pilcher et al., 2008). The clear segregation of four growth habits (dwarf, semi-dwarf, intermediate and vigorous) enabled mapping of *Dw1* gene to a 2.5 cM region at the top of LG5 of 'M.9', between RAPD marker NZraAM18_700 and microsatellite marker CH03a09. *Dw1* was suggested to be a major component in dwarfing given that most of the dwarfing or semi-dwarfing rootstocks contained a dwarfing allele of this locus; however, the rootstocks of some vigorous phenotypes also possessed this locus, hinting that additional loci may be involved in conditioning the dwarfing phenotype. These markers need to be tested in

other rootstocks and scion cultivars for their robustness in different genetic backgrounds (Brown, 2012).

In addition to rootstocks, interstocks can also lead to scion dwarfism that is associated with the length of the interstock (Parry and Rogers, 1968) and the position of budding (Parry, 1986). Dwarfing in scion cultivars can be achieved by bark grafting as well. This dwarfism is bark length dependent. Bark from a vigorous tree when grafted inverted, can confer a dwarfing effect that is stronger than interstock or dwarfing rootstock (Lochard and Schneider, 1981).

Different theories have been put forward to explain the dwarfing effects of the rootstocks at a molecular level. While consensus is still lacking, much research effort has been focused on the synthesis, transport and metabolism of plant hormones.

Auxin: Bark of the fruit trees contains many phenolic compounds. Mono-phenols (e.g.: phlorizin, one of the most abundant phenols in the bark), act as cofactors of IAA oxidation and are growth inhibitors; polyphenols inhibit IAA oxidase and tend to enhance growth. The amount of phlorizin or total phenol is lower in the dwarfing rootstocks as compared to the invigorating rootstocks (Faust, 1989). Yet, the bark of the more dwarfing rootstocks caused a higher rate of auxin destruction (Gur and Samish, 1968; Soumelidou et al., 1994; Kamboj et al., 1996) suggesting that rates of basipetal auxin transport were lower in dwarfing than in invigorating rootstocks. In inverted barks, downward translocation of IAA was also decreased (Antoszewski et al., 1978).

Li et al. (2012) demonstrated that IAA accumulated extensively in 'M.9' interstock, which could be a result of inability of downward IAA transport. As suggested by the auxin transport-

canalization[2] model, it is possible that grafted dwarfing rootstocks/interstocks decrease the PAT (polar auxin transport) capacity, which lowers the amount of auxin that transports downwards.

CK: CKs have been identified in the xylem sap of apple trees with trans-zeatin being the most dominant compound most of the year. Total CK level is highest before budburst (February) and start to decline after leafing out, while reaching a base level in July (Tromp and Ovaa, 1990). These spring bud-burst triggers are believed to be shoot-, rather than root-derived (Cook et al., 2001).

Columnar cultivars 'Maypole' and 'Tuscan', which are characterized by reduced branching, had higher shoot zeatin riboside (ZR) concentration than that of standard cultivar 'McIntosh' in June (Watanabe et al., 2004). *In vitro*, apple cultivars differ in their requirements for optimum CK (benzyladenine, BA) concentration for shoot multiplication (Lane and McDougald, 1982).

In dwarfing apple rootstocks, cytokinin concentration in the xylem sap below the graft union was at least four-fold higher than above the graft union (Jones, 1983).

Measuring the contents of endogenous zeatin as well as the expression of *IPT3*[3] at different times of the year and in different scion/interstock/rootstock combinations and treatments ('Red Fuji', 'M.9' and *Malus x micromalus* Makino, bridging and substitutions) showed greater amounts of zeatin and expression of *IPT* in invigorating trees in the growing season: early June to mid-August (Li et al., 2012).

Li et al. (2012) argued that dwarfing center of 'M.9' rootstock was in the roots instead of the stem, and that the graft unions had no obvious influence on hormone transport as IAA and zeatin

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[2] Auxin transport, rather than the concentration, out of the bud is crucial for bud activation (Li and Bangerth, 1999).
[3] Isopentenyl transferases, a key enzyme of cytokinin synthesis.

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content as well as *PIN1* expression level recovered to vigorous tree levels after 'M.9' rootstock and interstocks were substituted either below or above the original graft union. After root substitution, IAA level recovered more slowly than zeatin. In conclusion, the authors claimed that insufficient supply of CK to the aerial part of the plants caused by lowered synthesis capacity of cytokinin in the roots of 'M.9' might be the reason for apple scion dwarfing.

GA: Following [^{3}H]GA$_4$ application to the xylem of grafted apple trees or trees with 'M.9' (dwarfing) interstock had less [^{3}H]GA$_4$ uptake and a decreased proportion of radioactive materials transported to the shoot tips and leaves than did trees with 'M.M.115' (non-dwarfing) interstock (Richards et al., 1986). The graft union and neighboring tissues of the 'M.9' interstock also held a much greater amount and a higher proportion of the radio-active materials applied. The transport of the GA appeared to be reduced with insufficient GAs arriving at the shoot apex.

Bulley et al. (2005) demonstrated that when the level of bioactive GAs in the scion variety 'Greensleeves' was reduced by down-regulation of GA$_{20}$-oxidase, the dwarfing effect was not corrected by grafting the scion onto an invigorating rootstock ('M.M.106' and 'M.25').

Using PCR, Xu et al. (2010) investigated the transport of apple endogenous *GAI* mRNA between rootstock (*Malus xiaojinensis*) and scion ('Fuji'). The appearance of *GAI* mRNA in their graft partners led the authors to conclude that *GAI* mRNA moved both upward and downward in grafted trees. In situ hybridization detected *MdGAI* mRNA in the phloem but not the xylem.

Pruning and Training

The main purposes of training, which has been well described by Costes et al. (2006), are as follows:

> A rapid achievement of a developed canopy structure to reach orchard maturity and maximum fruit production within a few years; 2) an optimal capture of light to optimize carbon gain and fruit yield per hectare; 3) a fair distribution of intercepted light within the aerial system of the tree to minimize the spatial heterogeneity of local vegetative growth and fruit quality; and 4) management of tree shape and fruit load with minimum pruning.

Training may start early in the orchard and the goal for young tree manipulation is to shorten the juvenile/non-productive phase. Once the system has established, the focus shifts to balance reproductive and vegetative growth (Costes et al., 2006).

Best training practice should minimize the need for human intervention. The natural growth and fruiting habit of a tree has to be taken account when training is performed, especially at the young tree age (Costes et al., 2006). Not only limiting undesired reaction but also taking the advantage of the genetic variability will lead to early and more regular yield, and homogeneity of fruit quality (Lauri et al., 2009).

Manipulation of branch angle is an important tool in managing high density plantings in modern orchards (Robinson, 2011). It is often used to allocate branches properly within certain management systems and to reduce vegetative growth of the branch and promote flowering. Effects of shoot bending are time and cultivar related (Lauri and Lespinasse, 2001), but no consensus has being reached regarding how it affects flowering and fruiting. According to

Robinson (2011), branches that are more upright lead to vigorous growth and little flowering; those more horizontal produce less growth, heavy flowering and high fruit size and quality; branches with angles below horizontal produce almost no terminal growth but small spurs and small fruits.

Five genotypes ('Ariane', 'Fuji', 'Braeburn', 'Gala' and 'Granny Smith') with different 1-year-old shoot dimensions (on 'Pajam' rootstock) were chosen to study the physiological effects of bending. Bending performed in the proximal zone stimulated vegetative growth of buds that would normally stay latent. Bending also decreased hydraulic conductance in the lower face of the shoots, which could be the reason for increased lateral abortion in this region (Han et al., 2007).

Pruning is used for building tree structure and optimizing the light penetration within the canopy (Costes et al., 2006). Plants' reactions to pruning differs based on cultivar (Fumey et al., 2011), pruning techniques (thinning and heading cuts) as well as position and timing (dormant and summer pruning) of the action (Fumey et al., 2011; Miller and Tworkoski, 2003). In general, pruning is a dwarfing process although growth is stimulated in the vicinity of the cut (Miller and Tworkoski, 2003).

Fruiting

Manipulating the fruiting of a tree also has effects on tree architecture. The competition and correlation between vegetative and reproductive growth revolve around the distribution of limited biomass. Each year, fruiting affects the distribution of carbon hydrates and water economy; in the long run, heavy yields affect tree vigor cumulatively. Excessive cropping leads

to biennial bearing (Miller and Tworkoski, 2003). A study of a 6-year-old 'Golden Delicious' apple tree showed that fruit load affected secondary growth mainly after primary growth ceased (Lauri et al., 2010).

Given the strong effect of fruiting on the growth of trees and architecture, it is of vital importance for a fruit grower to balance vegetative and reproductive growth. Branch pruning combined with thinning is the key control strategy for regulating fruit load (Costes et al., 2006).

PGRs (plant growth regulators)

Except for rootstocks, PGRs are probably the most efficient, effective and economically viable technique for modifying plant architecture (Elfving, 1988). The effectiveness of a PGR application is determined partially by how well the chemical is absorbed by the plant, which is affected by the environment, tree vigor and age, the dosage, timing in the growing season and the apple cultivars used (Roper and Stang, 1990).

Prohexadione-calcium, registered as **Apogee®** in US and **Regalis®** in Europe, is an effective growth inhibitor in apples. It blocks the conversion from GA_{20} to GA_1, thus inhibiting shoot elongation (Evans et al., 1999). The compound can be absorbed by foliar application and moves acropetally to the growing points of apple shoots (Evans et al., 1999). An unexpected beneficial side effect of Apogee is a low incidence of fire blight due to less succulent vegetative growth (Norelli and Miller, 2004).

Ethylene, the only known gaseous plant hormone, inhibits shoot elongation and favors caliper (diameter of the tree) development. Together with auxin, it can inhibit lateral bud development.

Ethephon (2-Chloroethylphosphonic acid) is commercially used to reduce shoot growth and releases ethylene when applied. **ReTain** (aminoethoxyvinylglycine hydrochloride, AVG) interferes with ethylene synthesis (Roper and Stang, 1990; Miller and Tworkoski, 2003).

Promalin (6-benzyladenine, 6-BA + GA$_{4+7}$) and **CYC** (cyclanilide, auxin transport inhibitor) are used commercially for inducing lateral braches in the nurseries (Robinson, 2011). Spraying treatments of equal mixture of 6-BA and GA$_{4+7}$ at a concentration of 1,500 mg/L were the most efficient in improving seedling branching formation of 'Fuji Nagafu' No.2 on 'M.26'interstock and *M. robusta* Rehd. rootstock (Zhang et al., 2011). Other products to promote branch formation include **Maxcel** (active ingredient 6-benzyladenine) and **Tiberon** (cyclanilide) (Robinson, 2011).

Although ABA (abscisic acid) is shown to be a growth inhibitor and can inhibit shoot growth, there are no chemically synthesized forms for commercial use.

Chemicals that regulate fruiting can also affect vegetative growth indirectly. Effects of fruit load on vegetative growth and tree architecture is discussed in Costes et al. (2006).

Advances of our knowledge in plant architecture regulation may lead to the development of natural plant hormones as PGRs for growth suppression (Miller and Tworkoski, 2003). The newly discovered branching hormone, strigolactone (Beveridge and Rameau, 2010), will be a good candidate.

Genes Control Apple Tree Architecture

Fine tuning of plant architecture is achieved by regulation of synthesis/catabolism of plant hormones, or of their transduction signal pathways (Busov et al., 2008). How plant hormones regulate branching and plant height/stem elongation is reviewed in Chapter 4. Genes that strongly affect plant form and stature are summarized in Table 1 in Busov et al. (2008). Many of the plant architecture regulatory genes are transcription factors (Busov et al., 2008; Wang and Li, 2006), such as: *ANT* and *AGROS* (Hu et al., 2003; Hu et al., 2006; Mizukami, 2001), TCP-domain proteins (Li et al., 2005), *GRF* and *AN3* (Horiguchi et al., 2005), GRAS and MYB-proteins (Keller et al., 2006; Müller et al., 2006) and *TFL1* (Ratcliffe et al., 1999; Shannon and Meeks-Wagner, 1991).

In apple, RNA-Seq technology was used to analyze the expression profiles of young shoots from standard and columnar trees (progeny of 'Fuji' x 'Telamon') and 5,327 unigenes were found to have at least a two-fold difference in transcription level. Among those, 287 were plant architecture related: 60% for regulating branch formation, 20% for plant height, and 15% for plant architecture formation (Zhang et al., 2012). Among these 106 are GRAS transcriptional factors (including *DELLA*) and 31 were mapped to chromosome 10 of apple genome, a region where *Co* was mapped to (Hemmat et al., 1997; Tian et al., 2005). The fact that some of the genes encode key enzymes in GA, auxin and BR (brassinosteroid) pathway demonstrates the importance of plant hormones in regulating apple tree architecture (Zhang et al., 2012).

Bulley et al. (2005) suppressed the expression of a gene encoding the gibberellin (GA) biosynthetic enzyme GA-20 oxidase (by sense and anti-sense over-expression) to reduce the levels of bioactive GAs in a scion variety. There was a significant reduction of height and the scion remained dwarf after grafting on rootstocks. Application of GA_3 reversed the effect.

Using PCR Xu et al. (2010) investigated the transport of apple endogenous *GAI* mRNA between rootstock (*Malus xiaojinensis*) and scion ('Fuji'). The appearance of *GAI* mRNA in their graft partners led the authors to conclude that *GAI* mRNA moved both upward and downward in grafted trees. In situ hybridization detected *MdGAI* mRNA in the phloem but not the xylem.

Research suggests that there is a significant conservation of gene function between *DELLA* proteins[4] from apple and *Arabidopsis*. Over-expressing *Arabidopsis gai* in 'Gravenstein' and 'McIntosh' apple led to reduced growth in-vitro and in the greenhouse, with reduction in stem length, internode length and node number (Zhu et al., 2008). Six endogenous *DELLA* proteins were identified from an apple EST database by Foster et al. (2007). The *MdDELLAs* clustered into 3 pairs (*MdRGL1a/b, MdRGL2a/b and MdRGL3a/b*) and their mRNA was highest in summer-arrested shoots tips and in autumn vegetative buds. Transgenic *Arabidopsis* expressing *MdRGL2a* had smaller leaves and shorter stems, took longer to flower under short days and exhibit a reduced response to exogenous GA$_3$.

Mhgai1, an artificially generated GA-insensitive allele based on *MhGAI1* (from the tea crabapple *Malus hupenhensis* Redh. var. pingyiensis) reduced plant stature and fruit-set-ratio when ectopically expressed in tomato. Transgenic tomato rootstocks (with *Mhgai1)* led to dwarf phenotype in wild type (WT) tomato scions. *Mhgai1* transcripts could be detected in leaves and stems of WT scions but not in fruits and flowers. The authors suggested *Mhgai1* could be a useful target for genetic improvement of dwarfing rootstocks in apple (Wang et al., 2011).

Kotoda et al. (2002b) cloned the *MdTFL* gene from apple cultivar 'Jonathan' and detected its expression in vegetative but reproductive tissues (Kotoda et al., 2006). Suppression of *MdTFL*

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[4] Named for the conserved order of amino acids at N-terminus. D: aspartic acid (Asp); E: glutamic acid (Glu); L: leucine (Leu); A: alanine (Ala)

expression by antisense in apple reduced the juvenile phase and induced precocious flowering (Kotoda et al., 2002b) whereas overexpression could lead to late flowering phenotype (Kotoda and Wada, 2005). This gene has not been examined in columnar material, which is precocious.

Mimida (2009) identified four TFL1/CEN-like genes (*MdTFL1, MdTFL1a, MdCENa and MdCENb*) from tissues of apple cultivar 'Fuji' and 'Jonagold'. Expression analysis and transformation study (into *Arabidopsis)* suggested that *MdTFL1* and *MdTFL1a* function redundantly in vegetative tissues in both juvenile and adult phases, probably as flowering repressor and vegetative meristem identity regulator; *MdCENa* presents in both vegetative and reproductive proliferating tissues (fruit receptacles, cultured tissues and roots) so it may not be involved in the transition from juvenile phase to adult phase. *MdCENb* was silenced in most of the organs, probably due to the polyploidy of apple. In *vitro*, *MdTFL1* can be induced strongly by cytokinin throughout newly generated shoots and in apices of shoots on medium containing CK and auxin (Mimida et al., 2011). It is possible that CK induces *MdTFL1* expression to maintain the juvenile form at the base of the plant whereas CK and auxin determine the fate of shoot apical meristem (vegetative or reproductive).

Apple *FLOWERING LOCUS (FT)*-like genes *MdFT1* and *MdFT2* identified in tissue samples of adult apple cv. 'Fuji' and 'Jonathan' (Kotoda et al., 2010) were mapped to different linkage groups with partial homoeology (LG 12 and LG 14). Although both genes led to early flowering when expressed ectopically in *Arabidopsis by Agrobacterium-mediated* transformation, they had different expression patterns in apple: *MdFT1* expressed in apical buds of fruit-bearing shoots of adult phase whereas *MdFT2* mainly expressed in reproductive organs including flower buds and young fruits. *MdFT1* could potentially promote flowering by regulate other genes' expression as it was also shown to alter the expression level of *MdMADS12*.

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Yeast two-hybrid screens revealed an interaction between *MdFT1* and transcription factors *MdTCP2*, *MdTCP4 and an Arabidopsis VOZ1* (vascular plant One Zinc finger protein-1)-like protein, *MdVOZ1*. *MdFT2* also interacted with *MdVOZ1* and *MdTCP2*. Overlapping expression domains (mostly in fruit tissues) for *MdTCP2, MdVOZ1* and *MdFT1/2* further suggested protein interaction *in vivo*. Constitutive expression of *MdTCP2/4 and MdVOZ1* in *Arabidopsis* affected plant size, leaf morphology and the formation of leaf primordial on the adaxial side of cotyledons. The authors suggested that by interacting with *TCP-* and *VOZ-* family proteins, *MdFT1* or/and *MdFT2* might affect leaf and fruit development through regulating of tissue proliferation and formation of new tissues (Mimida et al., 2011).

FT gene from *Arabidopsis thaliana* induced rapid flowering (1.5 to 2 months after inoculation to seed cotyledons just after germination) in 33% of the apple seedlings infected with *FT*-Apple latent spherical virus (ALSV). Half of the precocious flowers had functional parts and pollen from one flower was able to pollinate flowers from 'Fuji' and produced seeds. However, expression of *MdFT1by* the ALSV vector under the same conditions did not induce early flowering in apple*,* which might be a result of limited expression of *MdFT1* by ALSV vector or a stronger activity of *FT* in inducing flowering in apples (Yamagishi et al., 2011).

Expression of *MdTFL1* was subject to changes of fruit load (with or without thinning) and shading. It could be reduced by application of KODA (9, 10-octadecadienoic acid). No clear changes of *MdFT1* expression level were observed for these factors (Kittikorn et al., 2011).

Studying expression patterns of homologous fragments of *FLO/LFY* and *AP1 (AFL and MdAP1,* respectively) in apple cv. 'Jonathan' revealed that *AFL* was expressed in reproductive and vegetative organs whereas *MdAP1*, which was identified as *MdMADS5,* expressed specifically in

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sepals concurrent with sepal formation (Kotoda et al., 2000). *AFL* may be more involved in floral induction as its transcription increased about two month earlier than *MdMADS5*. Overexpression of *MdMADS5* in *Arabidopsis* led to a heritable early flowering phenotype, suggesting a similar function of *MdMADS5* to *AP1,* although the mechanism of flower-bud formation in apple might be different from that of *Arabidopsis* (Kotoda et al., 2002a).

 Over expression of the *LFY* gene from *Arabidopsis* induced columnar-like phenotypes (shortened internodes and a significantly reduced length of re-growth of shoots) in the apple cultivar 'Pinova' (Flachowsky et al., 2010).

BREEDING APPLE TREES WITH IDEAL ARCHITECTURE

More and more breeding programs are integrating traits related to morphology and architecture into breeding and selection. Two complementary genetic approaches exist to modify apple tree size and architecture: the scion component and the rootstock component (Byrne, 2012).

Scion Breeding

Major goals of scion breeding include: high fruit quality coupled with pest/disease resistance (scab, fire blight and powdery mildew); tree habit for precocity, high productivity and annual bearing; and nutritional components and excellent postharvest traits to facilitate storage and processing (Brown, 2012).

While early efforts were more focused on rootstocks regarding vigor and architecture control, more and more breeders have shown interest in scion breeding for the same purpose and the

approaches range from selecting growth type with better branching habit and increased spur formation within the standard plants to develop new cultivars with unique tree architecture (Byrne, 2012).

For 2,700 fruit cultivars released between 1990 and 2000, fifty-six had unique growth types, the most common being dwarves and spur type cultivars (Byrne, 2012). The most promising growth types suitable for high density modern orchards appeared to be columnar type and spur growth habit, both of which allow better light penetration, require less pruning and potentially deliver greater yield efficiency (Byrne, 2012). Columnar apples with scab resistance have been released in Romania (Braniste et al., 2006) and Latvia (Ikase and Dumbras, 2004).

Short internodes, which are often associated with precocious bearing, together with low vigor and wide angle branching also makes an extremely desirable tree type (Zagaja and Faust, 1983).

Major breeding programs by country are outlined in Brown and Maloney (2003). Unique apple growth types and architectural studies for scion breeding are reviewed in Chapter 2.

Rootstock Breeding

Ideal rootstocks should be easy to propagate, resistant/ tolerant to abiotic and biotic stress and free from suckers and burr knots; they should also be able to control scion vigor to required level and induce precocious and abundant cropping (Webster and Wertheim, 2003). Much effort of rootstock breeding has been focused on horticultural important traits of productivity, dwarfing and precocity whereas certain programs emphasize on characteristics such as abiotic (cold hardiness) and biotic (fire blight, replant disease) stresses (Brown, 2012; Robinson, 2011).

Rootstock breeding programs are being conducted in many parts of the world. The first rootstock breeding program was established by East Malling Research Station (UK) in 1917, where the 'Malling Merton' series, among the best known and most widely grown rootstocks to date, were developed (Brown and Maloney, 2004). The breeding program at Cornell University's New York State Agricultural Experimental Station (Geneva, NY) was initiated in 1953 and the CG (Cornell Geneva) apple rootstock series were developed here. Currently, this is a joint program between Cornell University and the United States Department of Agriculture (USDA). Led by Dr. Fazio, the program is developing a MAS (marker-assisted selection, also known as "marker-assisted breeding") protocol to streamline the breeding process in rootstocks (Fazio et al., 2011). Other rootstock breeding programs are reviewed by Brown and Maloney (2004) and Robinson (2011).

Breeding Strategy

Apple is allopolyploid, but behaves functionally like a diploid (2n = 34). It has a relatively small genome, estimated at 742.3 Mb/haploid, and 17 linkage groups (Brown, 2012). The total number of genes predicted for the apple genome, 57,386, is the highest reported among plants so far (Velasco et al., 2010). Apples have a gametophytic self-incompatibility system and require cross-pollination via insects as pollinators (Brown, 2012). This breeding system is controlled by an extracellular ribonulease, S-RNase (encoded by a highly polymorphic S-locus on the pistil), and another S-locus gene, the pollen S-gene (possibly encode F-box genes), which is tightly linked to the S-RNase (Minamikawa et al., 2010; Sassa et al., 2010).When pollen tubes elongate through styles, if they share the same S-alleles with the pistils on which the pollen germinates, they will be attacked by the cytotoxic protein-S-RNase. Eighteen different S-alleles have been

differentiated in apple as of 2009; with S2, S3, and S9 the most frequent (Pereira-Lorenzo et al., 2009).

Due to apple's slow growth, long juvenile phase and strong self-incompatibility, it usually takes 10-15 years to release a new variety by traditional breeding and can be up to 50 years if an introgression of a trait from a wild relative is involved (Liebhard et al., 2003).

MAS may greatly increase the efficiency and effectiveness in plant breeding compared to conventional breeding methods by substituting complex field trials with molecular tests and selecting (of genotypes) at early seedling stages (Xu and Crouch, 2008).

MAS eliminates unreliable phenotypic evaluation associated with field trials, which often need to be conducted at particular times of the year at specific locations, or are technically complicated. It allows testing for specific traits where phenotypic evaluation is not feasible (e.g. quarantine restrictions may prevent exotic pathogens to be used for screening) or selection of traits with low heritability (Xu and Crouch, 2008). MAS is also useful when to combine multiple genes simultaneously (pyramiding) or avoid the transfer of undesirable or deleterious genes (linkage drag) particularly when integrate genes from wild species (Collard and Mackill, 2008; Tanksley, 1993; Ye and Smith, 2010)

Commonly used molecular markers include: AFLP (amplified fragment length polymorphisms); RFLP (restriction fragment length polymorphism); RAPD (random amplified polymorphic DNA); SSR (simple sequence repeats, microsatellite); SNP (single nucleotide polymorphism) and SCAR (sequence characterized amplified region, Collard et al., 2005; Staub et al., 1996).

Using specific 15-30 bp primers, SCARs designed from nucleotide sequences established in cloned RAPD fragments linked to a trait of interest (Collard et al., 2005). By using longer PCR

primers, SCARs do not face the problem of low reproducibility generally encountered with RAPDs. Obtaining a co-dominant marker may be an additional advantage of converting RAPDs into SCARs (Brown and Maloney, 2003a). Features of common molecular markers and their general usages are list in Table 1-3.

In apple, isozymes were the first markers to be used in breeding for germplasm assessment; RAPDs are the most frequently used markers mostly for map construction and linkage analysis whereas SSRs have been used for cultivar identification and genetic analysis (Brown and Maloney, 2003a). Apple breeders need markers that are easy to use and inexpensive. While many molecular markers have been developed in apple, few of them have been used due to insufficient funding, lack of knowledge of their robustness and significant technical, logistical, informational and sociological barriers (Brown, 2012; Iezzoni et al., 2010).

RosBREED, a transnational and transdisciplinary project dedicated to the improvement of US rosaceous crops, is aiming at establishing a sustainable platform for an efficient MAB pipeline that focuses on fruit quality traits in order to enhance new cultivar adoption, enlarge market potential and increase consumption of Rosaceae fruits (Iezzoni et al., 2010).

Table 1-3. DNA markers for map construction and linkage analysis (Brown and Maloney, 2004; Dale Young, 1999; Xu and Crouch, 2008).

Marker	PCR-based?	Dominance	Allelic	Transfer-ability	Features/Usage
AFLP	Y	Co-dominant (quantitative)	Y	N	Highly sensitive and reproducible; may yield up to 30 polymorphic dominant markers per primer combination; heterozygous and homozygous can be distinguished by the intensity of the bands; generate maps for QTL analysis; extend current maps; saturate maps in high resolution mapping
RAPD	Y	Dominant	N	N	Map construction and linkage analysis, most frequently used in apples
RFLP	N	Co-dominant	Y	Y	More informative than RAPD; require more DNA than PCR based methods; labor intensive and hard to automate; differentiate cultivars (limited usage); mark genes of interest (limited usage); MAS and evolutionary study; fill in linkage map gaps
SSR		Co-dominant		N	Abundant, highly polymorphic and reproducible; most efficient in constructing frameworks of genetic linkage maps; labor intensive and can be expensive; distinguish homozygous and heterozygous; aligning different linkage maps; synteny analysis
Isozymes					Germplasm assessment
SCAR	Y	Dominant/ Co-dominant			Linkage analysis; do not face the problem of low reproducibility generally encountered with RAPDs
SNP		Co-dominant			Highly polymorphic; identification can be automated thus be used in high throughput analysis; has huge advantages over other markers for MAS program

For linkage map construction, backcrossing is an unrealistic option for apple, due self-incompatibility and inbreeding depression. Therefore genetic analysis in apples is typically performed in the full-sib progeny of a single cross, which is also the base population for selection in breeding. Both parents of a cross are expected to display a high level of heterozygosity, thereby allowing markers to be found that are heterozygous in one or both parents (double pseudo-testcross).

Co-dominant markers like RFLPs and isozymes are very useful with regard to transferability to other apple cultivars but are rather labor intensive and /or require large amounts of high quality DNA (Liebhard et al., 2003). Additionally, although being theoretically co-dominant, in practice they segregate to a large extent as dominant markers. The use of PCR-based markers, like SSR (large number of alleles present in apple) and AFLPs offer a simple and fast approach towards new and transferable maps. They require only a little amount of DNA and can easily be automated. AFLP has successfully been used in apple to saturate maps, however the transferability is solely depend on SSRs.

Since the publication of the first map ('White Angle' x 'Rome Beauty') in apple (Hemmat et al., 1994), there are currently maps for at least 50 scion cultivars with different marker densities (Brown, 2012). Major scion cultivar linkage maps are summarized in Table 1-4. Maps have also been constructed for rootstock selections ('M.9', 'Robusta 5' and 'Ottawa 3') (Brown, 2012; Celton et al., 2009).

Table 1-4. Genetic maps of apple scion cultivars.

Reference	Seed parent	Pollen Parent	Pop Size	Markers used	Features
Hemmat et al., (1994)	'White Angle'	'Rome Beauty'	56	Isozymes, RAPDs RFLPs	First linkage map in apple; White angel: 253 markers, 24LG, 950 cM; Rome Beauty: 256 markers, 21LGs, 13 LGs are homologous
Conner et al., (1998)	'Wijcik'	NY 75441-58	172	RAPDs	
	'Wijcik'	NY 75441-67	114	Isozymes,morpho-logical markers (Vf, Co, Rf, Ma)	
Maliepaard et al., (1998)	'Prima'	'Fiesta'	152	RARDs, RFLPs, AFLPs, isozymes, SSR, SCAR	
Liebhard et al., (2003)	'Fiesta'	'Discovery'	267	AFLPs, RAPDs, SSRs, SCARs	Greatest genome coverage; spanned for 1140 and 1450 cM in the parent maps; a saturated reference map; co-dominant markers allow transfer
Kenis and Keulemans (2005)	'Telamon' (Co)	'Braeburn' (Std)	257	AFLP, SSR	Two parents maps constructed (with contrasting fruit quality), 16 homologous LGs

Table 1-4. Continued.

Reference	Seed parent	Pollen Parent	Pop size	Markers used	Feature
N'Diaye et al. (2008)	'Discovery'	'TN10-8'	676	Markers 1046 SSR 159	Consensus map based on 4 populations
	'Fiesta'	'Discovery'			
	'Discovery'	'Prima'			
	'Durello di forli'	'Fiesta'			
Igarashi et al. (2008)	'Ralls Janet'	'Mitsubakaido' (wild relative)	83		New EST markers from a cDNA of 'Fuji'
	'Delicious'	'Mitsubakaido'	72		
Wang et al. (2011)	'Royal Gala'	PI 613988 (*M. sieversii*)	188	SSR	*M. sieversii* map: 180 SSR markers, 1387 cM; 'Royal Gala': 190 SSR markers, 1283.4 cM; four scab resistance loci were mapped

MAS are aiming at selecting a phenotype based on the genotype of markers (Dale Young, 1999). To achieve this goal, the target gene of QTL of interest must have favorable alleles in cis with a novel marker allele and the selection of the marker allele will assure the selection of the target allele in most, but not all backgrounds. This usually requires a map distance smaller than 1 cM. Larger populations (>1,000) are needed to increase the resolution of the map and more markers are required to saturate the maps. After the high resolution maps are constructed, marker validation and conversion are often performed (Collard et al., 2005).

Once markers that are tightly linked to genes or QTLs of interest have been identified, prior to field evaluation of large numbers of plants, breeders may use specific DNA marker alleles as a diagnostic tool to identify plants carrying the genes or QTLs (Xu and Crouch, 2008). Markers and QTLs identified for growth and architecture related traits are reviewed in Chapter 2.

However, MAS has been ineffective in improving polygenic complex traits which small effect genes (Kumar et al., 2011) and few quantitative traits have been fully described in terms of DNA markers.

The weakness of MAS resides in the fact that MAS is a two-step process: the identification of QTLs and the estimation of their effects. The reliability of the first step is often times in question given its poor representation of allelic diversity from bi-parental populations, the possibility of missing many small effects QTLs by stringent significance thresholds (Janine et al., 2010) and its robustness across different genetic backgrounds (Kumar et al., 2011). Although association mapping (Rikkerink et al., 2007) can be used to mitigate the situation, the practice "retains the disadvantage of biased effect estimates and therefore poor prediction of line performance" (Jannink et al., 2010).

With the availability of apple genome sequence ('Golden Delicious') and high throughput genotyping technology that lead to the identification of abundant of SNP markers, genomic selection can be an attractive alternative in apple breeding. Instead of looking for association between a single loci and the trait of interest, genomic selection seeks simultaneous estimation of all markers. It develops a model from a training population, which is both phenotyped and genotyped (Jannink et al., 2010). It then takes in genomic data from a candidate population to calculate the GEBV (genomic estimated breeding value). Although GEBV says nothing about the underlying genes that regulate the trait, it is the best predictor for breeding value. Key drivers for genomic selection (GS) include genetic architecture of traits of selection, population size and structure, genetic evaluation system, density of SNP markers, and extent of linkage disequilibrium (Kumar et al., 2011). Predictions based on genomic selection have been shown to be accurate in both stimulations and empirical studies in plants and animals (reviewed in Jannink et al., 2010).

The next challenge for GS will be developing high throughput and high dimensional phenotyping platform to capitalize the genomic information gained from apple genome sequencing (Kumar et al., 2011).

Transgenics

With the advance of our knowledge in the mechanisms of important characteristics such as dwarfing and precocity in bearing and the availability of apple genome sequence (Velasco et al., 2010), genetic engineering can be used to incorporate major genes into new varieties directly,

which may lead to the elimination of rootstocks thus dramatically change the fruit production system (Robinson, 2011).

Agrobacterium-mediated transformation is the major way to produce transgenic apples and large amount of work has been dedicated to standardize tissue culture protocols for apple improvement, which are reviewed by Bhatti and Jha (2010).

Modification of plant growth and architecture have been sought both through transformation of rootstocks (e.g.: *rol A/B/C*) (Gambino and Gribaudo, 2012) and scion cultivars. Examples include but not limited to: integration of *rolA* gene into vigorous apple rootstock that led to reduced plant height and shortened internode length (Zhu et al., 2001); modification of GA biosynthesis (by down regulation of GA20-oxidase) in the grafted apple scion allowed the control of tree stature independent of the rootstock (Bulley et al., 2005); and overexpression of *Arabidopsis gai* gene in apple led to reduced plant size (Zhu et al., 2008).

Apple scion cultivars that have been transformed include but are not limited to: 'Granny Smith', 'Gala', 'Royal Gala', 'Delicious', 'Golden Delicious', 'Elstar', 'Pink Lady', 'Jonagold', 'Cox's Orange Pippin', 'Fuji', 'Greensleeves', 'McIntosh', 'Orin' and a scab resistant cultivar 'Florina'(Brown and Maloney, 2004) .

Apple selection 1 (a hybrid of 'Fuji' x Co-op 18) has a unique tree form with reduced stature due to reduced internodes at the shoot tips and many lateral branches of the same length from the occurrence of repeated forks. It is able to flower and set fruits, a feature rarely seen in reduced vigor types and crucial for genetic studies.

By crossing Selection 1 with cultivars having contrasting architectures (standard and columnar), two progenies (805 and 806) were generated. The seedlings were engaged in detailed

architectural analysis (Chapter 2), GA metabolism analysis (Chapter 3) and exogenous hormone application analysis (Chapter 4).

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CHAPTER 2

ARCHITECTURAL ANALYSIS OF TWO APPLE PROGENIES WITH DIFFERENT ARCHITECTURAL TYPES AS PARENTS

ABSTRACT

The present study aimed at investigating the interaction of different architecture components in two apple (*Malus ×domestica* Borkh.) F_1 progenies (805 and 806) derived from a common parent (Selection 1) crossed with two breeding selections with contrasting architectures (one standard habit and one columnar). Phenotyping, including both tree topology and geometry, was performed. Forked branching and reduced internode length were transmitted from Selection 1 to some of its progeny. Clear segregation of dwarf plants was observed in the progeny 806 in the first growing season. These dwarfs resembled 'sturdy dwarf' suggested by Alston (1976) and were controlled by two recessive genes (d_1, d_3). Fewer columnar plants and many more intermediate types were present in progeny 805 than expected, a manifestation of the interaction of different architectural types. Traits to characterize different apple tree architectures are discussed.

INTRODUCTION

High yield performance of fruit crops results from the integration of various components. To optimally manage the system at both the orchard scale and the tree scale, an accurate knowledge of growth, branching and flowering processes within the tree canopy, i.e., plant architecture, is

required. Plant architecture refers to the aerial 3-dimensional tree structure. It is the expression of equilibrium between endogenous growth processes and exogenous constraints exerted by the environment at any given time.

By precise morphological observation and appropriate quantitative methods, architectural analysis provides a detailed, multilevel, comprehensive and dynamic approach to plant development. It allows researchers to understand the endogenous processes and separate them from the plasticity of their expression from external influences (Barthélémy and Caraglio, 2007). Shoot branching is a major determinant of plant architecture, governing many aspects of form, function, efficiency and adaptation.

Fruit tree architecture influences fruit quality, planting density, production and labor requirements. Architectural analysis of fruit trees will not only aid the acquisition of knowledge about tree development and intra-species variation, but to a more applied perspective, to help to select and improve cultivars to enhance the overall orchard performance (Costes et al., 2006).

The current main goal of introducing architectural traits in breeding programs is related to the control of tree size and form (to facilitate high density planting and better light utilization), to an early and regular bearing and to branching patterns (to reduce cost associated with pruning and training (Costes et al., 2004).

Apple Tree Architectural Types

Lespinasse (1977) classified apple trees into four architectural types based on qualitative traits, namely growth habit, distribution of branches and fruiting position. Later this classification was

modified by Lespinasse (1992) (Figure 2-1). Type I is characteristic of columnar trees with reduced lateral branching; type II trees mainly grow short shoots and bear fruits on spurs in alternate years; type III trees have a more open canopy and medium to long shoots type; IV is characterized by a weeping habit and tip bearing, bearing fruit at the ends of branches. This type is distinctive from the weeping phenotype found in many crabapples (Segura et al., 2006).

Type I
'Wijcik McIntosh'

Type II
'Starkcrimson Delicious'

Type III
'Golden Delicious'

Type IV
'Granny Smith'

Figure 2-1. Four architectural types in apple (Lespinasse, 1977; Lespinasse, 1992).

Distinctive Apple Tree Forms

Distinctive apple tree forms of research interest include dwarf, columnar, compact and weeping. Spur habit is often discussed as a component of these forms. The genetics of apple tree habit was reviewed by Fideghelli et al. (2003).

   Dwarf scion plants (not rootstock-induced dwarfism): Dwarf apples normally have a stature less than 2 m and a small canopy. Utilizing genetic analysis of several large progenies, Decourtye (1967) concluded that the dwarf character in 'Golden Delicious' progeny was controlled by a single recessive gene (*n*). Alston (1976) classified dwarf apple plants into 'early dwarf' (controlled by two recessive genes d_1 and d_3 and an additional d_4); 'crinkle dwarf' (controlled by a single recessive gene) and 'sturdy dwarf' (determined by recessive genes at two loci and possibly other minor genes).

Early dwarf becomes apparent about 4 weeks after germination. They normally grow no more than 40 cm, with very short internodes and rarely survive the winter. Alston (1976) suggested this type of dwarf is controlled by two recessive genes d_1 and d_3 and an additional d_4. The "*n*" gene, possibly from 'Golden Delicious' and linked to the V_f gene for resistance to apple scab (Decourtye and Brian, 1967) for compact was re-designated by Alston as d_2 (Alston, 1970).

Crinkle dwarfs ranged from 300 to 600 mm in height at 2-years-old, with normal internodes and small, rounded crinkled leaves. They were suggested to be controlled by a single recessive gene transmitted from the parents 'Irish Peach' and 'TSR1T187', which also transmitted early dwarf in some progeny.

Sturdy dwarfs were reported grow to more than 1 to 1.5 m in three growing seasons. They are characterized by very short internodes, much branching and long juvenile phase. Alston (1970)

suggested recessive genes at two loci and that some other minor genes involvement in sturdy dwarf.

Tang et al. (2001) suggested that isozyme E9 (band 9), believed to be under the control of two complementing genes (A_B_), could be used for early selection of 1 or 2-year-old seedlings for dwarf apple types with an accuracy of 90%.

In dwarfing rootstocks, the *Dw1* gene was suggested to be a major component in dwarfing with additional modifying loci and was mapped to a 2.5 cM region at the top of LG5 of 'M.9', between RAPD marker NZraAM18_700 and microsatellite marker CH03a09 in a study of 'M.9' x 'R.5' involving 146 seedlings (Pilcher et al., 2008).

Columnar habit: Columnar apple trees have reduced branching and axillary buds that mostly grow into spurs. Little difference exists between the tip and base diameters of the branches. Secondary branches are narrow angled, almost parallel to the main shoot (Hemmat et al., 1997). Studying sixty 3-year-old hybrid seedlings derived from the cross 'Fuji' x 'Waltz' revealed that apple trees with columnar character had larger leaf area, heavier fresh leaf weight, greater leaf number, shorter internode length and larger height/thickness ratio (Wen et al., 2002). However, columnar types with internodes of different lengths exist, as observed in material at Geneva, NY.

'Wijcik McIntosh', a natural mutant of 'McIntosh', was suggested to be controlled by a single dominant gene by Lapins (1976), but frequently less than 50% columnar offspring were noted (Blazek, 1992; Meulenbroek et al., 1999; Tian et al., 2005; Kenis and Keulemans, 2007; Zhu et al., 2007), suggesting modifier genes. In a review of spur-type growth habits, Looney et al. (1983) discussed 'Wijcik' in regard to it genetics, physiology and field performance.

'Wijcik' has been used in breeding programs to develop cultivars and to study architectural traits (Blazek, 1992; Tian et al., 2005; Kenis and Keulemans, 2007; Zhu et al., 2007). Conner et al. (1998) conducted QTL studies in 172 F_1 plants from a cross of 'Wijcik' (*Coco*) x NY 75441-58 (*coco*). Associations between markers and traits for growth and development were detected (height increment, internode number, internode length, base diameter increment and base diameter) and variations of many traits were associated with the region where *Co* is located. Kenis and Keulemans (2004) also made a cross between cultivars with contrasting architecture: 'Telamon' (*Coco*) x 'Braeburn' (*coco*). With two parental linkage maps constructed, *Co* gene was mapped on LG 15 of 'Telamon'. Again, a large cluster of QTLs for growth were observed in this region.

Many attempts have been made to identify markers associated with *Co* phenotype. Moriya et al. (2009) tested published markers that closely linked to *Co* gene in 170 columnar type plants from 18 crosses, and concluded that SSR marker CH03d11 was the most reliable (100%) in selecting this phenotype while SCB82670 was not linked to *Co*. The authors also constructed linkage maps of the *Co* genomic region with three mapping populations ('Fuji' x 8H-9-45, 'Fuji' x -12786 and 'Fuji' x 'Tuscan') and mapped *Co* in the first two. Baldi et al. (2010) narrowed down the genomic region carrying the *Co* gene to 340 kb and identified some putative genes.

Table 2-1. Genetic markers identified for columnar phenotype (*Co*).

Reference	Marker	Type	cM	Conversion	Cross	Co/Std
Hemmat et al., (1997)	*SSR^{Co}	SSR			'Wijcik' (*Coco*) x NY 75441-67 (*coco*)	40/42/44 [Z]
Kim et al., (2003)	*$WB82_{670}$	RAPD	1.8	$SCB82_{670}$	'Fuji' (*coco*) x 'Tuscon' (*Co*)	69/41
	$P459_{800}$	RAPD	10.8			
	SSR^{Co}	SSR	19.1			
Tian et al., (2005)	*S14412600	RAPD			'Fuji' (*coco*) x 'Telamon' (*Co*)	55/51
	*S1142682	RAPD		$SCAR_{682}$		
	*S10321200	RAPD				
	*S1065437	RAPD				
	*S13311200	RAPD				
	*S1425900	RAPD				
	*E-ACT/M-CTA346	AFLP		$SCAR_{216}$		
	CH02a10	SSR	22.7			
	CH03d11	SSR	3.77			
	SSR^{co}	SSR	26.5			
	COL	SSR	31.9			
	P459800	RAPD	18.4			

*: Newly discovered markers as compare to the test of markers identified by others; [Z]: Co/intermediate/standard

Table 2-1. Continued

Reference	Marker	Type	cM	Conversion	Cross	Co/Std
Zhu et al., (2007)	*UBC811-1300	ISSR			'Fuji' (*coco*) x 'Telamon' (*Coco*)	60/60
	*UBC812-750	ISSR				
	*UBC813-1250	ISSR				
	*UBC814-950	ISSR				
	*UBC817-950	ISSR				
	*UBC818-1000	ISSR				
	*UBC834-900	ISSR				
	*UBC836-800	ISSR				
	*UBC845-900	ISSR				
	*UBC846-750	ISSR				
	*UBC855-1200	ISSR				
	*UBC868-1100	ISSR				
	*UBC880-900	ISSR				

*: Newly discovered markers as compare to the test of markers identified by others.

Differential analysis of cDNA among the columnar cultivar 'Waltz', standard cultivar 'Fuji' and their seedlings revealed 1163 differentially expressed fragments (Zhu et al., 2007). Eleven of them were confirmed, cloned and sequenced. Among those, two fragments A7 and A16 were highly homologous to cytochrome P450 monooxygenase in maize and α-expansin 2 in *Trihysaria versicolor* in amino acids, respectively. The authors suggested that gibberellins (GAs) whose biosynthesis is regulated by P450 monooxygenase may influence columnar habit.

The expression profiles of young shoots from standard and columnar trees (progeny of 'Fuji' x 'Telamon') were also analyzed by RNA-Seq technology (also called Whole Transcriptome

Shotgun Sequencing, WTSS) and 5,327 unigenes[5] had a two-fold difference in transcription level (Zhang et al., 2012). Among those, 287 were plant architecture related: 60% for regulating branch formation, 20% for plant height, and 15% for plant architecture formation. Among the 287 unigenes, 106 are GRAS[6] transcriptional factors (including *DELLA*)[7] and 31 were mapped to chromosome 10 of apple, the region where *Co* was mapped (Hemmat et al., 1997; Tian et al., 2005). The fact that some of the genes encode key enzymes in GA, auxin and brassinosteroid pathway demonstrates the importance of plant hormones in regulating apple tree architecture.

MdGAI (*GA insensitive*) was cloned from the shoot tips of 'Lujia 5' and was expressed in both vegetative and reproductive tissues. While real-time PCR demonstrated that *MdGAI* was always expressed in the shoot apices of both columnar and standard trees during the growing season, columnar apple trees always had a higher expression level (Liang et al., 2011).

Over-expression of the *LFY* (*LEAFY*) gene from *Arabidopsis* induced columnar-like phenotype (shortened internodes and a significantly reduced length of shoot re-growth) in transgenic lines of the apple cultivar 'Pinova' (Flachowsky et al., 2010).

Spur habit: Spur habit, a term used to describe a tree with a prolific development of short fruiting branches (growth unit less than 5 cm), produces trees which are precocious in bearing. The discovery of spur type 'Delicious', a variety with frequent spur-type sports, marked the beginning of the use of spur-habit in fruit production. They are moderate compact types with shorter extension shoots due to shorter internodes, more fruiting nodes per branch and greater

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[5] Unigene is an NCBI database of the trancriptome. Each entry is a set of transcripts that appear to stem from the same transcription locus

[6] A plant-specific protein family, named after the first three members: *GIBBERELLIC-ACID INSENSITIVE (GAI)*, *REPRESSOR of GAI (RGA)* and *SCARECROW (SCR)*

[7] Named for the conserved order of amino acids at N-terminus: D: aspartic acid (Asp); E: glutamic acid (Glu); L: leucine (Leu); A: alanine (Ala).

leaf area per spur. However, morphological features of spur types may vary with the cultivar (Pratt, 1990). Commercial spur types share an internode length of 22-25 mm (Faust, 1989). Columnar is an extreme spur type, with many axillary buds developing into spurs.

While the dwarfing component of the spur-type habit is very evident with some 'Delicious' strains, it is less obvious in other strains. This led to the speculation that spurriness and dwarfness were related but quite different physiological/morphological traits. In Looney's research (1983), 'Morspur Mac' and 'Starkspur Ultra Mac', the least vigorous strains, differed significantly in the degree of spurriness. Spur type was suggested to be inherited separately from internode length as shortened internode length was observed in spur type 'McIntosh' and 'Delicious' but not in spur type 'Rome' and 'Granny Smith' (Walsh and Miller, 1983).

Blazek (1983; 1985; 1992) suggested spur habit is under polygenic control (great variability in degree of spurring in the seedlings): no difference in vigor for spurred and non-spurred seedlings was observed in his study of 2,000 seedlings from an incomplete diallel consisting of 41 crosses of 11 cultivars and additional 1800 seedlings of 22 progenies of different cultivars. Parents transmitting spur type include spur type cultivars ('Starkspur Golden Delicious', 'Starkrimson Delicious') and cultivars with prolific spur development ('Bláhas Orange', 'Idared', 'Cox Orange' and 'Dukát') and the highest percentages were seen in progeny of 'Starkspur Golden Delicious' x 'Starkrimson Delicious'. However, 'Starkspur Golden Delicious' didn't transmit spur-type related features to its progenies in Arasu (1967)'s research. Alston and Watkins (1973) believed the spur habit in 'Redspur' and 'Starkrimson' was the result of a single recessive gene. It is possible that different mechanisms are involved for spur habit from different genetic sources.

In progenies from 'Rich Beauty', 'Yun Green', 'Golden Delicious' and 'Red Delicious', spur type was shown to have a 'spindle' stem (swelling in the mid-portion of the stem) in the first year and all the spindle seedlings (100%) had the ninth band of peroxidase isozyme in the starch gel electrophoresis while 'tower' type seedlings didn't, which can be used for pre-selection for spur-type apples (Zhang et al., 1982)

Compact habit: Compact apple trees have a combination of small size, short internodes and high spurriness (Faust and Zagaja, 1983) and are characterized by darker and thicker leaves and thicker internodes (Arasu, 1967). An example of a compact tree is 'Starkrimson'. Based on Blazek (1983), this habit is a simply inherited recessive character but affected by two or more modifying genes. Seedlings from crosses 'Granny Smith Standard' and 'Granny Smith Compact' with the same female parent exhibited a continuous variation of compact type, implying the inheritance of this trait was polygenicly controlled (Lespinasse et al., 1985).

Lapins (1969) suggested the compact growth types can be distinguished in two-year-old seedlings by examining the number of side shoots, internode length and ratio of length to diameter of one-year-old shoots.

Weeping habit: Weeping trees are the least studied among all the distinctive growth habits. The weeping habit of crabapples with the origin of *Malus baccata* was suggested to be under the control of a single dominant gene, but the sample size was very small (Sampson and Cameron, 1965). Weeping habit is different than type 4- or tip-bearing habit. Bendokas et al. (2012) also studied weeping and concluded that high lamina angle to the shoot in the juvenile phase is a morphological marker of weeping trees and offspring of weeping trees have long to moderate internodes.

More information about genetics of distinctive apple tree forms can be found in Alston (1999)**.**

Architectural Analysis in Apples

Lauri et al. (1997) studied growth and branching of seven cultivars representing four apple architectural types (Type I: 'Oregon Spur Delicious'; type II: 'Reine Des Reinettes'; type III: 'Golden Delicious', 'Melrose' and 'Jonagold'; and type IV: 'Granny Smith' and 'Red Winter') after two years of growth. Their results showed that, for a given cultivar, axis slenderness was positively correlated with internode length, with both characteristics related to a reduced number of growing points through an 'extinction' mechanism.

To determine interdependence between main shoot growth and branching, architectural analysis of 1-year old apple seedlings ('Telamon' x 'Braeburn') according to main shoot growth and sylleptic [8] branching characteristics was conducted by De Wit et al. (2002). Genetic control at the level of sylleptic shoot length, number and position was observed. Length, number and position of the sylleptic shoots were used to classify branched trees into different architectural groups. It was argued that besides the apical meristem, differences at the level of roots also played an important role in sylleptic shoot formation, as important plant hormones are produced in the roots (e.g.: cytokinins).

De Wit et al. (2004) crossed 'Telamon' (heterozygous for *Co*, used as seed parent) with trees with different architectures: 'Braeburn' (normal), 'Sunrise' (slender), 110 (columnar phenotype, but without the *Co* gene) and measured main axis and branch growth characteristics during and

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[8] Continuous development of a lateral from a terminal meristem to establish a branch, without an evident intervening period of rest of the lateral meristem.

after the first two growing seasons. A continuum from trees with few and short shoots to trees with many long shoots was observed in the 209 seedlings from all the crosses. The authors argued that apical dominance and apical control appeared to be positively correlated and branch length and number of the progeny followed the same trend as the male parent.

Costes et al. (2003) studied within-tree architectural development of the apple cultivars 'Fuji' and 'Braeburn' over 6 years. The quantity of primary growth, number and nature of axillary shoots and meristem death were studied. A decrease in vegetative growth as trees aged, along with an increase in the probability of flowering from the center of the tree to the periphery was observed, which confirmed the morphological gradients (determined by the topological location in the comprehensive architecture of a plant and the ontogenetic stage of the organism) and the physiological age of the buds (newly produced short shoots that bear flowers are physiologically old, but the main axis that contains vigorous growth units and annual shoots are physiologically young).

The number of branches in the third growth season was different on F_1 individuals that originated in several crosses between apple cultivars with different architectural ideotypes: spur: 'Starkrimson', 'Golden spur', semi-spur: 'Liberty', weeping: 'Florina' (Sestras et al., 2004). The authors argued that this difference was dependent on the genotype of genitors, especially on their architectural types. As both GCA (general combining ability) and SCA (specific combining ability) contributed to the number of branches expressed per tree, certain varieties (e.g.: 'Goldenspur') with negative GCA might be used to generate seedlings with low number of branches.

Segura et al. (2006) used one-year-old apple progeny derived from 'Starkrimson' and 'Granny Smith' as a model to study architectural traits. Among all the geometric and topological traits measured, mean internode length and number of axillary shoots for the trunk, as well as conicity [(base diameter-tip diameter)/height], cord bending (direct distance between the base and tip of the axillary shoot) and number of axillary shoots at order 3 (main axis is order 1, axillary shoots on main axis are considered order 2) were selected for partitioning progeny into architectural groups. This resulted in groups of trees of relatively similar branching pattern due to the traits selected having high heritability, low genetic correlations with each other, putative pomological interest and ease of measurement.

Lauri (2007) studied branching process of two apple cultivars with contrasting architectures and flowering habits: 'Pitchounette' (upright growth with vigorous shoots and higher branching frequency with a strong pattern of alternate flowering) and 'Chantecler' (low vigor and branching frequency, regular pattern of flowering). He suggested the acrotony concept (increased vigor of the vegetative proleptic branches, from the proximal to the distal part of the parent growth unit, a main factor governing tree as opposed to shrub development) should be extended beyond a length-based criterion and integrated into a more general conceptual framework in which the organo-genetic potential of the axillary meristem increases from the proximal to the distal part of the annual shoot, leading to greater branching density, larger offspring, and a greater propensity for flower bud formation over consecutive years.

In Segura et al. (2007)'s study of 123 seedlings from the cross 'Fiesta' x 'Discovery', bud break was mapped to LG 8, which is homologous to LG 7 in Conner et al. (1998) where one of the QTLs for bud break was mapped. Five QTLs were mapped for mean internode length and longest internode length: those on LG 3, 4 and 15 correspond to LG 3, 7 and 10 in Kenis and

Keuleman (2004); the one on LG 10 corresponds to LG 10 in Conner et al. (1998). Two non-epistatic QTLs for cord bending and one strong QTL for basis angle were also identified: the latter co-localizes on LG 10 with the QTL detected for length of the longest internode on LSAS (long sylleptic axillary shoots) close to the genomic region for *Co,* which confirms pleiotropic effects of this locus on architecture traits in apples.

In another study by Segura et al. (2009), of 125 progeny of 'Starkrimson' x 'Granny Smith', two QTLs co-localized on LG 17 for number of internodes both at the whole tree scale and LSAS; QTLs for tip diameter at different scales (whole tree, on trunk and on long proleptic axillary shoots) were found on LG 3; and on LG 7 four QTLs for tip diameter at different axes and one QTL for increment of base diameter were located.; On LG 13, a genomic region previously associated with sylleptic and proleptic shoots development, five QTLs for number of axillary shoots and the percentage of branching nodes were identified. Although many architectural traits mapped to different linkage groups, co-localization of some traits on LG 10 close to the *Co* locus has confirmed the importance of this locus on architecture in apples. Biennial bearing QTLs on LG10 were also located close to this genomic region (Guitton et al., 2012).

A biennial bearing study of 114 individuals from a cross of 'Starkrimson' (strong biennial bearer) and 'Granny Smith' (regular bearer) revealed co-localization[9] of vegetative growth traits and QTLs for biennial bearing, suggesting common molecular controls for tree architecture and biennial bearing (Guitton et al., 2012).

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[9] QTLs for branching intensity were found to co-locate with QTL clusters for biennial bearing on LG4 and LG13, as well as with QTLs for flower and fruit production on LG1; *CCD8* (petunia) was located at the border of the QTL cluster for inflorescence yield on LG15; a QTL for mean internode length of proleptic axillary shoots was co-located with a biennial bearing QTL on LG4.

Mutations for plant form that are manifested in a unique appearance (phenotype) have been useful for discovering genes affecting plant form in many crops. Unfortunately many unusual phenotypes in apple fail to set fruit, making their use in genetic studies impossible.

A unique offspring was observed in a progeny having 'Fuji' as one parent and a scab resistant selection as the other parent. This selection, NY 88 (designated Selection 1) is of reduced height due to reduced internode length. This seedling also has many lateral branches of the same height and a slightly bushy appearance characterized by multiple forking, ending with rosetted shoot tips. More importantly, it sets fruit of good quality, which enables genetic studies.

Two progenies (805 and 806) were created using Selection 1 as the seed parent, and two pollen parents with contrasting plant architectures were selected. Plants in population 806 clearly segregated into dwarf and standard plants in the first growing season. These genetic dwarfs were morphologically distinct from Alston's 'early dwarf' and 'crinkle dwarf' but resemble 'sturdy dwarf'. They had bushy tree forms caused by enhanced lateral branching and also showed rosetting of the leaves at the shoot tips. There was meristem abortion followed by growth of new laterals forming forked branches. This type of dwarfism is seen in progenies from Selection 1 but also in other materials such as in progeny of 'Goldrush' x 'Orin'.

To characterize the dwarf plants in population 806 and gain knowledge of interaction of different architectural components, phenotyping as to growth and architecture was conducted across three growing seasons (2008-2010). Features of dwarf and standard plants are compared; interaction of different tree forms that manifested in population 805 is discussed.

MATERIALS AND METHODS

Crosses of Selection 1 (NY 88, a hybrid of 'Fuji' x Co-op 18) were conducted in 2007. All the seeds harvested in 2007 were stratified in the refrigerator (4°C) for 90 days and planted in pots in the greenhouse in January 2008 with a day temperature of 21°C and night temperature of 17°C.

806 is a cross between Selection 1 and NY 97-1, a quality parent that has a standard tree form. NY 97-1 is a hybrid of two popular commercial cultivars. From the 74 fruits set, 541 seeds were collected. A subset of 120 seeds was planted in a flat and the seedlings were inoculated with a mixture of races of apple scab *Venturia inaequalis* (Cooke) Wint. to assess if selection for scab resistance would affect the segregation of architectural traits. Of the 120 plants screened, 51 plants were classified as resistant (43%). This is much higher than usual, which is about 20-26%, and this subset was designated 806-screened (806-S). Susceptible plants were discarded. For the remaining 421 seeds, 330 plants germinated successfully and were designated 806-unscreened (806-U). Germination rates of apple frequently exceed 90%, so the low percentage of germination may suggest the existence of some sub-lethal genes as suggested by Gao and van de Weg (2006).

Population 805 is a cross between Selection 1 and an advanced breeding selection NY CO-002. NY CO-002 is a spur type columnar (*Coco*) tree with a reduced number of branches. This selection resulted from a cross of a commercial cultivar x NY CO-16 (*Coco*). The cross to create progeny 805 resulted in 16 fruits and 93 seeds. After radicle emergence, 87 plants were planted in the greenhouse. Among these, 3 did not emerge and 2 plants died the first month, a loss of 5.8%.

Plants were grouped in the greenhouse based on appearance and characterized by height, node number, internode length, base diameter and tip diameter (Table 2-2). Unusual features such as early branching or unusual leaf attributes were also recorded.

These plants were later planted into the field. The 806-S population was planted in Research South Orchard 9; progeny 805 and the 806-U populations were planted at the Gates East orchard at NYSAES in Geneva, NY. Seedlings were on their own roots and allowed to grow naturally with no pruning or training. Pests and diseases were controlled as necessary.

Seedlings were further characterized in August. The branching pattern and branch number were recorded schematically for each plant in these progenies. The existence of spurs was also recorded. Plants were classified into six different architectural groups based on these sketches (Table 2-3). In the second and third growing season, height, base and tip diameter were measured for dwarf plants in progeny 806.

In 2009, the number of shoots was counted for dwarf plants in progeny 806 also, as well as the number of vigorously growing shoot tips and those were infected by powdery mildew.

Table 2-2. Parameters measured or calculated for apple seedlings in the greenhouse/field.

Parameters	Method/Formula
Geometry	
Height (cm)	Measured from the first true leave to the highest growth point
Mean internode length (mm)	Average of 3 internodes measured in the middle portion of the plant, and on different branches when possible
Tip Diameter (mm)	Measured below the highest growth point
Maximum Diameter (mm)	Measured at the thickest part of the plant
Base Diameter (mm)	Measured below the first true leaves
Mean Diameter (mm)	Average of base, tip and maximum diameters
Slenderness	Height/Mean Diameter
Conicity	(Base Diameter –Tip Diameter)/Height
Topology	
Growth	
Node number	Counted
Branching	
Number of laterals	Counted

Table 2-3. Classification of architectural groups for progeny 806 in the first growing season (2008).

Groups	Classification
1	A single leader with lateral branching
2	A single leader without lateral branching
3	A forked apex without lateral branching
4	A forked apex with lateral branching
5	A slanted leader without lateral branching
6	A slanted leader with lateral branching

Canopy was measured at the point where it had the largest horizontal spread with two measurements diagonal to each other. The area of this transversal surface was calculated as ½ (measurement 1 x measurement 2) cm^2.

Mature and fully expanded leaves were collected from progeny 806-U (13 standard plants, 11 dwarf plants) and 805 (5 standard plants and 5 columnar plants) in 2009. Fresh weight (g) /leaf area (cm^2) ratio was calculated.

Data was statistically analyzed using JMP9$^®$ (SAS Campus Drive, Building T, Cary, NC). Parameter means of different groups were compared with Tukey-Kramer HSD test at $\alpha = 0.05$. Data set was submitted to Principle Component Analysis (PCA) and Factor Analysis to identify the parameters that capture most of the difference in the data set. Regularized discriminant analysis was used to determine if seedlings can be classified into pre-defined classes based on measured variables. Cluster analysis was performed to find natural grouping among seedlings.

RESULTS

Population 806

In mid-April, 2008, plants in population 806 started to vary in height, with smaller plants also having narrower leaves and greater internode length reduction (Figure 2-2). Lateral branching was detected in some of the dwarf plants, which is rare for apple seedlings of this age (Figure 2-3). In May, plants in 806 were sorted into different groups (S-small, M-medium and L-large) based on height and overall phenotype (Table 2-4).

Figure 2-2. Dwarf plants in progeny 806-U with internode reduction (picture taken 3 months after germination in the greenhouse).

Figure 2-3. Dwarf plants in progeny 806-U with early branching (picture taken 3 months after germination in the greenhouse).

Table 2-4. Grouping of plants in 806-U based on height in the first growing season (2008).

Groups	Range (cm)	Number of plants	%
S	<16	42	12.7
M	16<, <=25	39	11.2
L	>25	249	75.4
Total		330	100

With 330 plants in 806-unscreened population, 288 were standard plants (249 tall and 39 medium heights) and 42 were dwarf (13%). This roughly follows a 7:1 ratio of two homozygous recessive genes (Table 2-5).

Table 2-5. Segregation of dwarf plants in 806 progeny in the first growing season (2008).

	Observed		Expected		X^2	df	P value
	Standard	**Dwarf**	**Standard**	**Dwarf**			
806-U	288	42	288.75	41.25	0.02	1	>0.05
806-S	34	17	44.63	6.38	9.97	1	<0.01

In the second growing season, most of the plants stayed true to their 2008 classification. Branches from the first growing season still served as the basic scaffold of the plants. In population 806-U, eight plants from the M group and two plants from the L group were switched to the S group, while two plants from the S group grew out of their category (dwarf/small) into the M and L respectively. For 806-S population, although the number of the standard and dwarf plants stayed the same, one plant from 2008 M group moved to the S group and one plant from the S group became a standard plant (Table 2-6). Standard plants that grew very little in the second growing season were re-classified into the S group and remained dwarf.

Table 2-6. Number of dwarf seedlings in 806 unscreened (806-U) and screened (806-S) populations in year 2008 and 2009.

Population	Group	2008	2009
806-U	Dwarf	42	50
	Standard	288	280
806-S	Dwarf	17	17
	Standard	34	34

Dwarf plants in the 806-U population looked bushier compared to standard plants, with leaves that were smaller, thicker and darker in color. Dwarf plants in population 806 also were very susceptible to powdery mildew [*Podosphaera leucotricha* (El. & Ev.) Salm.]. Many shoot tips were infected and this may have delayed their development (Figure 2-4).

Figure 2-4. Dwarf seedling from 806-screened population with a powdery mildew

For mature leaves collected in the second growing season (2009), standard plants in progeny 806-U had a significant higher leaf fresh weight g/cm^2 compared to dwarf plants. Within each category this ratio had little fluctuation (standard plants: 0.030 ± 0.00067 g/ cm^2; dwarf plants: 0.028 ± 0.00073 g/ cm^2).

No significant difference was identified for leaf area/fresh weight ratio between standard and columnar plants in progeny 805, although in Wen et al. (2002), a heavier fresh leaf weight was identified for columnar plants.

No significant difference was found between standard plants from progeny 806-U and 805.

In the growing season of 2009, dwarf plants in 806-U had a significantly higher number of shoots/plant and vigorous shoot tips/plant compared to that of 806-S (Table 2-7) in which the dwarf plants either had none or only one vigorous growing shoot tip.

Table 2-7. Counts of shoot tips of dwarf plants in progeny 806 in 2009.

Population	Total number of shoots	Vigorous shoot tips	Ratio of vigorous shoot tips[z]	Infected shoot tips	Ratio of infection[y]
806-U	$21.04 \pm 1.05a$[x]	$8.20 \pm 0.85a$	$0.40 \pm 0.04a$	$10.47 \pm 0.97a$	$0.50 \pm 0.04b$
806-S	$8.00 \pm 1.78b$	$0.18 \pm 1.45b$	$0.03 \pm 0.06b$	$7.00 \pm 1.64b$	$0.90 \pm 0.07a$

[z] : vigorous shoot tips/total number of shoots; [y] : infected shoot tips/ total number of shoots; [x] : Means followed by different letters (a, b) are significantly different at $\alpha = 0.05$ for Student's-T test.

For some dwarf plants in 806-U (no special similarity was observed among these plants) that were infected by powdery mildew, certain shoot tips grew out of the infection but this was not observed in 806-S. Although dwarf plants in 806-U had a significantly higher number of infected shoot tips/plant, dwarf plants in 806-S had a much higher infection rate, which could be attributed to their low vigor. The infection probably further stunted their growth.

Within the group of dwarf plants in 806-U, different tree forms were observed, as was the horizontal canopy spread, which ranged from 74.7 cm^2 to 1751.6 cm^2. Dwarf plants in 806-U had a significantly wider horizontal canopy spread than that of 806-S (609.3 ± 52.1 cm^2 and 120.6 ± 90.2 cm^2, respectively). This was expected given the low vigor of the plants in 806-S.

In the third growing season, four dwarf plants bore fruits (Table 2-8). No significant similarities were found in their architecture (Figure 2-5). However, except for progeny #3, the other 3 dwarf plants were taller than 75% of the dwarfs in progeny 806, suggesting higher vigor could contribute to early fruiting.

Table 2-8. Features of 806 dwarf plants in progeny 806 that bore fruits in the 3rd growing season (2010).

Progeny number	Number of fruits	Height (cm)	Architecture type (2010)[z]
3	17	109.7	Traditional with more open canopy
10	> 100	146.8	Chaotic
16	1	134.1	Traditional but larger and bushier
27	19	131.2	Two growth segment

[z] **:** Details of architecture classification of dwarf plants in progeny 806 in 2010 are in Chapter 4.

Figure 2-5. Architecture of dwarf plants from progeny 806 that bore fruits in the 3rd growing season (2010).

806 – unscreened Population (806-U)

Classification Based on Height

Height, Internode Length and Node Number

Comparisons of internode length and node number across these groups demonstrated that the small stature of dwarf plants resulted from a combination of shorter internode length and fewer nodes per plant. The reduced internode trait of the Selection 1 parent was transmitted to some of its progeny. The internode mean length in the L group was about two times that in the S group.

In 2011, the tallest dwarf plants had 50% growth reduction compared to standard plants while the shortest dwarf plants had about 70% growth reduction. Fruiting was observed on seven dwarf plants in 2011 and also on some standard habit progeny.

Although architectural complexity increases as the tree age, quantitative measurements and analysis were mostly focused on the first growing season to see if trees could be categorized at a young stage. Throughout the analysis, dwarf plants were labeled as 'S', and standard plants were classified into 'M' and 'L' groups based on height.

The node number in the S group ranged from 1 to 3 and peaked at 2 (61.9%), the L group had a much larger range (2 to 7.5 nodes).

Stem Diameter

Dwarf and standard plants differed significantly in base and mean diameter but not tip diameter. However, plants in the S group still had thicker tips than plants in the L group (Table 2-9). Maximum diameter was measured because for some plants this did not occur at the base of the plant but at a lower part of the trunk. To test whether the base diameter and maximum diameter were significantly different for plants in different groups, paired T-tests were conducted using JMP9 software. No significant differences were detected between base stem diameter and maximum stem diameter in population 806-U (Tukey-Kramer HSD, $\alpha = 0.05$).

Table 2-9. Stem diameter measurements in population 806-U in the first growing season (2008).

Groups	Plant Number	Base Diameter (mm)	Tip Diameter (mm)	Max Diameter (mm)	Mean Diameter (mm)
L	249	5.58 ± 0.75a	2.42 ± 0.72b	5.78 ± 0.68a	4.59 ± 0.60a
M	39	4.36 ± 0.64b	2.86 ± 0.69a	4.82 ± 0.76b	4.01 ± 0.57b
S	42	3.54 ± 0.46c	2.86 ± 0.57a	4.52 ± 0.75b	3.64 ± 0.50c

[z] : S: small, M: medium, L: large based on plant height; [x] : Means followed by different letters are significantly different at $\alpha = 0.05$, Tukey-Kramer HSD test.

Lateral Branching

Seedlings in the L and S groups differed significantly in lateral formation (Table 2-10) the first growing season. More than 60% of the S plants did not have any lateral branches, while plants in the M and L groups were more evenly distributed across groups with different number of laterals.

Tree Form

Slenderness and conicity are important parameters to describe overall tree form. Slenderness in our study was defined as the height of the plant divided by the mean diameter. Dwarf plants had shorter and thicker stems (Table 2-11). In populations 806-U, the slenderness across all three groups were significantly different (Tukey-Kramer HSD test, $\alpha = 0.05$). Conicity, defined as (Base Diameter –Tip Diameter)/Height, was significantly different between plants of L and S groups (Tukey-Kramer HSD test, $\alpha = 0.05$).

Table 2-10. Number of lateral branches in population 806-U in the first growing season (2008).

Groups	# of plants	# of lateral branches					Average
		0	**1**	**2**	**3~5**	**6**	
S[z]	42	26	9	3	4	0	0.5b[x]
M	39	11	13	7	8	0	0.9ab
L	249	75	50	63	51	10	1.5a
Total	330	112	72	73	63	10	1.0

[z]: S: small, M: medium, L: large based on plant height; [x]: Means followed by different letters are significantly different at $\alpha = 0.05$, Tukey-Kramer HSD test.

Table 2-11. Slenderness and conicity in population 806-U based on height groups.

Groups	Number of plants	Slenderness[Z]	Conicity
L	249	9.17 ± 2.0a	0.08 ± 0.02a
M	39	5.29 ± 0.86b	0.07 ± 0.03ab
S	42	2.79 ± 0.86c	0.06 ± 0.07^{x}b

[Z]: Slenderness = height/mean diameter; conicity = (base diameter – top diameter)/height; S: small, M: medium, L: large; Means followed by different letters (a and b) are significantly different at $\alpha = 0.05$, Tukey-Kramer HSD test; [x]: Progeny #2 and #4 have the lowest (-0.18) and the highest (0.27) conicity among S plants in 806-U, which led to the large standard deviation in the S group. When these 2 plants were excluded from the analysis conicity's mean for S group was 0.65 ± 0.47.

Classification Based on Architecture

Most of the plants in population 806-U were in architectural groups 2 (39%) and 4 (27%). 35% of all plants had split apical meristems (divided into two) in the first growing season (Table

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2-12). The majority of the dwarf plants are type 1 and 3, whereas type 5 and 6 are least likely. However, dwarf plants did demonstrate a slightly higher than average rate of forked branching (38% versus an average of 35%, Table 2-13). More than half of the dwarf plants didn't have lateral branches in the first growing season, a much higher rate than that of the M (26%) and L groups (12%). This might be explained by the stronger apical dominance resulting from the proximity of the apical meristem to the axillary meristem in the dwarf group caused by small stature. Although dwarf plants that did branch in the first growing season, branched much earlier in the greenhouse compared to their standard equivalents.

Table 2-12. Classification of plants in population 806-U based on architectural groups.

Groups	Number of plants	Single leader		Forked apex		Slanted leader	
		1	2	3	4	5	6
		W/o laterals	W/ laterals	W/o laterals	W/ laterals	W/o laterals	W/ laterals
S	42	12	14	10	6	0	0
M	39	4	19	6	4	0	6
L	249	22	95	11	78	4	39
Total	330	38	128	27	88	4	45

Table 2-13. Classification of plants in population 806-U based on existence of forked apex and lateral branches in the first growing season (2008).

Groups	Number of plants	Forked apex		Lateral formation	
		Yes	No	W/	W/o
S	42	16	26	20	22
M	39	10	29	29	10
L	249	89	160	212	37
Total	330	115	215	261	69

Parameters were compared across different architectural groups and plants from architecture type 5 were excluded from the comparisons as there were only 4 plants in this group.

Height, Node number and Internode Length

Group 4 and 6 had significantly taller plants than group 1 and 3; plants from group 4 and 3 differed most in height although they shared a forked apex. This is consistent with the previous analysis, as group 4 and 6 had a lower percentage of dwarf plants than group 1 and 3. The height difference was a result of both internode length and node number.

Stem Diameters

Base diameters differed significantly between architectural groups with (group 2, 4 and 6) and without (group 1 and 3) lateral branches during the first growing season (Tukey-Kramer HSD, α = 0.05).

Tree Form

Slenderness differed significantly among groups with lateral branches (group 2, 4 and 6) and those without lateral branches (group 1 and 3) during the first growing season (Tukey-Kramer HSD, α = 0.05). However, there were no significant differences in conicity.

Split Apex

Plants with a forked apex in the first growing season (group 3 and 4) had significantly fewer laterals (0.95 laterals /tree) than seedlings without forks (group 1 and 2, 1.70 laterals /tree).

Lateral Branching

When measurements were compared among plants that had sylleptic shoots in the first growing season versus plants that didn't, the only parameter not significantly different was tip diameter (t-test, Prob > |t| = 0.5792) and conicity (t-test, Prob > |t| = 0.4907).

Forked Branching

Selection 1 has a distinct tendency to form multiple forks on adult trees and transmitted this trait to some of its progeny. In the second growing season, plants in population 806-U had formed forks due to meristem abortion as opposed to the first season where apex meristems split and gave rise to two or more axes with equal or similar vigor.

Examining a subset of population 806-U (137 plants), a greater incidence of forked branching was more evident in dwarf plants than in standard plants (Table 2-14). Plants in the S group had a significantly higher number of forks per plant compared to the plants in the L group, while the M plants were not statistically different from either group (Tukey-Kramer HSD test, α = 0.05).

To understand whether the forking tendency differs by plant architecture, the same subset of plants were compared across different architectural groups regarding number of forks by Tukey-Kramer HSD analysis. As expected, architecture type 3 and 5 had significantly more forks than architecture type 1 in the second growing season.

Table 2-14. Number of forks per plant in population 806-U in the second growing season based on height groups.

	L	M	S
Number of plants	72	25	39
Number of forks/plant	$1.90 \pm 1.16b^z$	$2.20 \pm 1.38ab$	$2.59 \pm 1.87a$

^{z}: Means followed by different letters (a, b) are significantly different at α = 0.05 for Tukey-Kramer HSD test.

Spur

About 50% of the plants in population 806-U had spurs in the first growing season, suggesting a single dominant gene. This spur habit is from the quality parent, as selection 1 is not a spur type. Among dwarf plants, only 24% had spurs in the first growing season, compared to 54% in the L group. Four dwarf plants reported as having spur habit in 2008 did not show this phenotype in 2009, suggesting an error in phenotyping or growth of the spurs into laterals. Of the 50 dwarf plants in the second growing season, 38 had spurs (76%); those that didn't mostly belonged to group 1 and 2 and had a single leader. Some dwarf plants that did not have spurs in the first growing season developed spurs in the second growing season.

A higher ratio of plants that had spurs developed sylleptic shoots in the first growing season ($R^2 = 0.019$, Pro > Chi-square = 0.016).

Pyramidal Shape

'Pyramid' described a branching pattern at the top of apple seedlings where the length of the lateral branches increased with distance from the apex. A subset of population 806-U (136 plants) was characterized for this branching pattern (Table 2-15) and classified into pyramidal, intermediate and standard groups. Pyramidal form didn't occur in the S group, there was only 4% in the M group, yet 28% of the L group plants displayed this phenotype.

Table 2-15. Classification of pyramidal phenotype in a subsection (136 plants) of apple progeny 806-U.

Groups	Number of plants	Pyramid	Intermediate	Standard
L	72	20	23	29
M	25	1	7	17
S	39	0	8	31
Total	136	21	38	77

Statistical Analysis

PCA and Factor Analysis

The data set was submitted to PCA analysis and parameter 'number of forks' was excluded as only a subset of the population was characterized for this trait.

Base on the eigenvalues and the scree plot, four principle components explained 88% of all the differences in the population. For the first component, 'height' has the biggest loading with 'base diameter' a close second. Tip diameter has the largest loading in the second component while conicity in the third. The number of lateral branches in the first growing season loads most heavily on the fourth component.

To understand the difference of the data set other than 'height', PCA analysis was performed again without this parameter. Similar results were obtained except that base diameter has the biggest loading on the first component. In both situations, plants in 806-U are well separated

based on the first two components, suggesting that dwarf plants differ from standard plants not only in height.

To better conceptualize what parameters explain most of the difference in the data set, four components were used to carry out the factor analysis with Principal Component/Varimax Method. With rotated factor loading, slenderness, mean diameter, conicity and number of lateral branches had the highest loadings for the four factors, respectively.

Discriminant Analysis

Discriminant analysis is a way to determine if objects can be classified into pre-defined classes based on measured variables. JMP offers Linear Discriminant Analysis (when variables are uncorrelated, it uses the same covariance for all the groups), Quadratic Discriminant Analysis (uses different covariance for different groups, but suffers from small data set) and Regularized Discriminant Analysis (a compromise between the previous methods). Regularized Discriminant Analysis was used throughout the study given that many parameters are highly correlated.

To determine whether the dwarf and standard plants naturally separate based on measurements, analysis with lambda = 0.1 and gamma = 0.1 was performed. With a misclassification rate of 5%, 15 out of 330 plants were misclassified. One plant from S and M group respectively, was misplaced into each other's group; and 13 L plants were misclassified as M plants.

Carrying out the analysis with the architectural types as the pre-defined classes, misclassification rates were higher than 60% for different combinations of lambda and gamma.

For the forked apex in the first growing season, when lambda = 0.1 and gamma = 0.1, 111 plants were misclassified, a rate of 40%. More than half of the plants (96 out of 166) that didn't have forked apex in the first growing season were misplaced.

For lateral formation, when lambda = 0.5 and gamma = 0.3, the analysis gave the smallest misclassification rate (22%), also unacceptable. Among 61 plants that were misclassified, the misclassification rate for both categories was similar.

Cluster Analysis

Hierarchical cluster analysis with the Ward method (the distance between two clusters is the ANOVA sum of squares between the two clusters added up over all the variables) based on standardized data was performed.

When seedlings were classified into six clusters, no correlation was observed between this clustering and the architectural classification in 2008 (R^2 =0.07, Prob > Chi-square <0.0001, chi-square suspect, 20 cells have expected count less than 5).

Clustering was more meaningful when seedlings were classified into four clusters. Height, mean internode length, base diameter and mean diameter were all significantly different across groups with Tukey-Kramer HSD at α = 0.05. Plants in cluster 1 (58 plants) had small stature, thin stems and were least slender. Cluster 3 (92 plants) and cluster 4 (136 plants) had plants that were tall with thick stems. Plants in cluster 3 had the highest mean number of lateral branches. Cluster 2 was intermediate but with the widest tip diameter among all groups.

When seedlings were grouped into three clusters, all the plants from S and M groups were classified into cluster 1 (102 plants), which had the largest tip diameter but ranked the lowest in

all other parameters. Cluster 2 (92 plants) and 3 (136 plants) differed significantly in height, diameters and number of lateral branches, with cluster 2 having the highest mean for lateral branches among all three clusters.

806 –screened population (806-S)

Classification Based on Height

One flat of 120 seedlings was screened with apple scab (*Venturia inaequalis*) and the 51 resistant seedlings were designated as the 806-screened (806-S) population and planted in the field. The susceptible plants were discarded.

Of these 51 plants, 19 were classified as L, 15 as M and 17 as S (Table 2-16), which was a higher ratio of dwarf plants compared with progeny 806-U, but not when calculated as the percentage from the 120 plants screened with scab. As the susceptible plants were discarded, it was not possible to observe the segregation of dwarf plants in this sub-group, but frequently dwarf types are resistant due to their thicker leaf surface (Kellerhals et al., 1993).

Table 2-16. Grouping of plants in 806-S based on height in the first growing season.

Groups	Range (cm)	Number of plants	%
S	<40	17	33.3
M	40<, <=65	15	29.4
L	>65	19	37.2
Total		51	100.0

Height, Internode Length and Node Number

Tukey-Kramer HSD analysis at $\alpha = 0.05$ revealed significant differences in height for all groups. Similarly to 806-U, the small stature of the dwarf plants in 806-S was a combined result of shorter internode length and fewer nodes.

Stem Diameter

Dwarf plants in population 806-S had a much smaller base diameter, but a similar tip diameter compared to standard plants. Their stems were more uniform from the base to the tip (Table 2-17).

Table 2-17. Stem diameter measurements of plants in 806-S in the first growing season (2008).

Groups	Number of plants	Base Diameter (mm)	Tip Diameter (mm)	Max Diameter (mm)	Mean Diameter (mm)
L	19	$10.68 \pm 2.37a^z$	$2.44 \pm 0.65a$	$10.75 \pm 2.37a$	$7.66 \pm 2.06a$
M	15	$10.93 \pm 1.61a$	$2.31 \pm 0.50a$	$11.24 \pm 1.88a$	$8.16 \pm 1.07a$
S	17	$6.53 \pm 1.69b$	$2.80 \pm 0.64a$	$6.62 \pm 1.64b$	$5.31 \pm 1.25b$

[z]: Means followed by different letters (a, b) are significantly different at $\alpha = 0.05$ for Tukey-Kramer HSD test.

Lateral Branching

As in population 806-U, dwarf and standard plants in 806-S also differed significantly in number of lateral branches. Interestingly, seedlings in 806-S had more lateral branches across all height groups compared to 806-U (Table 2-18) but the scale of this branching was reduced in both size and length due to less plant growth development. Resistant seedlings are subject to greater stress than seedlings that receive full fungicide treatments and this may have delayed development, since plants appeared a year younger in growth despite their being the same age.

Table 2-18. Number of lateral branches per plant in population 806-S in the first growing season (2008).

Groups	Number of plants	% of plants with certain number of laterals			
		0	**1**	**2**	**3~5**
S	17	59%	35%	0%	6%
M	15	59%	20%	7%	20%
L	19	32%	21%	21%	26%
Total	51	47%	10%	10%	8%

Tree Form

The mean slenderness of the L group was double that of the S group. Unlike 806-U, where the slenderness was significantly different across three height groups, in 806-S, it was comparable for M and S groups (Table 2-19).

Table 2-19. Tree form of plants in population 806-S based on height groups.

Groups	Number of plants	Slenderness^z	Conicity
L	**19**	$11.1 \pm 4.8a$	$0.10 \pm 0.04b$
M	**15**	$6.9 \pm 1.0b$	$0.16 \pm 0.03a$
S	**17**	$4.78 \pm 2.0b$	$0.16 \pm 0.06a$

^{z} : Slenderness = height/ mean diameter; conicity = (base diameter –tip diameter)/height; letter a, b and c demonstrates whether the values are significantly different at $\alpha = 0.05$ for Tukey-Kramer HSD test.

Classification Based on Architecture

For 51 plants in the 806-S group, 40% were architectural type 2; about 20% were type 1 and 4 respectively. 10% were type 3 and 10% type 6. There were no plants in type 5 (Table 2-20).

70% of the plants in 806-S had lateral formation in the first growing season, which was less than population 806-U (79%). However, 35% of plants had forked apex, the same as 806-U (Table 2-21).

Table 2-20. Classification of plants in population 806-S based on architectural groups.

Groups	Number of plants	Single leader		Forked apex		Slanted leader	
		1	**2**	**3**	**4**	**5**	**6**
		W/o laterals	W/ laterals	W/o laterals	W/ laterals	W/o laterals	W/ laterals
S	17	2	8	3	3	0	1
M	15	3	5	2	4	0	1
L	19	5	7	0	4	0	3
Total	51	10	20	5	11	0	5

Table 2-21. Classification of plants in 806-S based on the existence of forked branching and lateral branches in the first growing season (2008).

Groups	Number of plants	Forked apex		Lateral formation	
		Yes	No	W/	W/o
S	17	6	11	12	5
M	15	6	9	10	5
L	19	4	15	14	5
Total	51	16	35	36	15

No obvious correlation was evident between the different architectural groups and the height groups, although there were no L plants in group 3 and a relatively larger proportion of L plants in group 6. This was different from 806-U, where there was a correlation between these two types of classification. Dwarfs in 806-S were more uniform than that in 806-U.

When compared across the different architectural groups or based on the existence of lateral formation during the first growing season, none of the parameters were significantly different. The only parameter that differed, based on the existence of a forked apex in the first growing season, was node number.

Spurs

In the first growing season, only 25% of all plants in 806-S had spurs as compared to 50% in population 806-U and 805. Of the 17 dwarf plants in 806-S, none had spurs in this first season, although 9 (52%) had spurs the subsequent year. This may have been due to delayed plant development in this location.

Statistical Analysis

PCA and Factor Analysis

Performing PCA analysis, 3 components were sufficient to explain the major differences in the 806-S data set. PC1 explained 53.3% of the difference and the majority of the parameters have high loadings on the first PC, which is not surprising given that most of them are highly correlated. Interestingly, tip diameter contributes to the difference in a different dimension: it has the highest loading on the third component and holds a different sign from most of the other parameters in the PC1. Conicity loads heavily on PC2, and number of lateral branches is another major parameter for PC3 other than tip diameter.

To explain the differences among the data in a more intuitive way, factor analysis was performed with three factors using Principle component/Varimax method.

After rotation, differences are shouldered more evenly between factor 1 and factor 2; each explains more than 30% of the difference with factor 3 explains 14%.

For factor 1, stem diameters are important parameters: mean diameter, base diameter and maximum diameter all have loadings larger than 96%. Slenderness gets the most loading on factor 2, with conicity comes as a close second, but with a different sign. Then it is height, node mean and node number. For factor 3 tip diameter has the highest loading, with number of lateral branches comes as the second but with a different sign.

When height was excluded from the parameters, PCA analysis gave comparable results. Three components were sufficient to explain the major difference of the data set. Base and maximum

diameter have the highest loadings on the first component; conicity is the most important parameter for the second component; tip diameter for the third.

The score plots of the first two components (with and without height) are similar, demonstrating height parameter can be represented by correlated measurements.

Discriminant Analysis

To determine whether seedlings in population 806-S naturally separate into pre-determined classes, Regularized Discriminant Analysis was performed. Lambda (shrinkage to common covariance, specifies how to mix the individual and group covariance matrices) and gamma (shrinkage to diagonal, specifies whether to deflate the non-diagonal elements, the covariances across variables) were adjusted to achieve best results.

For plant height classification, with lambda = 0.5 and gamma = 0.1, 2 plants out of 51 were misclassified, a misclassification rate of 4%. One L plant was misclassified into M group and 1 M plant misclassified into S group. Given the low misclassification rate and the canonical plot, where plants from different height groups were well separated, the set of descriptors used in the experiment was sufficient in differentiating the standard plants from the dwarf plants population 806-S.

To test whether the plants group into the architecture group designated based on the parameters measured, discriminant analysis with lambda = 0.1 and gamma = 0.1 was performed. With 16 plants out of 51 misclassified, the test gave a misclassification rate of 31%. Plants in group 4 and 6 were correctly classified. In group 4, 7 of 11 plants were misclassified.

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Regarding lateral formation, a misclassification of roughly 30% was observed based on lambda = 0.6 and gamma = 0.1. All misclassifications involved plants with lateral formation the first season. This suggests that the parameters can be used to accurately predict plants that have lateral formation and certain plants that had laterals shared similar features with plants that didn't. In the progeny of 'Telamon' x 'Braeburn', De Wit et al. (2002) found many non-branched trees expressed similar main shoot growth to branched trees.

For forked apex in the first growing season, the misclassification rate was 24%. Most of the misclassification was with plants with a forked apex.

Cluster Analysis

Hierarchical cluster analysis with Ward method was performed on standardized data. When seedlings were grouped into three clusters, correlation with classification based on height was R^2 = 0.49; Prob > Chi-square < 0.0001. These three clusters were significantly different for slenderness, base diameter and mean diameter. For the 17 plants in cluster 1, 16 were from S group and 1 from M group. Plants in this cluster had the smallest stature, internode length and fewest nodes; although their mean and base diameters were the smallest as well, they were the least slender, suggesting more horizontal growth proportionally.

Cluster 2 had 10 plants from L group, 12 from M group and 1 from S. Plants in this cluster had the thickest base and mean diameter and the most lateral branches. Plants in cluster 3 were tallest, although not significantly different from cluster 2. With medium stem diameters, they were among the most slender plants. Over three growing seasons, dwarf plants in both 806-U

and 806-S became more slender. Dwarf plants in population 806-U have been growing much more vigorously than that of 806-S (Table 2-22, Table 2-23).

Table 2-22. Change of dwarf plants in population 806-U across three growing seasons.

Year	Height (cm)	BD[z] (mm)	TD (mm)	MD (mm)	Slenderness[y]	Conicity
2010	108.4a	24.8a	3.0a	13.9a	7.7a	0.21a
2009	57.0b	15.3b	2.8a	8.5b	6.3b	0.24a
2008	45.6c	3.6c	2.4b	5.9c	3.2c	0.07b

[z]: BD, base diameter; TD: tip diameter; MD: mean diameter; [y]: Slenderness = height/ mean diameter; conicity = (base diameter –tip diameter)/height; letter a, b and c demonstrates whether the values are significantly different at α = 0.05 for Tukey-Kramer HSD test.

Table 2-23. Change of dwarf plants in population 806-S across three growing seasons.

Year	Height (cm)	BD[z] (mm)	TD (mm)	MD (mm)	Slenderness[y]	Conicity
2010	54.5a	11.4a	2.4a	6.9a	7.5a	0.19a
2009	38.2ab	8.7b	2.6a	5.7b	6.2ab	0.19a
2008	25.7c	6.6c	2.8a	5.4b	4.9c	0.16a

[z]: BD, base diameter; TD: tip diameter; MD: mean diameter; [y]: Slenderness = height/ mean diameter; conicity = (base diameter –tip diameter)/height; [x]: Letter a, b and c demonstrates whether the values are significantly different at α = 0.05 for Tukey-Kramer HSD test.

Population 805

Classification Based on Columnar vs. Non-columnar

Only one dwarf plant was detected in population 805 in the first growing season, and out-crossing must be ruled out to determine if this is a true progeny. The lack of dwarf plants in progeny 805 suggested the lack of recessive dwarfing alleles in the columnar parent. Traits of columnar plants started to be more clearly manifest in the second growing season (2009). The columnar parent is heterozygous for dominant *Co* gene so a 1:1 ratio for columnar vs. non-columnar plants is expected, although less than 50% of columnar plants are often observed, perhaps due to modifying genes.

Only fourteen typically columnar plants and many intermediate types (41%) were rated (Table 2-24) in progeny 805, suggesting strong interaction between *Co* gene and other modifying genes. If we combine plants from group C, IC and I together to form a loose columnar group and plants from IS and S together as the standard group, we obtain a ratio of 42: 38 (Table 2-25).

Table 2-24. Breakdown of plants in progeny 805 based on columnar vs. non-columnar plant habit.

Total	C^z	IC	I	IS	S
80	14	14	14	5	33

^{z} **:** C: columnar; IC: intermediate, more inclined to columnar; I: intermediate; IS: intermediate, more like standard. Progeny # 48 is dwarf and is not included, # 67 is a specific situation where a side branch is dominant, and this was also not included.

Table 2-25. Segregation of columnar plants in progeny 805.

Observed		Expected		X^2	df	P value
Co[z]	Non-Col	Co	Non-Col			
42	38	40	40	0.2	1	>0.05

[z] : Co: columnar, Non-Co: non-columnar.

Height, Internode Length and Node Number

Standard and columnar plants differed significantly in height (Prob > |t| = 0.0015), with columnar plants about 20% shorter on average. Plant height in the intermediate group was not significantly different from the other groups. Plants in 806-U had a mean height of 35.3 ± 14.4 cm, comparable to 805's 37.7 ± 17.2 cm. Height difference between columnar and standard plants in 805 was solely conferred by difference in number of internodes and all seedlings had internode length comparable to that of L plants in progeny 806 (Table 2-26).

Stem Diameter

None of the diameter parameters were significantly different across groups although columnar habit usually is characterized as having thick stems (Table 2-27).

Table 2-26. Comparison of mean internode length in different groups in progeny 806 and 805 (2008).

806-U		806-S		805	
Groups	Internode mean (mm)	Groups	Internode mean (mm)	Groups	Internode mean (mm)
L[z]	9.4a[y]	L	10.4a	S[x]	10.8a
M	6.8b	M	8.6b	I	9.7a
S	4.3c	S	4.9c	C	9.4a

[z]: L: large, M: medium, S: small; [y]: internode mean is compared within each population and values followed by different letters (a, b, c) are significantly different at α = 0.05 for Tukey-Kramer HSD test; [x]: S: standard, I: intermediate, C: columnar.

Table 2-27. Stem diameter measurements of plants in population 805 based on columnar vs. non-columnar groupings.

Groups	Number of plants	Base Diameter (mm)	Tip Diameter (mm)	Max Diameter (mm)	Mean Diameter (mm)
C[z]	14	6.52 ± 0.54a[y]	2.69 ± 0.70a	6.76 ± 0.56a	5.32 ± 0.36a
I	33	6.16 ± 0.65a	2.65 ± 0.78a	6.34 ± 0.66a	5.05 ± 0.50ab
S	34	6.23 ± 0.65a	2.39 ± 0.57a	6.25 ± 0.94a	4.95 ± 0.47b

[z]: C: columnar, I: intermediate, S: standard; [y]: a, b and c demonstrates whether the values are significantly different at α = 0.05 for Tukey-Kramer HSD test.

Lateral Branching

No difference was observed for later branching between standard and columnar plants.

Tree Form

Columnar is an architectural type that is perceived thicker than the standard type, which was confirmed by the data (Table 2-28).

Table 2-28. Tree form of plants in population 805 based on classification as columnar, intermediate or non-columnar (standard- S).

Groups	Number of plants	Slenderness^z	Conicity
C^y	14	$7.85 \pm 1.9b^x$	$0.10 \pm 0.03a$
I	33	$9.43 \pm 2.4ab$	$0.08 \pm 0.02b$
S	34	$10.75 \pm 2.6a$	$0.07 \pm 0.01b$

^{z} : Slenderness = height/ mean diameter; conicity = (base diameter – top diameter)/height; ^{y} : C: columnar, I: intermediate, S: standard; ^{x} : a, b and c demonstrates whether the values are significantly different at $\alpha = 0.05$ for Tukey-Kramer HSD test.

Classification Based on Architecture

Seedlings in population 805 were classified into the same 6 architectural groups as population in the first growing season (2008). Architectural types 5 and 6 did not occur in 805. As in population 806-U, most 805 seedlings were in group 2 (55%) and group 4 (32%), so that the majority of the plants (87%) had their axillary buds released in the first growing season, forming sylleptic shoots (Table 2-29).

Comparing architecture classification in 2008 and the columnar versus non-columnar classification in 2009, the distribution of plants as to columnar versus not columnar were roughly

similar across different architectural groups, except that there were no columnar plants in architectural group 3.

Table 2-29. Distribution of columnar and non-columnar apple seedlings in population 805 in six architectural groups**.**

Groups	1	2	3	4	5	6	Total
C[z]	1	10	0	3	0	0	14
IC	2	6	1	4	0	0	14
I	0	7	1	6	0	0	14
IS	1	4	0	0	0	0	5
S	2	16	2	13	0	0	33
Total	6	43	4	26	0	0	80

[z] : C: columnar; IC: intermediate, more inclined to columnar; I: intermediate; IS: intermediate, more like standard. Two atypical plants were excluded: Progeny # 48 (dwarf) and # 67 (had a lateral branch became dominant, thus was not classified into any architectural group).

When parameters were compared across the 2008 architectural groups, only the maximum stem diameter and conicity were significantly different. Group 2 had the largest maximum diameter among all groups, significantly different from group 1. While architectural types 1 and 2 had tip diameters larger than types 3 and 4, this was not statistically significant. This difference was intuitively reasonable, as in type 3 and 4 the apical meristems were divided into two.

When plants were grouped based on the existence of a forked apex in the first growing season (2008), those identified as columnar in 2009 had less forked branching in 2008 compared to intermediate and standard plants, but this was not statistically significant.

Forked Branching

In the second growing season (2009), the majority of the 81 plants in population 805 (96.2%) had 1 to 3 forks. Only 1 plant (intermediate) had no forks (1.2%) and 2 plants (1 columnar and 1 standard) had 4 forks. The columnar plants tended to have more forks, which was unexpected given the strong apical dominance associated with columnar habit (Table 2-30).

Table 2-30. Number of forks per plant in population 805 based on columnar vs. non-columnar classification in the second growing season (2009).

Groups	Number of plants	Number of forks/plant				
		0	**1**	**2**	**3**	**4**
C[z]	14	0	5	4	4	1
I	33	1	13	16	3	0
S	34	0	18	10	5	1
Total	81	1	36	30	12	2

[z]: C: columnar, I: intermediate, S: standard.

However, a forked columnar architecture was also observed by Hu et al. (2009) in peach (*Prunus persica* Batsch.). Hybridization of the semi-dwarf peach tree 'A72' (*Nn*) with columnar (*br/br*) resulted in all columnar trees (*br/br*). Traits of 'A72', specifically forked branching (FBR), shorter stature, and late bloom were observed on these columnar trees. Using forking as a discriminator, the progeny separated into the expected 1*NN*: 1*Nn* ratio. The authors suggested that FBR is inherited as a monogenetic trait with incomplete dominance.

Tukey-Kramer HSD test was performed with $\alpha = 0.05$ by designating fork number as a continuous variable. However, there were no significant differences.

Spurs

Despite 805's columnar parent being an extreme spur-type, we didn't observe more seedlings with spurs in the first growing season, rather, 50% comparable to that of population 806-U. Among 14 plants that developed into columnar habit in the second growing season, 7 had spurs in 2008.

Statistical Analysis

PCA and Factor analysis

Principle component analysis on population 805 identified 4 components with eigenvalues larger than 1 and a fifth component with an eigen value of 0.92 (progeny # 48 was excluded from the analysis, as we are not sure whether it is a true progeny). The first 4 components explained 77% of the variance within the population cumulatively. Node number had a higher correlation with

height (0.58) than internode mean length (0.46), which is consistent with previous results suggesting that the height difference in progeny 805 is conferred by difference in node number.

Examining the loading matrix for each principle component, slenderness has the highest loading (0.95) for the first component, height is second (0.81); diameter variables load heavily on the second component, with base-diameter first (0.90) and maximum diameter second; tip diameter has the highest loading for the third component (0.67) with conicity second but in a different direction (-0.65); the number of lateral branches and number of forks load similarly on the fourth component (0.57 and 0.55 respectively).

To make these results more intuitive, factor analysis (Principal component/Varimax and 4 factor rotation) was performed. After rotation, height and slenderness have similar high loadings for the first factor with node number third; diameter mean, maximum and base diameter all having high loadings for the second factor the same sign. The loadings for the second factor suggest that mean diameter is the more powerful differentiating parameter than any individual diameter parameter, with tip diameter least important. Conicity loads most heavily on the third factor while number of forks and mean internode length load similarly for the fourth factor, but with a different sign.

Discriminant Analysis

To determine whether the plants in progeny 805 group naturally as to columnar versus non-columnar or the six architectural groups in the first growing season, regularized discriminant analysis was performed since some parameters are highly correlated.

For columnar versus non-columnar, with lambda equals 0.3 and gamma 0.1, the analysis misclassified 41 plants out of 81 total, a misclassification rate of 51%. Half of the misclassified plants were intermediate types: 12 were classified into columnar while another 10 as standard. This was different from our grouping based on visual observation where 14 plants were classified as true intermediate types, another 14 were intermediate more similar to columnar and 5 more similar to standard.

For architectural types in 2008, with lambda = 0.1 and gamma = 0.5, the misclassification rate was about 30%. For the 6 plants in group 1, 2 were classified into group 2 and 1 to group 3, a misclassification rate of 50%; for the 45 plants in group 2, 29 were correctly classified, 1 to group 1, 9 to group 3 and 6 to group 4; plants in group 3 were all correctly classified; group 4 had a misclassification rate of about 20%.

Since lateral formation in the first growing season was important in shaping overall tree form, discriminant analysis was performed to determine whether plants with and without sylleptic shoots in the first growing season naturally separate. With lambda = 0.1 and gamma = 0.1, the misclassification for the data set was about 23%. There was misclassification in both groups, and about half of the plants that did not have lateral formation in the first growing season were misclassified.

For forked apex, with lambda = 0.2 and gamma = 0.5, 20 out of 81 plants were misclassified (25%). For 30 plants that had forked apex in the first growing season, 5 plants were wrongly placed in the other group; among 51 plants that didn't have this feature in the first year, 15 plants were misclassified. This suggests that some plants that didn't have the forked apex demonstrated similar growth characteristics to plants with forked apex.

Cluster Analysis

To further understand how plants naturally group in population 805, hierarchical cluster analysis with Ward was performed on standardized data.

When seedlings were classified into four clusters, no correlation was observed between this classification with the architectural grouping in 2008 (R^2 = 0.048, Prob > Chi-square = 0.67). Comparing parameter mean across these four clusters with Tukey-Kramer HSD (α = 0.05), slenderness is the only parameter that was significantly different for all these four groups. Cluster 1 has 6 plants that are the least slender with small stature, thick stem and few nodes. Cluster 4 is the group that is most slender, with 31 plants that are tall and slim. Cluster 2 and 3 had 26 and 18 plants, respectively with the slenderness ratio intermediate. Mean internode length contributed less than node number to this clustering and the number of lateral branches in the first growing season and the number of forks also were not high contributors.

DISCUSSION

Dwarf Plants in Progeny 806

Plant height is not only a decisive factor that affects plant architecture, but it is also an important trait that can contribute to crop yield in some crops. Our apple progeny Selection 1 has reduced stature due to reduced internode length. It has a unique forking phenotype and also bears fruit which enables genetic study. This is rare in many dwarf segregants.

Hybridization of Selection 1 and a quality parent with a standard tree form resulted in unexpected segregation of dwarf plants in the seedling stage. After transplanting to the field in

the first growing season, all the plants survived the winter. They were different from the 'early dwarf' suggested by Alston (1976) in both phenotype and survivability. They also differed from the small, crinkled leaves and normal internode length of Alston's crinkle dwarfs.

The segregation of our dwarf plants roughly followed a 1:7 ratio, leading us to speculate that this dwarfing trait is controlled by double recessive genes. Alston (1976) also suggested a two recessive gene control for 'sturdy dwarf', a dwarf type that most resemble ours phenotypically. Dwarfing was shown to be under the control of two complementary genes (A_B_) in Tang et al. (2001)'s study of 10,158 apple seedlings coming from different varieties and species. Lammerts (1945) reported a semi-dwarf peach trait ('bushy') that was also inherited as a double recessive gene ($bu_1bu_1bu_2bu_2$).

If dwarf plants in 806-U are designated as d_1d_1/d_3d_3, Selection 1 should be an intermediate dwarf type with the genotype of D_1d_1/d_3d_3 (d_3 gene complete dominant, d_1 incomplete dominant) or d_1d_1/D_3d_3 (d_1 gene complete dominant, d_3 incomplete dominant). The quality parent is normal in height but carrying the dwarfing alleles of these two genes with a genotype of D_1d_1/D_3d_3. There should be additional modifying genes; hence the plants carrying D_1d_1/d_3d_3 genotype in the progeny were not intermediate types like Selection 1 but normal in height.

Dwarf plants in progeny 806 looked 'bushier' than standard plants because of shorter internodes and forked branching. In peach (*Prunus persica*), Connors (1922) studied the inheritance of upright and spreading growth habits (upright 'Early Crawford', spreading 'Greensboro', and intermediate 'Elberta') and suggested some genes controlling the upright branching habit being homozygous dominant; spreading, homozygous recessive; and intermediate, heterozygous.

Leaves in dwarf plants had shorter petioles that tended to curl back and down towards the branches, resulting in branches covered by leaves. This also contributed to the 'bushier look'. In the 'bushy' type peach tree, the internodes are very short, and branch angles are narrow, these two traits contribute to the dense canopy (Scorza et al., 1984).

Shortened internodes are often the primary characteristic associated with dwarfed trees (Scorza et al., 1984; Westwood and Zielinski, 1966). It is seen in Selection 1 and was transmitted to dwarf plants in progeny 806.

Shortening the internodes in a fruit tree was believed invariably to lead to a reduction in vigor. However, low vigor trees with long internodes and high vigor tree with short internodes were observed by Zagaja et al. (1983) in the offspring of a sib-cross created by two short internode parent cultivars ('Goldspur Delicious' x 'Redspur Delicious'). The authors argued that internode length control was separate from control of vigor and growth.

Faust et al. (1983) suggested that shortening of the internodes was often, but not always, accompanied by increased spur development and that neither of those characters (short internode or spurriness) was always associated with tree vigor. Blazek (1983; 1985; 1992) suggested that spur habit was inherited separately from internode length. Among dwarf plants in 806-U, only 24% had spurs in the first growing season, compared to 54% in L group, demonstrating spurriness is controlled separately from dwarfism.

By studying the spatial variation of cell number and shape of two genotypes with contrasting internode length ('Starkrimson', 'Granny Smith') and four of their F_1 offspring belonging to the most extreme allelic classes, Ripetti et al. (2008) observed that difference in internode length primarily involved number of cells, while cell length played a secondary role. The research

results also demonstrated that variations in internode length were correlated to cell number but not to cell shape, namely cell lengthening was homogenous during internode development, which allowed the total number of cells to be estimated at the internode scale, thus opening up new possibility for a simpler tissue sampling method.

Dwarfism caused by node number reduction is normally associated with rootstocks and Pilcher et al. (2008) argued that the reduction in node number was a key difference between the dwarfing effects of some apple rootstocks and dwarfed or compact scion cultivars. However, our study demonstrated dwarfing in population 806 was a result of both shorter internode length and fewer nodes.

The forking trait from Selection 1 was transmitted to some of its progeny. Forks can usually be classified into four different categories (Drénou, 2000): 1) Temporary forks, which are normally evident in younger trees (under 6 m) that are grown under unfavorable conditions (e.g., weak light); 2) Recurrent forks that appear each year at the end of the trunk and reabsorb themselves after two or three years. This is a heritable trait in *Quercus robur* and *Q. petraea*; 3) Main fork marks the appearance of the main branches of the crown, a result of a progressive straightening of the trunk's branches. It can be either expressed later by the death of the apex, terminal flowering, or a balance between the trunk and other main branches. The appearance of a main trunk is generally preceded by a wave of forks appearing on the branches; 4) Accidental forks are caused by damage to the leader, which can be a result of either herbivores or environment.

The forking phenomenon in our study is a heritable and recurrent trait, related to the production of lateral branches of the same length following meristem death or abortion. It is evident in Selection 1 and many of its progeny, leading to bushy trees. Drénou (2000) argued this type of

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recurrent forking was an effect of sympodial growth and weak apical dominance. In selection 1, we need to determine if forked branching is controlled separately, linked to the dwarfing alleles or if it is a pleiotropic effect of the dwarfing genes.

Forking was also observed in progeny 805, with columnar plants unexpectedly having a higher tendency to fork. However, forked columnar (pillar types) were also observed by Hu et al. (2009) in peach. Hybridization of the semi-dwarf peach tree 'A72' (*Nn*) with columnar (*br/br*) resulted in all columnar trees (*br/br*). Traits of 'A72', specifically forked branching (FBR), shorter stature, and late bloom were observed on these columnar trees. Using forking as a discriminator, the progeny separated into the expected 1*NN*: 1*Nn* ratio. The authors suggested that FBR is inherited as a monogenetic trait expressing incomplete dominance.

When a flat of 120 plants were screened for scab resistance, 51 plants were classified as resistant (42.5%). Given that Selection 1 is heterozygous for V_f gene and the quality parent of 806 is homozygous recessive, a segregation of 1:1 for resistant seedlings should be obtained, but since at Cornell the type 3 reactions to scab are considered to be susceptible, this usually results in less than 26% resistant seedlings. Alston (1976) observed a surplus of susceptible plants and suggested a close linkage between the 'pale green lethal' (PGL) gene (*l*, recessive) and scab resistant gene V_f (9 cM) which will result in a low proportion of normal scab resistant seedlings when *l* allele is linked to V_f allele (repulsion). Gao and Van de Weg (2006) suggested that the distorted segregation of V_f resistance in their study was caused by two sub-lethal genes (*sl1, sl2*) and the phenotypes of the lethal seedlings were distinct from that of PGL.

Some of the plants classified as dwarf in 2008 grew out of their dwarf stature in the second growing season. A similar phenomenon was observed by Alston (1976) in early dwarfs and it

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was attributed to the dominant re-growth gene *G*. However, given the low ratio of the dwarf plants that grew out of dwarfism in our study, it is unlikely that this is due to a re-growth gene. Other possible explanations for this shift could be that these plants are standard plants with low vigor or rate of growth in the first season.

Standard plants that grew little in the second growing season were re-classified into the S group and remained dwarf in phenotype, suggesting these were dwarf plants with slightly higher vigor in the first season.

Interaction of Different Architectural Tree Forms

Interaction of different architectural components or genes affecting growth habits following hybridization would further expand our knowledge of apple architecture and be helpful to develop new growth types. Selection 1 was crossed with a columnar parent that was heterozygous for the dominant *Co* gene to gain understanding of interaction of different architectural components.

The dominant *Co* gene in apple leads to reduced lateral branching and in some cases a growth reduction, accompanied by an equally drastic reduction in internode length. It also reduces branching and stimulates abundant spur formation.

The expected 1 (*Coco*): 1 (*coco*) was not observed; rather many intermediate types were present. Other characteristics of columnar type, e.g.: thick stem, reduced internode length, reduced lateral branching were also compromised.

All columnar seedlings had internode length comparable to that of L plants in progeny 806 (Table 2-25), although columnar plants are usually characterized by reduced internode length. Since 805's parent Selection 1 also has reduced internode length at the tips, this may be a case of interactions of different architectural components. Some research has shown that the internode length of apple trees is generally reduced but can vary from very short to long (Zagaja et al., 1983). Columnar types are known to have reduced branching; however, when crossed with Selection 1, which has enhanced branching, this trait became less prominent in the progeny. The average number of side branches in 805 is 2.0, versus 1.0 in 806-U and 1.6 in 806-S (the standard plants in progeny 805 had more laterals than the columnar plants, but this was not statistically significant). The fact that descendants of Selection 1 by columnar branched even more than that of plants in progeny 806 suggests an influence on branching from the non-columnar parent. Although the columnar phenotype is mostly controlled by a single dominant gene, additional modifying genes may be involved as well (with genetic sources from Selection 1 in this case).

Scorza et al. (2002) studied interaction of different architectural components in peach (*Prunus persica (L.) Batsch*) by crossing trees with PI (pillar/columnar, monogenetic, incomplete dominance, *br/br*) habit with standard, compact, dwarf and pillar parents. *Br/br* derived from crosses between standard (ST) and PI (pillar) was recognized as an upright tree with narrower branch angles than ST trees but wider than PI trees. The combination of *br/br* and brachytic dwarf (DW, *dw/dw*) produced dwarf pillar (DWPI) trees. This dwarf columnar combination was not observed in our progeny.

The architectural classification in 2008 revealed that most plants were in groups 2 and 4, so that the majority of plants had lateral formation in the first growing season. Apples normally pursue a

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monopodial or single trunk growth before the production of reproductive organs. However 35% of plants in progeny 806-U had a split apex, suggesting a trait transmitted from the Selection 1 parent.

The limited number of architectural type 5 plants in 806-U and none in 806-S may be a result of lessened apical dominance caused by slanted leaders, where change in hormones led to a greater tendency for axillary bud release (Han et al., 2007). When vertical shoots were re-oriented to horizontal, Wareing and Nasr (1958) observed terminal shoot growth reduction and increase of lateral buds. Vertically oriented branches on fruit trees are usually more vigorous and less productive than branches oriented in a more horizontal position (Miller and Tworkoski, 2003). The high misclassification rate for architectural types in discriminant analysis could be attributed to the insufficiency of the data to classify the plants into architectural groups or the substantial environmental effects on these traits.

Traits to Characterize Apple Tree Architecture

Measuring plant structure is difficult and time consuming. A large number of variables can be collected (Costes et al., 2004): 1) the whole tree scale (height, trunk cross sectional area, volume; 2) the axes the tree is constituted of (length, proximal and distal diameters, number of leaves, flowers, and nodes); 3) the nodes, the leaves and the flowers.

In our study, measurements were taken for both tree geometry and tree topology to find a set of parameters that is minimal, yet able to best describe biological processes and sufficient to characterize/predict apple tree architecture. Segura et al. (2006) suggested the criteria for selecting parameters should be 1) ease of measurement; 2) low correlation among parameters;

and 3) high heritability. Based on these criteria, on the trunk level, Segura et al. (2006) selected mean internode length and number of sylleptic axillary shoots; on the axillary shoots, conicity, bending and number of sylleptic shoots on the third order.

The segregation of dwarf plants in progeny 806 has enabled us to examine traits to best characterize dwarf plants from standard ones. The low misclassification rate in discriminant analysis showed that parameters used in the study were sufficient to distinguish dwarf plants from standard plants. These two groups still naturally separated even when the parameter height was excluded, demonstrating that dwarfism as an architecture type, is a combined result of many features rather than only reduced height.

Internode length: Internode length is one of the four genetic characters identified by Faust (1989) that determine tree size (the other three being branch angle, the location of branching and the rate of shoot growth or the vigor of the tree, not characterized in the current study). It was one of the parameters used to group plants in 806-U in cluster analysis and 4th Factor in explaining the difference in the data set in progeny 805.

Segura et al. (2007) studied a wide range of variables accounting for tree architecture and showed that internode length was one of the most heritable characters among the numerous variables correlated to whole tree size. They argued that internode lengthening appeared to be a more stable process, whereas the emergence of new metamers/nodes was a process which allowed the plant to adapt to its environment. Furthermore, a strong QTL was detected for this character on linkage group 3 (Donini et al., 2007).

Stem diameter: Stem diameters were important in describing tree architecture in our study with mean diameter having the most differentiating power, and tip diameter the least. Base diameter

was used in clustering in both 806-U and 806-S populations. Stem diameter was heritable in a population of 'Fiesta' x 'Discovery' ($h_b^2 = 0.5$) (Liebhard et al., 2003). However, these geometrical measures were of low heritability ($h_b^2 < 0.2$) in Segura et al. (2007) in a study of one-year-old apple progeny derived from 'Starkrimson' and 'Granny Smith'. Heritability estimates are specific to the population and the environments analyzed, and are not valid in populations where pre-selection has occurred. Thus the discrepancy between the conclusions of Liebhard et al. (2003) and Segura et al. (2006) may be a combined results of different parents and the dwarfing rootstocks used ('M. 27' and 'M. 9', respectively), which are known to reduce tree vigor and stem diameters.

Slenderness: Slenderness was one of the most important parameters in describing apple tree architecture in our study. It was the 1st and 2nd factor in explaining seedlings' difference in 806-U and 806-S respectively; it was the only parameter to separate the clusters in progeny 805 and one of three parameters (base diameter and mean diameter the other two) in 806-S. Slenderness ratio (defined as annual shoot length/ basal diameter, on the branch level) was highly correlated with mean internode length (r = 0.86), distinguishing type IV apple cultivars from types I, II and III (Lauri et al., 1997).

Conicity was of less important than slenderness; however, Segura et al. (2006) suggested conicity was an important trait for describing shoot geometry, and predicting a shoot's propensity to bend together with internode length. Our findings may differ because conicity was measured on a whole tree scale in our study as compared to a branch scale in Segura et al.'s (2006) study.

Lateral formation: One important aspect of plant architecture is shoot branching. The number of laterals, their location and angle within the parent/main shoot are important traits for tree form and yield (Yildirim and Kankaya, 2004). Tree branching habits are under genetic control and genotype dependant (Yildirim and Kankaya, 2004; Segura et al., 2006, Sestras et al., 2004). 'Braeburn' and 'Granny Smith' naturally tend to branch more in the nursery (Yildirim and Kankaya, 2004). When used as parents, 'Golden spur' produced seedlings with a low number of branches; other varieties like 'Starkrimson' and 'Florina' had a tendency to produce branching seedlings (Sestras et al., 2004). The authors suggested that selection of the parents based on their genotype would result in rapid genetic gains.

De Wit et al. (2002) used these variables to separate 209 seedlings from three different crosses into four architectural groups. Segura et al. (2006) selected the number of lateral branches for partitioning progeny into architectural groups due to its significant correlations (from $r = 0.49$ to $r = 0.95$) with all other topological traits and it being easier to measure than other branching variables.

The development of shoot branches has two distinct steps: the initiation of the new AM (axillary meristem) and the outgrowth of the axillary buds with or without dormancy (Wang and Li, 2006). A further variation on pattern of branching concerns relative timing of growth of different axes (Turnbull, 2005): prolepsis is manifested as flushing of the main axis coincident with outgrowth of older pre-existing lateral buds or shoots; syllepsis is when lateral buds of the currently growing main axis grow out during the flush period in which they were formed. The cyclic growth of vegetative tissues is known as flushing.

Sylleptic formation was important in shaping the overall tree form in our study, especially the existence of lateral formation in the first growing season. In the second growing season, every plant had lateral branches, which means the difference in plants with or without lateral branches in the first growing season differed only in the timing of extension (axillary bud) rather than the organogenesis of the axillary meristem. However it was also possible that the axillary meristem was formed in the current growing season and followed by sylleptic shoots.

Architectural parameters are sometimes limited to the plants materials/genetic backgrounds studied. Their robustness needs to be tested under different genetic backgrounds.

We should also consider that the traits were analyzed at a juvenile phase. We need to determine which parameters will be useful in predicting tree form in adulthood and which ones are specific to seedlings. We regret that no detailed measurements of standard plants in the second and third growing seasons were taken, which would be valuable in testing the strength of predicting apple tree architecture from first year growth data.

Costes et al. (2004) argued that sylleptic branching could emerge as a possible predictor of both the entrance into the reproductive period and tree vigor. This was echoed by Yildirim and Kankaya (2004), who believed that, the differential in lateral branching at the young tree level could be an accurate predictor of the time at which a given cultivar will enter production.

Sylleptic branching could also be involved in further branching behavior of the adult tree since long sylleptic shoots on the one–year-old trunks often develop into scaffolds. Comparative studies of the sylleptic branching patterns of one-year-old trunks of a set of apple cultivars showed that these patterns can be used to cluster the genotypes, separating the most contrasting types (Guédon et al., 2003).

However, further investigation is required to clarify if the tree architecture after two years can be predicted from the branching habit of one-year old seedlings (De Wit et al., 2002). Analyzing 46 seedlings from a cross of 'Telamon' (*Co*) x 'Braeburn' (standard) in 2 growing seasons, De Wit et al. (2002) concluded that it was not possible to predict tree architecture based on the branching pattern of one-year-old seedlings, as 60% of trees that developed only a few short sylleptic shoots in the first year were classified into another group having many long branches after the second growing season. Hemmat et al. (1997) also suggested that columnar habit was difficult to identify in trees less than two years old in many progenies.

In our study, we also observed certain traits varied from season to season. Spur development in 2009 could not be fully predicted based on data from the first year and the columnar phenotype only became clearly manifest in the second growing season. Blazek (1990) suggested that the best time to identify spur type, compact and columnar type was in the third or fourth growing season. Meulenbroek et al. (1999) also argued that the selection of columnar type was most reliable at the end of the second growing season, although early selection (2 to 3 weeks after germination) was possible, but subject to a high rate of misclassification. The disadvantage of this is that large amount of space, time and money will be wasted on seedlings that are not desired and discarded at the end. Wen et al. (2002) suggested that leaf area, leaf fresh weight, leaf number, internode length, height/thickness ratio could be used as indexes to select against common-type apple trees (as compared to columnar ones) at early stage, but could not be used as indexes of middle-type apple trees. For compact types, Lapins (1969) argued that number of side shoots, intermodal length, and the ratio of length to diameter of one-year-old shoots could be used as distinguishers for two-year-old seedling trees.

The fact that d_1d_3 and d_4 genotype became apparent 4 weeks after germination, the sturdy dwarf genes at 8 weeks; G (re-growth gene) in the field at about 12 weeks and d_2 and the crinkle dwarf in the second growing season led Alston to speculate that "growth in apple seedlings may be determined by a series of gene specific to particular developmental stages" (Alston, 1976).

The remedy for this is to develop proper systems to characterize architectural features at the juvenile stage that are predictive for traits in more mature, fruiting trees or to analyze mature tree system directly. Segura et al. (2007) tried to develop a phenotyping strategy for four-year-old apple hybrids (50 genotypes from 'Starkrimson' x 'Granny Smith', replicated 3 times) that just entered maturity. Measurements and observations were focused on primary and secondary growth, branching and form. Eight quantitative variables were selected to represent the tree architecture at the beginning of maturity; however discrepancies were found when clustering seedlings with quantitative and qualitative traits, especially for intermediate groups.

Development of molecular markers associated with architectural traits of interest is another alternative. Robust and well tested molecular markers will speed up the breeding process and avoid the problem of selecting traits at the juvenile phase that are not able to predict the adulthood architecture. More extensive juvenile/adult studies will be required across different genetic backgrounds.

Controlling tree size is a major goal of introducing architectural traits into plant breeding programs. Compared to horticultural practices that traditionally confer dwarfism, genetic scion dwarfs might eliminate the compatibility problem in the use of dwarfing rootstocks, and could potentially increase production efficiency by reducing time, money and labor devoted to pruning and training by genetic selection of more open canopies.

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The current study of Selection 1 has allowed us to further identify and characterize dwarf plants that resemble Alston's 'sturdy dwarf' (1976), identify forking and reduced internode length as heritable traits and understand how these architectural components interact with standard and columnar tree form. It has laid a foundation for future marker and QTL analysis and possible candidate gene identification.

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CHAPTER 3

GIBBERELLIN (GA) FEEDING EXPERIMENT IN APPLE (*Malus* ×domestica)

ABSTRACT

[^{14}C]GA$_{12}$ was applied to shoots from the apple variety 'Redcort' on 'M.M.106' rootstock and to shoots from dwarf and standard seedlings on their own roots from progeny 806, a cross of 'Fuji' x Co-op 18. Twenty-one metabolites were identified and used as tracers for the purification of endogenous GAs. The existence of endogenous GA$_{12}$, GA$_{15}$, GA$_{53}$, GA$_{44}$, GA$_{19}$, GA$_{20}$ and GA$_3$ was demonstrated by GC-MS, an indication that [^{14}C]GA$_{12}$ was metabolized mainly through the 13-hydroxylation pathway. Dwarf and standard seedlings from progeny 806 produced similar metabolites from [^{14}C]GA$_{12}$, except for peak N, which was only detected in standard plants between 3 and 6 h. The metabolic rate in standard plants was almost double that of dwarf plants, which may be due to the low vigor of the dwarf seedlings or to low bioactivity of certain enzymes in the pathway.

INTRODUCTION

The objective of this section of the research was an attempt to determine whether apple tree morphology could be related to the content or metabolism of gibberellins, given that GAs are important regulators of shoot elongation and found to be important in fruit tree stature (El-Sharkawy et al., 2012). In this chapter, we examined the metabolic pathway of GA in apple vegetative tissues by applying radioactive [^{14}C]GA$_{12}$, a common precursor of all GAs in higher

plants, to the base of vigorously growing shoot tips. Twenty-one metabolites were identified during the process, including GA_{12}, GA_{15}, GA_{53}, GA_{44}, GA_{19}, GA_{20} and GA_3. To investigate the cause of the dwarf plants in progeny 806, GA metabolism in dwarf and standard apple plants from this population was compared, and they were found to follow similar metabolic patterns with different metabolic rates.

Apple selection 1 (a hybrid of 'Fuji' x Co-op 18) has a unique tree form with reduced stature due to reduced internodes at the shoot tips and many lateral branches of the same length from the occurrence of repeated forks (Figure 3-1). It is able to flower and set fruits, a feature rarely seen in reduced vigor types and crucial for genetic studies. A cross between this selection and a parent with good fruit quality (derived from 'Honeycrisp' x 'Gala') with a standard tree form, generated progeny 806. This cross was made to investigate whether the unique branching habit and the reduction of internode length in selection 1 would be transmitted to the progeny and to learn more about the genetic mechanism behind tree architecture and dwarfing to explore the possibility of using scion vigor reduction to enhance final tree form.

Figure 3-1. Forking in apple selection 1 (picture was taken in January 2012).

In progeny 806, there were 42 dwarfs in a population of 330 plants (13%) and they could be characterized at an early stage of growth in the greenhouse (Figure 3-2). These dwarf seedlings have enhanced lateral branching and a rossetting of the leaves at the shoot tips. Almost 95% of the dwarf plants formed forks in the second growing season, where meristem abortion was followed by growth of two or more new laterals forming a forked branch. These dwarf plants are morphologically distinct from the early dwarfs described by Alston (1976) (Figure 3-3).

Figure 3-2. Comparison of dwarf and standard plants in 806 population (picture was taken 4 months after growth in the greenhouse).

Figure 3-3. An apple seedling that is similar to that designated by Alston (1976) as "early dwarf".

The small stature of these dwarf plants, their reduced internode length and dark foliage are very similar to plants with defects in gibberellins (GAs) biosynthesis or response. GAs are a family of compounds defined by their structure (a group of dipterpenoids). They are a major group of plant hormones that affect many aspects of plant growth and development including stem elongation, determining flower sex, and promoting fruit growth and seed germination. However, GAs are best known for their significant effects on internode elongation in dwarf and rosette species (Sponsel, 2006).

GAs are synthesized in young shoot tissue and developing seeds (stage-specific). Most mutants deficient in GA biosynthesis are characterized by shorter stature and darker green, compact

leaves in comparison to wild type plants. Other phenotypes include defects in seed germination and flower and fruit development. Mutants impaired in GA signaling resemble GA biosynthesis mutants except that they cannot be rescued by GA application. Constitutive GA mutants are very tall and have paler green leaves, which mimics wild-type plants that are over-dosed with GA (Davies, 2004).

The identification, quantification and metabolism of GAs in apples have been investigated (Table 3-1). Numerous GAs have been identified in apple seeds. However, the analysis of the GA content from different groups often vary due to different cultivars examined, developmental stages used and techniques used, and evidence for GA metabolism in vegetative tissues of apple in vivo is still lacking and . GA_{19}, GA_{20}, GA_1, GA_8, and GA_{29} were identified by mass-spectrometry in vegetative apple tissue by Koshioka et al. (1985) and Steffens et al. (1992a) indicative of the early 13-hydroxylation pathway of GA metabolism. Stephan's group (1999a) also identified GA_1, GA_3, GA_4, GA_7, and GA_{34} in exudates (fruits with pedicels were placed on agar gel and incubated in the dark for 20 h at $20 \pm 2°C$) from developing fruits from several apple varieties ('Elstar', 'Golden Delicious', 'Jonica' and 'Spencer Seedless').

GA content from different groups often vary due to different cultivars examined, developmental stages used and techniques used, and evidence for GA metabolism in vegetative tissues of apple in *vivo* is still lacking. GA_{19}, GA_{20}, GA_1, GA_8, and GA_{29} were identified by mass-spectrometry in vegetative apple tissue by Koshioka et al. (1985) and Steffens et al. (1992a), indicative of the early 13-hydroxylation pathway of GA metabolism. Stephan's group (1999a) also identified GA_1, GA_3, GA_4, GA_7, and GA_{34} in pedicel exudates from developing fruits from several apple varieties ('Elstar', 'Golden Delicious', 'Jonica' and 'Spencer Seedless'). (To do this fruits with pedicels were placed on agar gel and incubated in the dark for 20 h at 20°C.).

Table 3-1. Gibberellins (GAs) identified in apple (*Malus ×domestica*) by different researchers.

Citations	GAs found	Plant Materials	Variety	Techniques used[z]
Motosugi et al. (1996)	GA_{15}, GA_{17}, GA_{18}, GA_{19}, GA_{23}, GA_{44}, GA_{53}	Xylem exudates	3 year old 'Fuji' on 'Marubakaidou' and 'M.26' rootstocks	HPLC (C-18 reverse phase); modified bioassay on dwarf rice*
Koshioka et al. (1985)	GA_{19}, GA_{20} and trace amount of GA_1 and GA_9	Leaf and buds	'McIntosh', 'Jonathan'	Si gel partition column chromatography-bioassay-HPLC- GC/MS
Steffens et al. (1992a)	GA_{19}, GA_{20}, GA_1, GA_8, GA_{29}	Shoot tips	Seedlings from a sib-cross obtained from 'Redspur Delicious' x 'Goldspur Golden Delicious'	HPLC, GC-MS
Halińska et al. (1987)	GA_4, GA_7, GA_9	Seeds (partially stratified)	'Antonovka'	GC
Oyama et al. (1996)	GA_4, GA_7, GA_9, GA_{17}, GA_{35}, GA_{54}, GA_{62}, GA_{80}, GA_{84}	Seeds 10 weeks after full bloom	'McIntosh'	GC-MS
Oyama et al. (1996)	GA_{17}, GA_{25}, GA_{45}, GA_{62}, GA_{63}, GA_{80}, GA_{84}	Seeds 14 weeks after full bloom	'McIntosh'	Bioassay on dwarf rice, GC-MS
Stephan et al. (1999a)	GA_1, GA_3, GA_4, GA_7, GA_{20}, GA_{34}	Fruit exudates at the time of flower induction (June)	'Elstar', 'Golden Delicious', 'Jonica,' 'Spencer Seedless'	LC-ESI-MS
Stephan et al. (1997)	GA_1, GA_3, GA_4, GA_7, GA_{20}, GA_{34}	Developing apple fruits	'Jonagold'	LC/ESI-MS
	GA_1, GA_3, GA_4, GA_7, GA_{20}, GA_{34}	Immature seeds	'Jonagold'	LC/ESI-MS
Kittikorn et al. (2010)	GA_1, GA_4	1-year-old shoots	30-year-old 'Fuji' on Malus *prunitoria* (Willd.) Borkh. rootstock	GC-MS

[z]: Only GC-MS or LC-MS provides unequivocal evaluation. The use of HPLC and bioassay alone provides only an indication of hydrophobicity of the GA. As GA_{17} is not biologically active it cannot be found by bioassay.

Apple Genetic Dwarfs

Dwarf apple plants occur naturally in certain breeding populations. Alston (1976) classified apple dwarfs into three different types: early dwarf, crinkle dwarf and sturdy dwarf and suggested a duplicate recessive inheritance. The plants in our study resemble sturdy dwarf.

Steffens et al. (1992b) studied the effect of high temperature, both in controlled growth chambers (constant 27°C and a 20-30-20°C ramped temperature regime) and in the orchard, on tissue culture-propagated dwarf hybrid apple trees selected from an orchard-grown population of 'Goldspur Delicious' and 'Redspur Delicious'. The ramped temperature regime resulted in short internodes, dwarf plants with small leaves similar to the orchard-grown dwarf trees. Orchard-grown dwarf plants (tissue cultured plants derived from dwarf seedlings from 'Goldspur Delicious' x 'Redspur Delicious') had a longer growing period than standard plants (Steffens et al., 1989b). These plants were not further reduced in size by paclobutrazol (a gibberellin biosynthesis inhibitor) treatment, nor rescued by exogenous GA_3 application (Steffens et al., 1989a). After further investigation of the GA content of these plants (Steffens and Hedden, 1992a), they suggested that shoot elongation of dwarf plants was sensitive to elevated temperatures both as a result of reduced responsiveness to GAs and because of a reduction in the concentration of GA_1, apparently, as the authors suggested, as a result of a lower rate of conversion of GA_{19} to GA_{20}. However, if a reduction in the bioactive GA level is partially the cause of the dwarfism, effects of exogenous application of GA_3 would have been expected.

Dwarfing Rootstocks

Dwarfing rootstocks are used commercially to reduce the vigor of apple scion cultivars. However, the exact interaction between scion and rootstock is unclear. Dwarfing caused by an overall reduction in bioactive GAs (achieved through the down regulation of a particular GA 20-oxidase gene by sense and anti-sense over-expression), where dwarfed plants showed both a reduced internode length and number (Bulley et al., 2005) is different from that due to grafting onto dwarfing rootstocks, in that dwarfing rootstocks do not necessarily reduce internode length; but rather they cause the cessation of vegetative growth earlier in the growing season, resulting in fewer nodes (Seleznyova et al., 2003). However as cell division in apices is also influenced by GAs (Davies, 2010) the form could reflect an early cessation of GA production.

Hooijdonk et al. (2005) grafted 'Royal Gala' apple scions to 1-year old rootstock liners of 'M.9' (dwarf), 'M.M. 106' (semi-dwarf), 'Merton 793' (invigorating) and 'Royal Gala' (very vigorous, own rooted scion). At the end of the first season, length and node number were similar on primary shoots, but 'M.9' seemed to limit the number of secondary shoots formed on primary shoots during the summer. Dwarfing apple rootstocks were suggested to limit root-produced GA_{19} supply to shoot apices of the scion. However, Bulley et al. (2005) demonstrated that when the level of bioactive GAs in the scion variety 'Greensleeves' was reduced by the down-regulation of GA_{20}-oxidase, the dwarfing effect was not corrected by grafting the scion onto an invigorating rootstock ('M.M.106' and 'M.25').

Applying $[^3H]GA_4$ to the xylem of grafted apple trees with 'M.9' (dwarfing) and 'M.M.115' (non-dwarfing) interstock respectively (Richards et al., 1986), trees with 'M.9' interstock had less $[^3H]GA_4$ uptake and decreased proportion of radioactive materials transported to the shoot tips and leaves. The grafted union and neighboring tissues of the 'M.9' interstock also held a

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much more amount and a higher proportion of the radio-active materials applied. The transport of the GA, as the authors suggested, appeared to be reduced and the insufficient GAs arrived at the shoot preferably moved to the apex. However, as GC-MS was not used for gibberellins identification, no information regarding the mobility of individual GAs can be drawn from the study.

Gene Function Studies

Genes that regulate key steps in GA synthesis and signaling pathways have also been investigated in apple. Kusaba et al. (2001) detected GA 20-oxidase from 'Fuji' apple primarily in immature seeds 1 to 3 months after full bloom. Zhao et al. (2010) cloned GA-20-oxidase (*MdGA20ox1*), 3-oxidase (*MdGA3ox1*) and 2-oxidase (*MdGA2ox1*) from different tissues[10] of 'Fuji' apple and demonstrated their enzymatic activity[11] in vitro with 17-17-[^{2}H$_2$] substrates. Expression analysis indicated that these genes are tissue-specific: *MdGA20ox1* is strongly expressed in immature seeds but rarely in other tissues; *MdGA2ox1* and *MdGA3ox1* are mainly expressed in flowers.

   DELLA proteins, named for the conserved order of amino acids (DELLA[12], using the standard one letter characterization of amino acids) at N-terminus, mediate gibberellin signal transduction. These proteins belong to GRAS family (a family of plant-specific proteins named after *GAI*, *RGA* and *SCR*, the first three of its members isolated) and are negative regulators in

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[10] Apical shoots with unfolded young leaves, terminal flower buds, flowers at balloon stage and full bloom, immature and mature fruits.

[11] MdGA20ox-MBP fusion protein can sequentially oxidize three times at C-20 of GA$_{12}$ and GA$_{53}$ and generate GA$_9$ and GA$_{20}$; MdGA3ox1-MBP converts GA$_{20}$ and GA$_9$ to GA$_4$ and GA$_1$; MdGA2ox1-MBP converts GA$_4$ and GA$_1$ to GA$_{34}$ and GA$_8$.

[12] D: aspartic acid (Asp); E: glutamic acid (Glu); L: leucine (Leu); A: alanine (Ala).

the GA signaling pathway (Sponsel and Hedden, 2004). The *Arabidopsis* mutant *gai* (GA insensitive) gene carries an N-terminal deletion of 17 amino acids that alters the activity of the protein. Six endogenous *DELLA* proteins were identified from an apple EST database by Foster et al. (2007). The *MdDELLAs* clustered into 3 pairs (*MdRGL1a/b, MdRGL2a/b and MdRGL3a/b*) and their mRNA was highest in summer-arrested shoots tips and in autumn vegetative buds. Research suggests that there is a significant conservation of gene function between *DELLA* proteins from apple and *Arabidopsis*. Transgenic *Arabidopsis* expressing *MdRGL2a* had smaller leaves and shorter stems, took longer to flower under short days and exhibit a reduced response to exogenous GA_3. Over-expressing *Arabidopsis gai* gene in apple cultivars 'Gravenstein' and 'McIntosh' led to reduced growth in-vitro and in the greenhouse, with reduction in stem length, internode length and node number (Zhu et al., 2008).

MdGAI was cloned from the shoot tips of 'Lujia 5' and shown to be expressed both in vegetative and reproductive tissues. While real-time PCR demonstrated that *MdGAI* was always expressed in the shoot apices of both columnar and standard trees during the growing season, columnar apple trees always had a higher expression level (Liang et al., 2011).

Using PCR Xu et al. (2010) investigated the transport of apple endogenous *GAI* mRNA between rootstock (*Malus xiaojinensis*) and scion ('Fuji'). The appearance of *GAI* mRNA in their graft partners led the authors to conclude that *GAI* mRNA moved both upward and downward in grafted trees. In situ hybridization detected *MdGAI* mRNA in the phloem but not the xylem.

Removal of Seed Dormancy

Apple seeds are unable to germinate right after fruit harvest even under optimal conditions. Instead, an after-ripening process, which proceeds under conditions of stratification, is needed to remove this dormancy. Depth of dormancy is measured by the rate of germination under precisely defined temperature conditions and the optimum temperature for intact seed stratification for apples ranges from 4 to 10°C over a 3 month period, depending on the cultivar (Lewak, 1981). This cold-stratification consists of three phases: removal of primary dormancy, a catabolic phase, and the period of growth initiation. Hormone balance has been suggested to play a controlling role in the process (Halińska et al., 1987), but to date no hormone changes have been found to account for the stratification as no hormone application can completely mimic the stratification process.

GA seed research was reviewed by Lewak (2011). Many GAs have been identified in apple seeds at different development stages, including GA_4, GA_7 and GA_9 (Oyama et al., 1996). However, GA_4 is the only GA that undergoes a marked increase during stratification (Sinska and Lewak, 1970) and this can be caused by either a release from its conjugate or 'de novo' synthesis, though this is probably unreliable given the detection techniques used and the state of GA knowledge at the time.

Using the incorporation of $[^{14}C]MVA$ to putative GA_4 in the absence and presence of a GA-biosynthesis inhibitor, Sinska and Lewak (1977) demonstrated that GA_4 biosynthesis is needed for the removal of seed dormancy.[13] Their research suggested that the last phase of cold

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[13] Isolated apple embryos after different times of culture were homogenized in 96% ethanol and the concentrated filtrate was separated by a thin-layer chromatography. The zone corresponding to GA_4 was eluted and the content of GA_4 was determined in the elute using the bioassay based on the induction of α-maylase in barely seeds and this was further confirmed when the GA biosynthesis inhibitor (AMO-1618), which causes a decrease in GA_4 content in partly stratified embryos, simultaneously inhibited the germination process.

stratification was not directly controlled by GA_4, supported by the lack of inhibitory effect of (AMO-1618) on GA biosynthesis, as well as germination of embryos isolated from seeds stratified for 60 to 85 days.

Sinska and Lewis (1977) also investigated the relationship between GA and seed dormancy. They observed a temporary rise followed by a decrease in putative GA_{4+7} during cold stratification and germination of embryos isolated from dormant seeds. GA_9 was absent in dormant embryos, but increased significantly during cold stratification of non-dormant embryos. They suggested that GA_{4+7} controlled some processes in the first two phases (cold-mediated removal of dormancy) and GA_9 was involved in the induction of normal seedling development (Halińska et al., 1987). By examining both the free and conjugated forms of GA_{4+7} at different stages, they concluded that the significant increase of these two hormones in the first 30 days of stratification may be a result of both biosynthesis and release of GA_{4+7} from their conjugates, while the decrease of concentration of GA_{4+7} after 40 days of stratification was probably caused by simultaneous conjugation and degradation.

Flower Initiation and Biennial Bearing

In apple, flower bud initiation occurs early in the growing season (5 to 10 weeks after full bloom) at the tips of meristems located on lateral spur shoots. Seeded fruits exert some inhibitory effects on flower bud initiation. Biennial bearing, the alteration of heavy fruiting during 'on years' and weak fruiting in 'off years', is only observed on seed-containing apple cultivars, but not on seedless cultivars.

Developing apple seeds are exceptionally rich in GAs. Steffens et al. (1992) found predominantly GA_4, GA_7 and GA_9 in apple seeds and a low amount of GA_3. A GA_3/GA_4 (8:1) ratio was observed in fruit exudates of 'Elstar', a cultivar with biennial bearing; while the ratio changed in favor of GA_4 (1:5) ratio in exudate of 'Spencer Seedless', a regular bearing apetalous cultivar (1997). The highest levels of GAs occur 6 to 10 weeks after full bloom, which is the critical time of flower initiation.

GAs are thought to be responsible for the inhibition of flower bud initiation and for the biennial bearing of individual spurs and even the whole tree (Ramírez et al., 2004). Several genes involved in the GA biosynthesis pathways were located in QTL cluster intervals[14] for biennial bearing (Guitton et al., 2012).

Exogenous GA sprays, especially GA_3 and GA_7, inhibited flower induction in the current year in 5 years old 'Delicious' on 'M.7' rootstock and also reduced flowering the following year (Unrath and Whitworth, 1991).

Native GAs may differ significantly in their effects on the inhibition of flower bud initiation. To establish the causal relationship between GAs in seeds and inhibitory effects of flower initiation, the type of GA in seeds, whether the GAs in bourse buds[15] are from seeds rather than fruits (as the pattern of GAs exported out of the fruits can differ from the GA content in the seeds), the nature of any change of GA level, and the comparative timing of changes all need to be considered (Looney, 1985).

When [^{3}H]GA_4 was applied to seeds within the fruit of 'Delicious', the ^{3}H-GA_4 moved out of the fruit into the spur tissue, particularly to the bourse bud (Ramirez, 2004). This transport is likely

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[14] LG1: MdGA20ox1a, MdGA3ox-like-b; LG10: MdGA2ox8a
[15] Bourse bud: the flower growth unit with leafy basal part and a distal floral part.

via the xylem (Hamilton and Davies, 1988; Lang, 1990). While Ramirez (2004) demonstrated that the GAs from the seeds can be transported to the bourse bud, the timing was not discussed, and the effects of GAs from the fruits were not excluded. Kittikorn et al. (2010) demonstrated that endogenous GA_1 and GA_4 content in apical buds of heavy crop load (HCT) apple trees were higher than in the fruit thinned treatment group. In addition, HCT GA levels were highest at 60 days after full bloom (DAFB), the time at which flower buds are initiated. They concluded that high endogenous GA (GA_1 and GA_4) levels are negatively correlated with flower bud formation in apples. When Tromp (1982) compared applied GA_3, GA_4 and GA_7 for their effects on apple tree flowering, GA_4 was the only GA that did not inhibit flowering. GAs were still identified in the fruit exudates from 'Spencer Seedless', which has no seeds as the source of GA (Stephan et al., 1997). Looney et al. (1985) even demonstrated that certain GAs can promote flowering on apple spur shoots. Stephan (1999b) also observed GA_4 as the main compound exported by fruits of cultivars with little or no biennial bearing, supporting the finding that GA_4 may promote return bloom.

A biennial bearing study of 114 individuals from 'Starkrimson' (strong biennial bearer) by 'Granny Smith' (regular bearer) revealed that candidate genes involved in flowering were not co-localized with the QTLs for production and alteration whereas genes related to control of amounts of the hormones auxin and GA co-localized. These results suggested that biennial bearing is not likely to be directly controlled by floral integrator or meristem identity genes; rather, their control by plant hormones might be the determining factor in the decision to flower (Guitton et al., 2012).

Fruit Development

Exogenous GA applications affect the russetting of growing fruitlets and the elongation of fruits (Unrath and Whitworth, 1991; Greene, 1993). A commercial spray of GA_{4+7} and BA (benzyladenine) at bloom is used to give 'Delicious' that distinctive elongation (lobing) at the bottom of the fruits. Russetting is a genetic disorder due to suberization of epidermal cells as a reaction to cuticle cracking during the first development of fruitlet and can also be affected by climate factors. Multiple applications of GA_{4+7} are used to reduce the incidence and severity of skin russeting in apples. By studying the GA content of fruitlets of different varieties and of different 'Golden Delicious' clones for two subsequent years, a close correlation between GA_4 content of growing fruitlets and apple skin russeting was confirmed (Di Lella et al., 2006), with the 3 to 4 weeks period after full bloom the susceptibility period for russet development.

MATERIALS AND METHODS

Plant Material

Vigorous shoots from 'Redcort' on 'M.M.106' rootstock (an invigorating rootstock) in the Ithaca orchard were collected on June 10th, 2011 for the examination of GA_{12} metabolism and for GA identification.

Another series of treatments examined apple progeny 806 that was generated in 2007 by crossing Selection 1, a hybrid of 'Fuji' x Co-op 18 with an advanced breeding selection ('Honeycrisp' x 'Gala') with good fruit quality and a standard tree form. All the seeds harvested in 2007 were stratified in the refrigerator for 90 days and planted in pots in the greenhouse in January 2008 with a day temperature of 21°C and night temperature of 17°C. Seedlings were

later transplanted into an orchard in Geneva, NY on their own roots. Vigorously growing shoots were collected from standard and dwarf progeny from population 806 in 2010, their third growing season, for GA biosynthesis pathway comparison. Seedlings used in this work were treated in May 2010.

[^{14}C]GA$_{12}$ Synthesis

[^{14}C]GA$_{12}$ was synthesized from [^{14}C]mevalonic acid, using a pumpkin endosperm extract by Anna Halinska and purified by Strata-X SPE and HPLC (Davies et al., 1986; Halinska et al., 1989). The identity of the [^{14}C]GA$_{12}$ fraction from HPLC was confirmed by the author using GC-MS (data not shown).

[^{14}C]GA$_{12}$ Application

Shoot tips of 'Redcort' were previously excised from orchard trees. Shoots about 25 cm in length were harvested. When brought back to the lab, they were cut under water just above the third fully expanded leaf below the apex (weighting between 2.5 to 4 g) and the base of the cut stem of each shoot tip was placed in a 1.5-mL of treatment solution polystyrene vial with a V-shaped bottom.

For shoots from 'Redcort', each vial contained 0.5 mL of water with 0.2 µCi [^{14}C]GA$_{12}$ (560 pmol, 190 ng) (Zhu et al., 1988). Cuttings were left in the light under fluorescent lamps at 5.17 µmoles m^{-2}.s^{-1} for 30 min, 1 h, 3 h, 6 h, or 48 h. There were three replicates for each time period. Water in 0.5 mL aliquots was added when the vials were nearly empty. Uptake occurred at an

average rate of about 1 mL/h, although varied due to shoot vigor. The shoots in the 48 h treatment were placed in a 100 mL beaker with water after they had been in the vial for 12 h.

Shoots from progeny 806, were similarly cut and placed in vials. Each vial contained 0.5 mL of water with 0.1μCi [^{14}C]GA$_{12}$ (280 pmol, 95 ng). Cuttings were left in the light for 15 min, 30 min, 1 h, 3 h, 6 h, 12 h, 24 h or 48 h. Water in 0.5-mL aliquots was added when the vials were nearly empty. The shoots in the 24 h and 48 h treatment were placed in a beaker with water after they had been in the vial for 12 h.

After the treatments the shoots were frozen in liquid nitrogen and stored at -80°C. There were two replicates for each time period and each experiment was conducted twice for both the standard and dwarf plants.

Gibberellin Extraction and Purification

All solvents used were HPLC grade. Glassware was baked at 500°C to destroy any contaminating GAs and then silanized with Aquasil (Pierce, Rockford, IL). Each frozen shoot tip was individually placed in 20 mL ice-cold 80% methanol and ground with a 2-cm head Polytron (Brinkman Instruments, Westbury, NY), which was rinsed twice with 80% methanol. The rinses were combined with the extract. The homogenate was left overnight at 4°C prior to vacuum filtration through Whatman filter paper with pad of filter aid (Highflo Super Cel®, Sigma-Aldrich, St. Louis, MO) followed by three-pad volume rinse of 80% methanol. The filtrate volume was reduced on rotary evaporator at 36°C to partially remove the methanol. 1 mL NH$_4$OH and 20 mL hexanes were added to each sample and the mix shaken vigorously to partition the chlorophyll into the hexanes. Then evaporation was resumed to precipitate

chlorophyll upon the removal of the hexanes, and then until all the methanol was gone and the volume was reduced to about 20 mL. The sample was acidified to pH 3 to 3.5 with acetic acid and vacuum filtered through filter-aid again with two-pad volume rinse of acidified water (0.2% acetic acid, pH 3.5).

A Strata-X, and later Strata-X-A/XL-A for improved results, SPE Cartridge 6 mL (Phenomenex, Torrence, CA) was washed with 5 mL of 100% MeOH and 10 mL of acidified H_2O. The sample was loaded via the reservoir on top of the cartridge. The cartridge was washed with flask rinse and 2 mL of acidified water and eluate was discarded. 4 mL 100% MeOH was used to wash the cartridge and eluate was collected in a polystyrene tube and stored in the freezer until further use.

As the methanol concentration was reduced in concentrated samples, a material precipitated in large amounts in filters or on attempts to inject into HPLC columns, frustrating the purification of any other than a minute amount of extract. This material was either a cream-colored precipitate or brown and oily. It was soluble in methanol and in methanol-water down to about 20% ethanol. Attempts to partition the GAs into acidic ethyl acetate, leaving the contaminating material in the aqueous phase, were unsuccessful, as the contaminant also partitioned into ethyl acetate. Dr. Lailiang Cheng suggested phlorizin as a possible identity for this material and this was supported by its material properties and its high concentration in the bark of apple trees (Gmelin, 1864; Faust, 1989; Gosch et al., 2010).

Phlorizin removal from later samples was accomplished using Strata-X-A and Strata- XL-A mixed function reverse phase anion exchange cartridges (the XL having a larger pore size suitable for larger samples or those with some very fine particulates) (Phenomenex). These

cartridges were washed with 1mL methanol and equilibrated with 1mL water. Bulked samples were diluted to less than 20% MeOH and buffered to pH 6 to 7. Then they were loaded to the reservoir on top of the Strata- XL-A cartridge and drawn through by vacuum. The eluate then was loaded to Strata-X-A and vacuum filtered through to ensure the capture of all the wanted GAs. Both columns were washed with 25% ammonium acetate followed by 100% MeOH with the eluate, containing the phlorizin, discarded. Lastly, both cartridges were washed with 5% formic acid and then 4 mL 100% MeOH. The eluate, containing the GAs, was combined in a tube and stored in the freezer until further use. This succeeded in removing the phlorizin and enabled HPLC purification.

HPLC purification

Each sample was evaporated to dryness under N_2 at 37°C and container was rinsed with 0.1 mL MeOH and transferred to a 0.45 µm centrifugal filter (Corning Spin-x, Lowell, MA) tube followed by two further rinses of 0.3 mL of H_2O with 0.2% acetic acid. The solution was then filtered by centrifuging at 8,000 rpm for 5 min. Each sample of ca. 0.7 mL was loaded onto an analytical C18 HPLC column (0.46 x 25 cm, Synergi 4u Hydro-RP 80A (Phenomenex) using a 1 mL injection loop, and run at 1 mL/min in an H_2O (containing 2 mL/ L glacial acetic acid) (A) to acetonitrile (B) gradient. The gradient used was: 27% B for 2 min, 27 to 33% B over 5 min, 33 to 35% B over 4 min, 35 to 70% B over 15 min, 75 to 100% B over 5 min, and holding at 100% B. The column eluate passed through an in-line radioactivity monitor (Tace 7140, Packard, Downers Grove, IL) equipped with a flow cell packed with insoluble scintillator beads (170 µL void volume). The efficiency of the monitor was approximately 10% for [14]C. An automatic

peak-detection circuit controlled an LKB fraction collector (Bromma, Sweden) and collected each individual peak for all the samples.

Identification of GC Retention Time for GAs with [²H] Standards

[²H]GA standards (GA$_1$, GA$_3$, GA$_4$, GA$_7$, GA$_8$, GA$_9$, GA$_{12}$, GA$_{15}$, GA$_{19}$, GA$_{20}$, GA$_{29}$, GA$_{34}$, GA$_{44}$, and GA$_{53}$) were obtained from Professor Lew Mander (Research School of Chemistry, The Australian National University, Canberra, Australia, synthesized by Mr. Tony Herlt). 50 ng of each GA were combined six at a time and methylated with a surplus of ethereal diazomethane (see appendix for diazomethane synthesis). The mixture was then dried and re-dissolved in methanol before transfer to 1 mL tapered glass vials (Chromcol, Fisher, Sun SRi™, Rockwood, TN), and dissolved in 2 µL pyridine and 10 µL BSTFA (bis-trimethyl silyl trifluoro-acetamide) containing 1% TMCS (trimethylchlorosilane) (Supelco, Bellefonte, PA). After 40 min in an oven at 80°C GC-MS analyses were performed with a Hewlett-Packard 5890A gas chromatograph (Palo Alto, CA) connected to a 5970B Mass Selective Detector. Samples (1 µL) were injected without splitting onto a 0.18 µm (L=20 m x I.D. = 0.18 mmdf) Zebron ZB-5MS column (Phenomenex). The temperature program for the GC was injection at 60°C, increasing to 240 °C at 30°C/ min, to 275°C at 4°C/ min and finally to 325°C at 30°C/ min. The injection of the mixture was analyzed in 'SIM' mode to monitor the most abundant ion and that ion +2 for each GA (Gaskin and Mac Millan, 1991).

Retention time obtained for each GA standard was used to adjust the GC program to monitor specific GAs at specific time windows to increase sensitivity. The standard mixture was run at the beginning of each day before analysis of the apple samples.

Identification of Endogenous GAs Using GC-MS

The same HPLC peak from different apple samples were bulked together and methylated with a surplus of ethereal diazomethane. The methylated samples were re-chromatographed using the analytical column and the same gradient (described above). This provided an extra purification, since the retention time of the methylated compounds was later than the non-methylated GAs.

Samples were dried and re-dissolved in methanol before transfer to 1 mL tapered glass vials (Chromcol, Fisher, Sun SRi™), and dissolved in 2 µL pyridine and 10 µL BSTFA (bis-trimethyl silyl trifluoro-acetamide) containing 1% TMCS (trimethylchlorosilane) (Supelco, Bellefonte, PA). After 40 min in an oven at 80°C, GC-MS analyses were performed with a Hewlett-Packard 5890A gas chromatograph (Palo Alto, CA) connected to a 5970B Mass Selective Detector. Samples (1 µL) were injected without splitting and run under the same temperature program as the standard mixture. The first injection of each sample was under the 'SIM' mode to monitor the most abundant ion for each GA plus 16 at the specific time window. After preliminary results were obtained, a second injection monitoring more (+16) ions from the GA spectrum was used to confirm the identity of the GA.

RESULTS

GC-MS Retention Times of [³H] GA Standards

The retention times of standard GAs varied slightly from run to run but largely were consistent. The ions monitored for each GA and their retention times from one sample run are presented in Table 3-2.

Table 3-2. Ions monitored and retention times of [³H]GA standards.

GA	Ions	R.T (min)
GA_9	298/300	10.818
GA_{12}	300/302	10.897
GA_{53}	448/450	12.064
GA_{20}	418/420	12.082
GA_4	284/286	12.245
GA_7	222/224	12.606
GA_{19}	434/436	13.217
GA_{34}	506/508	14.131
GA_1	506/508	14.149
GA_{29}	506/508	14.149
GA_3	504/506	13.921
GA_{15}	239/241	14.257
GA_8	594/596	14.960
GA_{44}	432/434	15.759

Metabolites of [¹⁴C]GA12 in 'Redcort'

Sixteen metabolites of [¹⁴C]GA_{12} were identified in 'Redcort' apple on 'M.M.106' rootstock (Figure 3-4). Generally, GA metabolism produces compounds of increasing polarity and as the metabolism progresses more polar compounds are produced and eluted from the HPLC (Davies et al., 1986). The feeding material [¹⁴C]GA_{12} was the last compound to elute. For convenience, major metabolites were designated with the letters A to U, corresponding to increasing polarity.
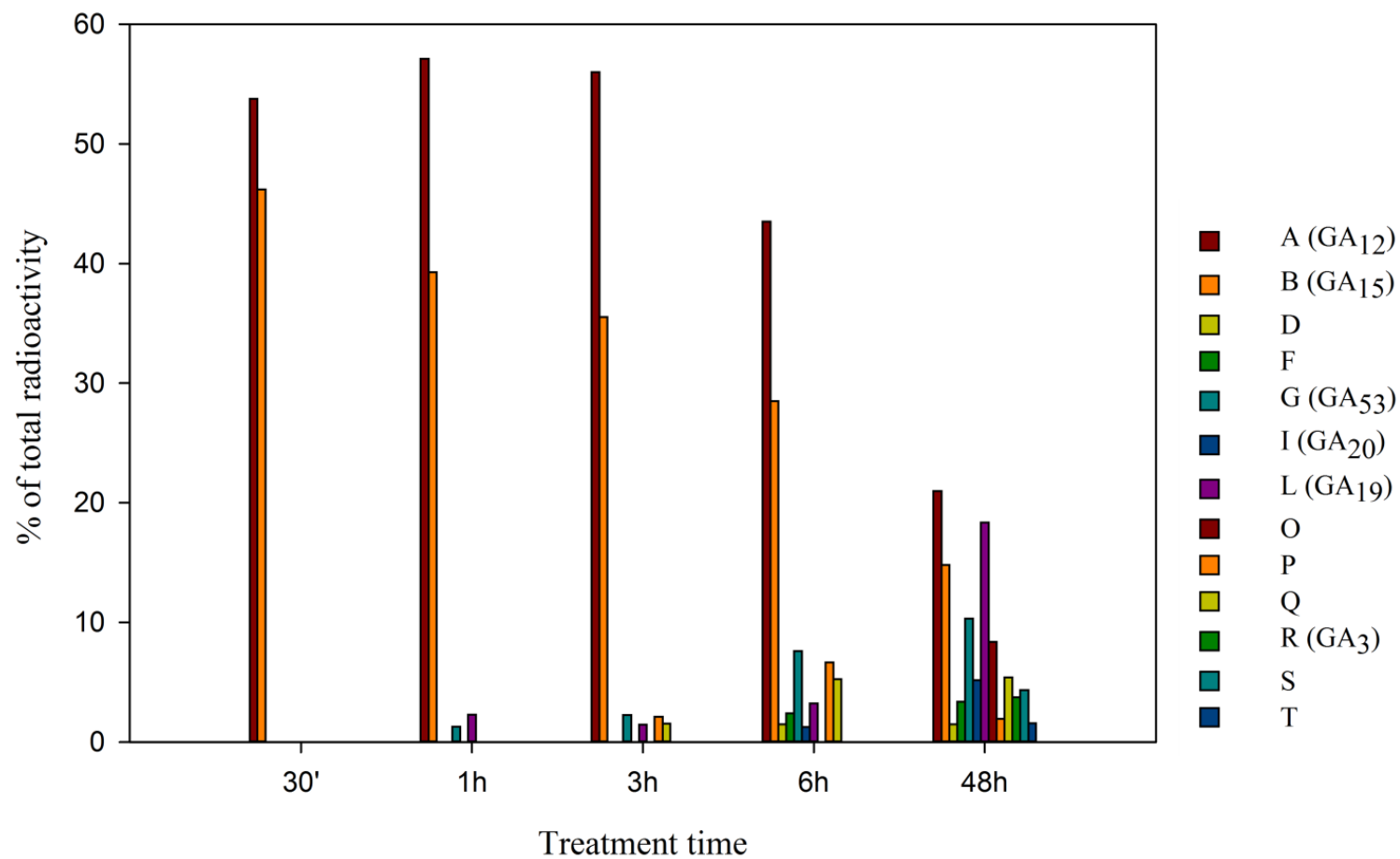
Figure 3-4. [^{14}C]GA$_{12}$ metabolism in 'Redcort' apple shoots from 30 min to 48 h. Radioactivity was calculated by HPLC peak area.

In 'Redcort', 50% of the feeding material A was metabolized quickly into peak B at 30 min. Other early metabolites started to be synthesized at 1 h. At 48 h, thirteen of the sixteen metabolites detected in 'Redcort' were evident in the HPLC chromatograph (Figure 3-5) and the major metabolites included peaks L, G, I, A and B. Samples were not collected between 6 and 24 h, so the initial appearance and trend of certain metabolites may have been missed.
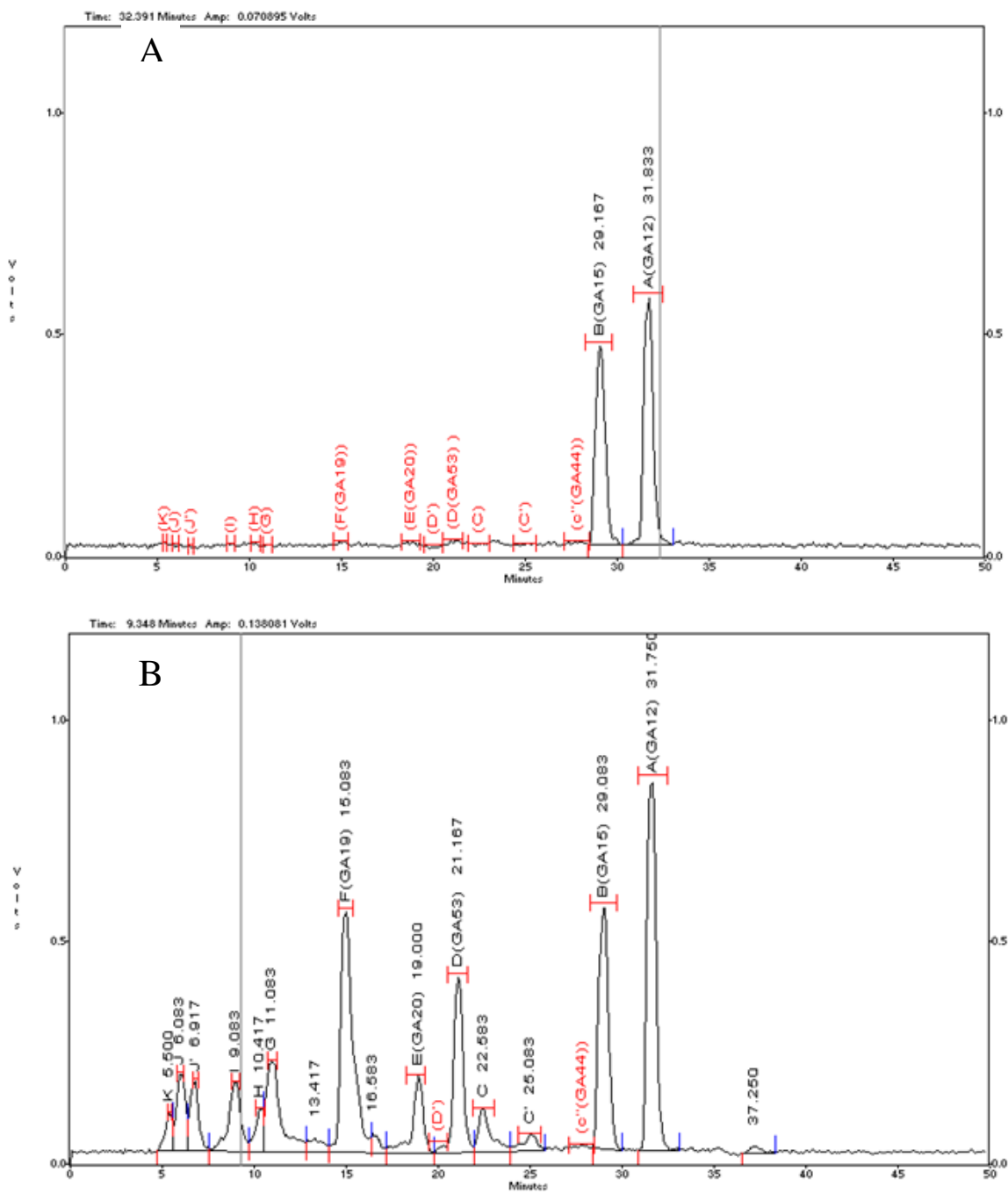
Figure 3-5. Metabolites of [^{14}C]GA$_{12}$ in 'Redcort' apple shoots at 30 min (A) and 48 h (B). Peaks with red letters were not detected.

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Peaks collected from the same Rt on HPLC were bulked together for methylation and further purification. Phlorizin tended to precipitate out at this stage forming a brown gluey fraction at the bottom of the containers. The supernatant of the concentrated sample was labeled sample 1 and the bottom fraction that went through phlorizin removal was labeled sample 2. These two samples were methylated and further analyzed by HPLC.

Twelve methylated metabolites were detected in sample 1 and nine in sample 2. Metabolites beyond peak L (O, P, Q, R and S) were all missing in sample 2 (Figure 3-6). Peak C (Rt 27.92), E (Rt 24.5) and H (Rt 20.00) were only detected in sample 2 but not in sample 1, nor were they detected in individual samples collected from each time point. A likely reason for the differences between sample 1 and sample 2 is that the columns used for sample 2 preparations were in acidic conditions whereas for sample 1 they were not. This led us to speculate that peaks (O, P, Q, R and S) were sugar conjugates[16].

Peak T was detected in individual 48 h HPLC sample but was not detected in the combined samples, possibly a result of its small quantity.

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[16] Conjugation is one strategy that plants use to regulate bioactive GA concentrations and conjugation to glucose is mostly found. GA conjugates were suggested to be present in the metabolites of GA_{12}-aldehyde in pea shoots from G2 line (Davies et al., 1986) and in the metabolites of GA_{12} from potato shoots (Van den Berg et al., 1995). However, these metabolites were also present in shoots from 806 which didn't go through the acidic SPE columns. To confirm whether these metabolites are GA-conjugates, base hydrolysis could be conducted to yield free GAs, which would then be further purified on HPLC and identified by GC-MS (Koshioka et al., 1983).
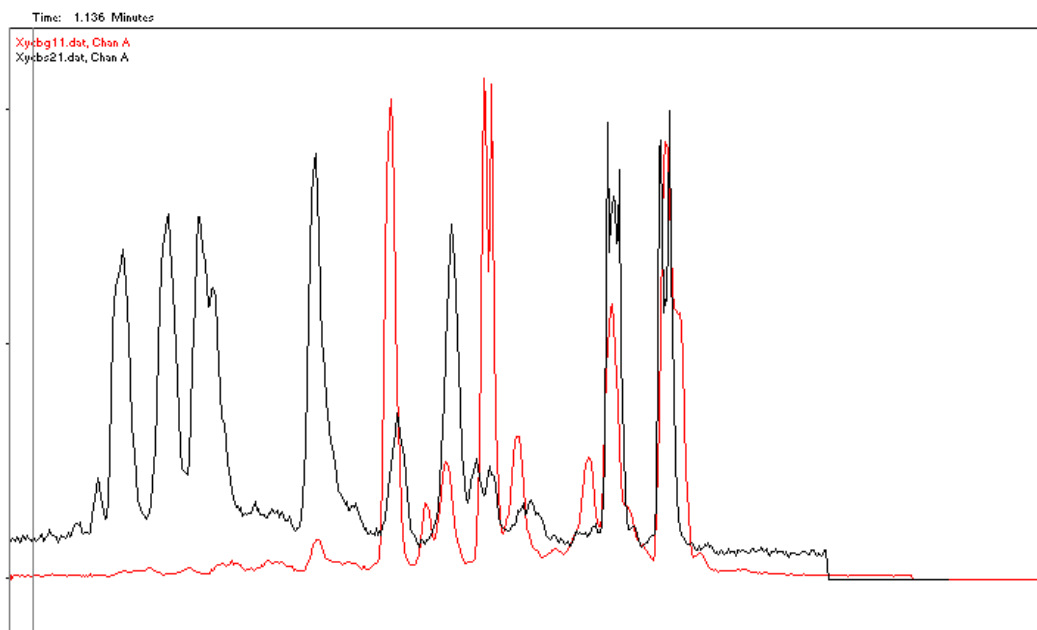
Figure 3-6. HPLC chromatographs comparison of 'Redcort' sample 1 and 2 (black: sample 1; red: sample 2).

Identification of GAs with GC-MS

By following the radioactive peaks, the endogenous GAs were co-purified. By running on GC-MS and searching for the appropriate ions of the endogenous and [^{14}C]GAs, GA_{12}, GA_{15}, GA_{53}, GA_{44}, GA_{19}, GA_{20}, GA_3 (in that metabolic order) were found to be present in shoots of the apple variety 'Redcort' on 'M.M.106' rootstock (Table 3-3).

The metabolic pathway of common GAs in plants is shown in Figure 3-7, those in apple with their metabolic pathway in Figure 3-8, and molecular structure of the detected GAs in Figure 3-9. The relative HPLC elution times of these GAs are consistent with results from Koshioka et al. (1983). The native and ^{14}C mass ions for all the GAs for which [^{2}H]GAs were available (e.g.,

GA$_4$, GA$_7$, GA$_1$, GA$_9$) were checked in every appropriate HPLC fraction without any others being found.

Table 3-3. Endogenous GAs in 'Redcort' identified with GC-MS.

Peak	GA	HPLC Rt* non-methylated (mins)	HPLC Rt Methylated (mins)	Ions monitored in GC-MS	GC-MS Rt	Reported KRI
A	GA$_{12}$	31.7	37.9	300/316, 328, 285, 241	10.897	2333
B	GA$_{15}$	29.1	35.5	239/255, 284, 312, 344	14.257	2605
G	GA$_{53}$	21.1	33.8	448/464, 416, 389, 373	12.082	2497
C	GA$_{44}$	27.9	34.42	432/448, 373, 238, 417	15.759	2786
L	GA$_{19}$	14.8	33.2	434/450, 402, 374, 345	13.217	2569
I	GA$_{20}$	18.4	24.9	418/434, 403, 375, 359	12.064	2482
R	GA$_3$	6.9	12.3	504/520, 475, 445, 311	13.921	2692

*: Retention time. After methylation, each peak may separate to multiple methylated peaks. The Rt for methylated peak is for the peak that was identified as GAs.

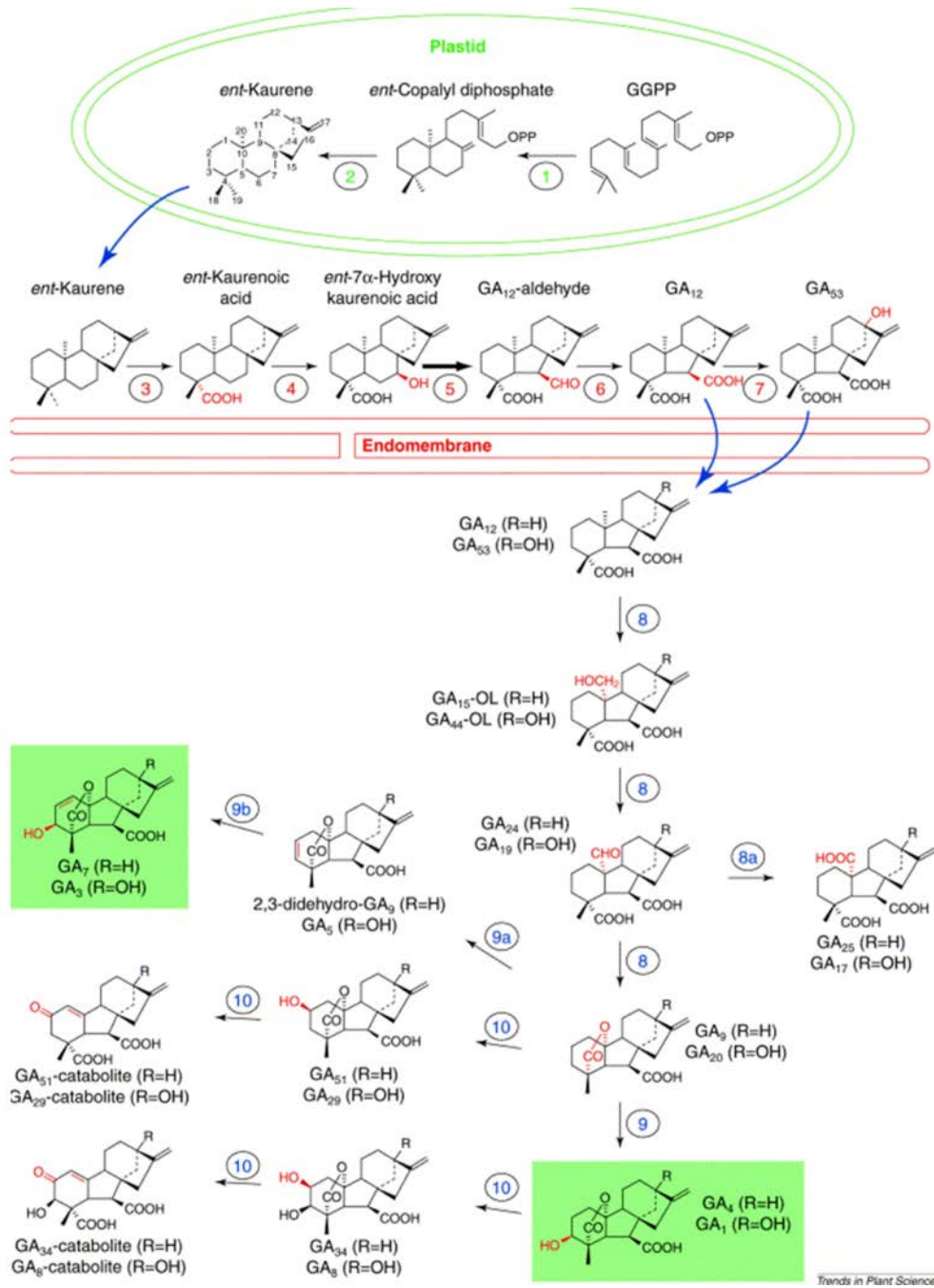**Figure 3-7.** GA biosynthesis pathway (Hedden and Phillips, 2000).
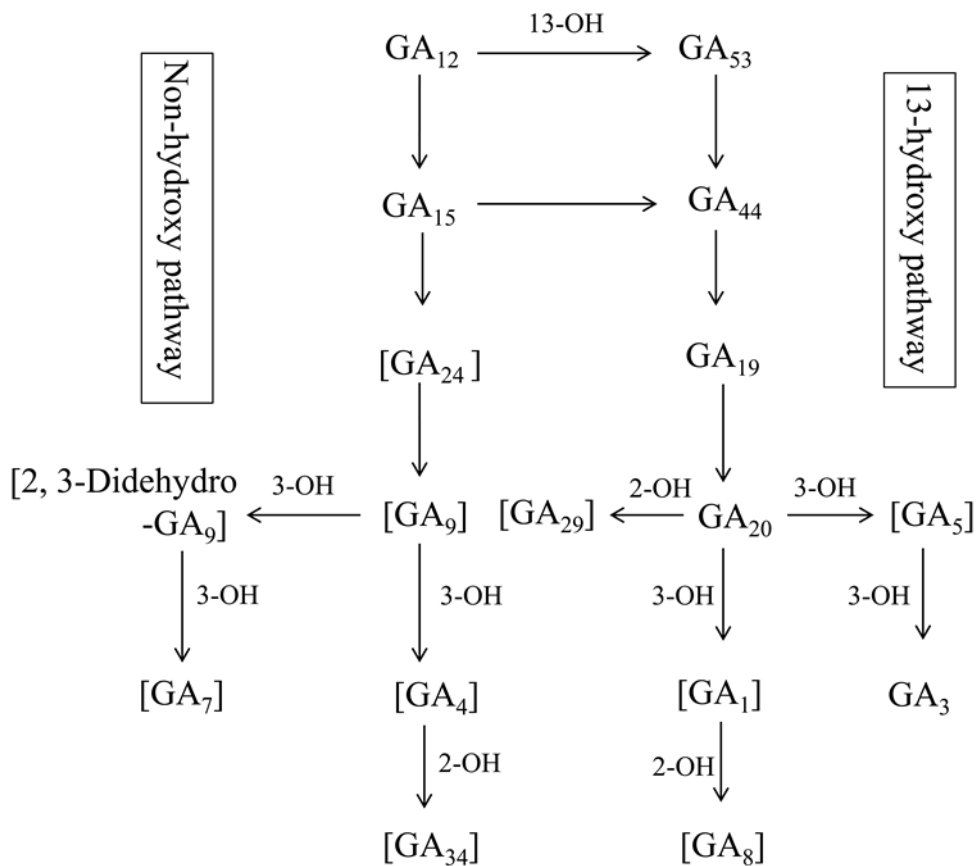
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Figure 3-8. Metabolic pathway of GAs identified in 'Redcort' apple shoots and other GAs detected by others (see Table 1) in apple. GAs in parenthesis were not detected in this study.
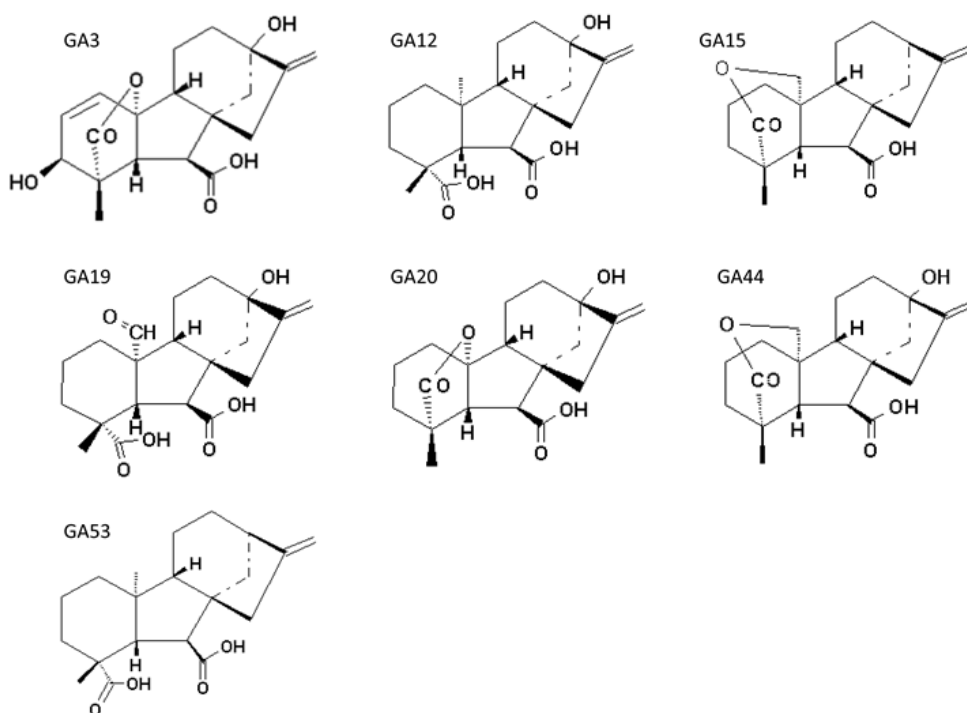
Figure 3-9. Structures of GAs identified in 'Redcort' apple shoots (http://www.plant-hormones.info/ gibberellins.htm).

During the study, GA_{12} and GA_{15} followed similar metabolic trends. GA_{12} was consumed to produce GA_{15} and GA_{53}, the latter was metabolized further to produce the bio-active GA_3. Based on the existence of GA_{20} and GA_3, GA_5 also should exist in shoots from 'Redcort'. GA_{53} and GA_{19} both were detected at 1 h, while GA_{20} was not detected until 6 h, a sign that GA_{19} to GA_{20} was a rate-limiting step. GA_3 was first detected at 48 h, but as no sample was collected adjacent to 6 h, the initial appearance of GA_3 could not be determined, nor whether GA_{20} to GA_3 was a fast or slow metabolic step (Figure 3-10). GA_{44}, the intermediate between GA_{53} and GA_{19}, was not detected in any samples from individual time periods, but its occurrence can be inferred from

the presence of both precursor and product. Its metabolism through to GA_{19} must therefore be fairly rapid.
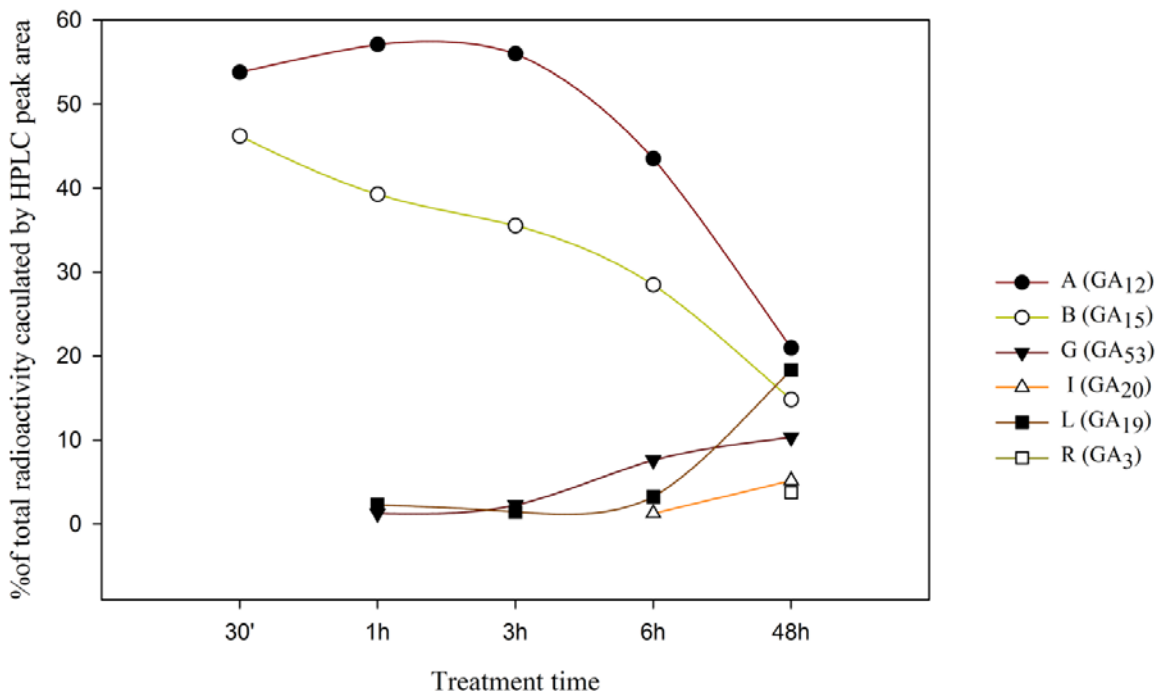
Figure 3-10. Metabolic trends of individual GAs in 'Redcort' apple shoots.

Every HPLC peak was specifically tested by GC-MS for the ions of every GA for which standards were possessed. Despite the presence of GAs on the non-hydroxy and 13-hydroxy pathways, the following GAs were not detected, in spite of their expected appearance: GAs 34, 9, 1, 29 and 8. Neither was there any trace of GAs 4 and 7, which are the most common GAs in apple seeds and fruits. We conclude that either these compounds were not produced or that they are present below the level of our detection (about 1ng/g), and if present as a radioactive metabolite their presence is transitory because of rapid further metabolism.

Comparison of Biosynthesis Pathway in Dwarf and Standard Apple Seedlings in Progeny 806

The reproducibility of HPLC runs enabled comparison among metabolites produced in 'Redcort' and progeny 806. The same peak numbering system for metabolites was used in 806 and eighteen [^{14}C]GA$_{12}$ metabolites were identified (Table 3-4). Among those, peaks J, K, M, N, T and U were not detected in 'Redcort'. Peak N only existed in standard plants between 3 and 6 h. Peak E was not detected in sample 1 from 'Redcort', but was detected in both standard and dwarf seedlings. Peak C and H, only detected from sample 2 in 'Redcort', were not detected in 806.

The major metabolite O was not identified as to GA, but it was the dominant peak after 12 h in 806-standard plants. In contrast, peak Q, G (GA$_{53}$) and L (GA$_{19}$) were all major metabolites for 806-dwarf seedlings at the second half of the time course.

Dwarf and standard plants in 806 demonstrated similar metabolic trends during the study (Figure 3-11 and Figure 3-12). Early and non-prominent metabolites started to appear between 15 min and 1 h. Most metabolites were seen between 3 and 12 h, then the number of metabolites started to decrease. Comparing typical HPLC chromatograph results from dwarf plants at 15 min, 6 h and 48 h, metabolites with more polarity were produced later in the time course when the feeding materials were almost consumed (Figure 3-13).

Table 3-4. Comparison of metabolites identified in different apple plant materials.

	GA	Peak	'Redcort'		806	
			Sample 1	**Sample 2**	**Standard**	**Dwarf**
1	GA_{12}	A	X	X	X	X
2	GA_{15}	B	X	X	X	X
3	GA_{44}	C		X		
4		D	X		X	X
5		E		X	X	X
6		F	X	X	X	X
7	GA_{53}	G	X	X	X	X
8		H		X		
9	GA_{20}	I	X	X	X	X
10		J			X	X
11		K			X	X
12	GA_{19}	L	X	X	X	X
13		M			X	X
14		N			X	
15		O	X		X	X
16		P	X		X	X
17		Q	X		X	X
18	GA_3	R	X		X	X
19		S	X		X	X
20		T			X	X
21		U			X	X
Total		21	12	9	17	18
			'Redcort': 16 peaks		806: 18 peaks	
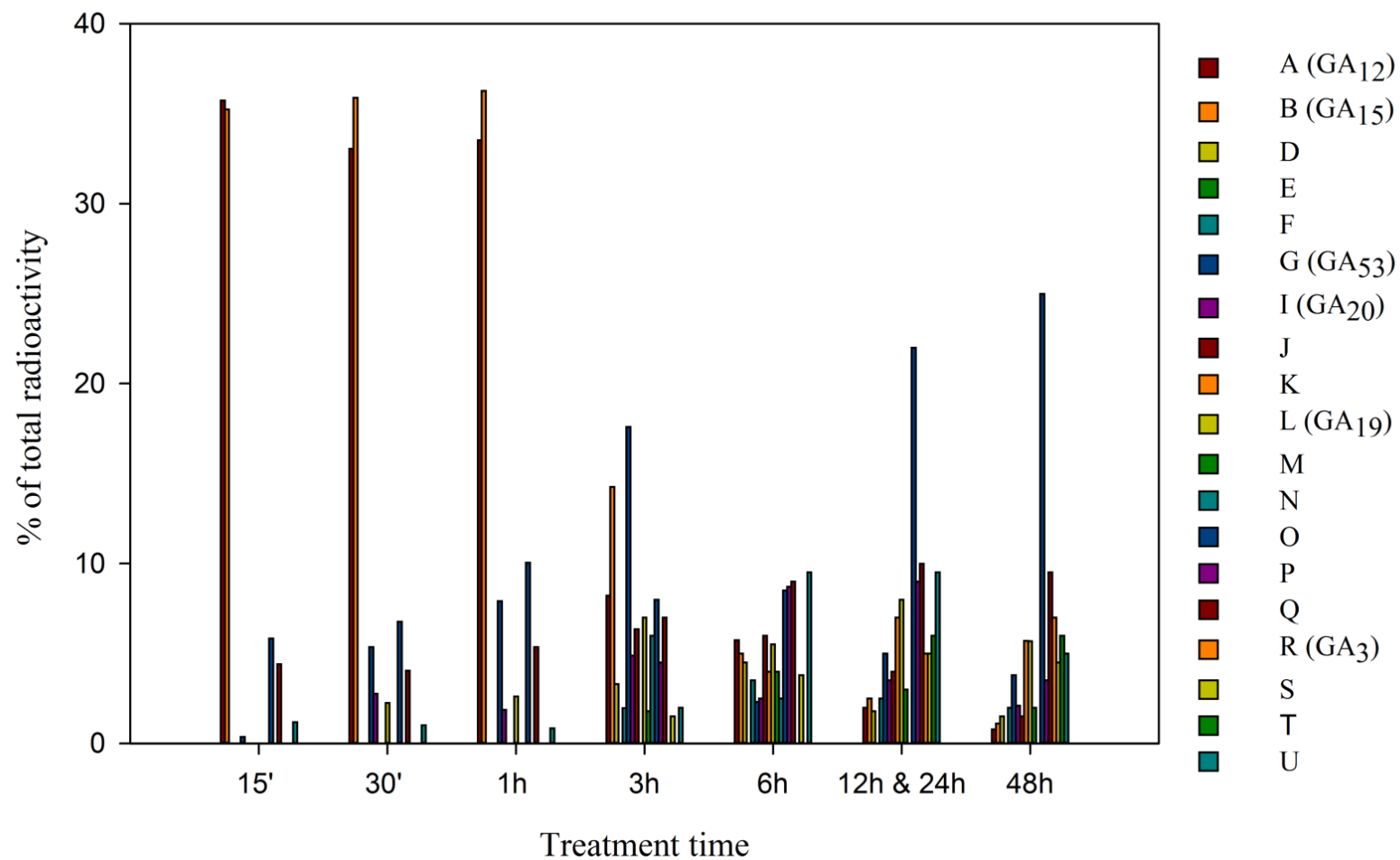
Figure 3-11. [^{14}C]GA$_{12}$ metabolism in standard apple seedlings from progeny 806.
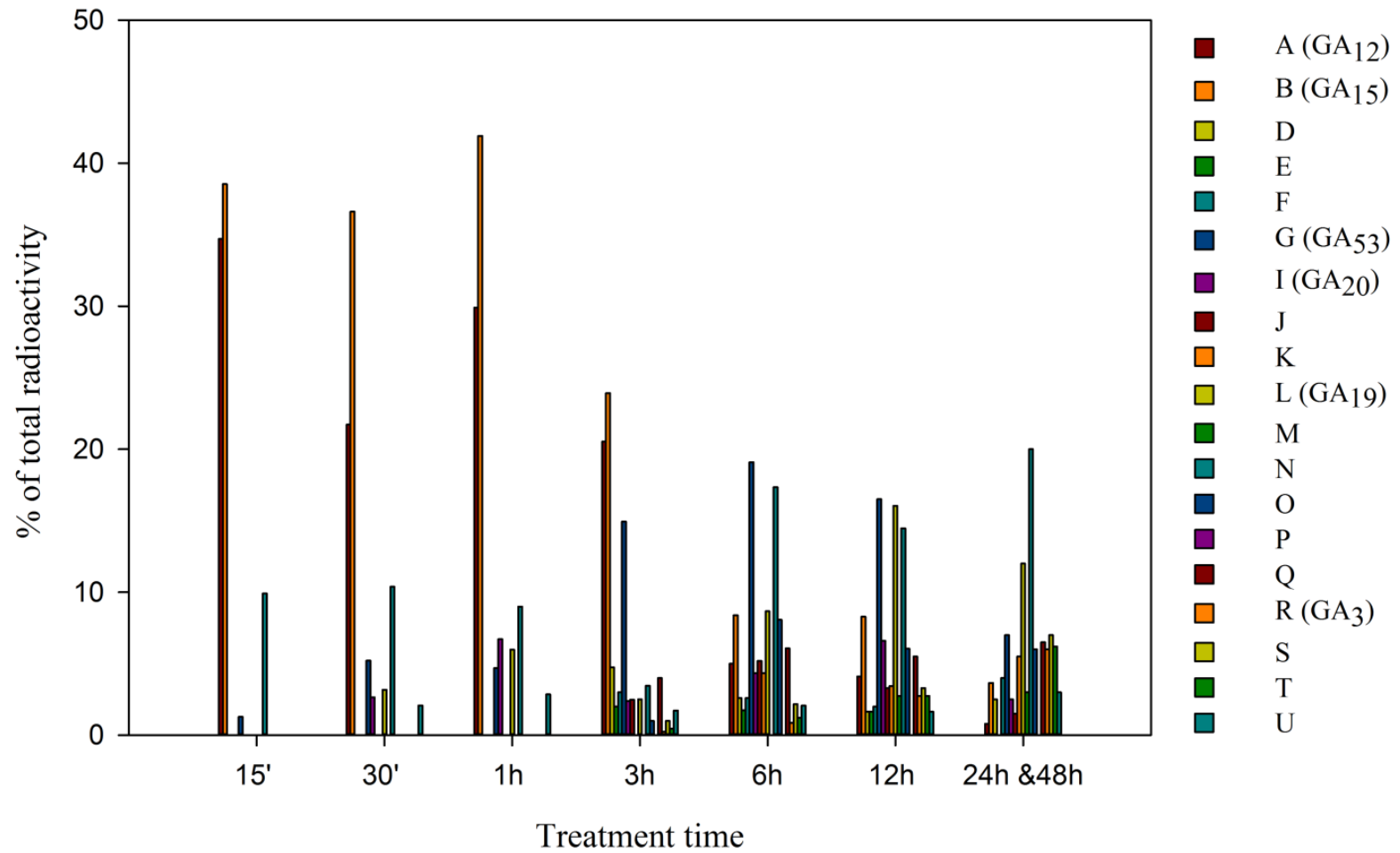
Figure 3-12. [^{14}C]GA$_{12}$ metabolism in dwarf apple seedlings from progeny 806.
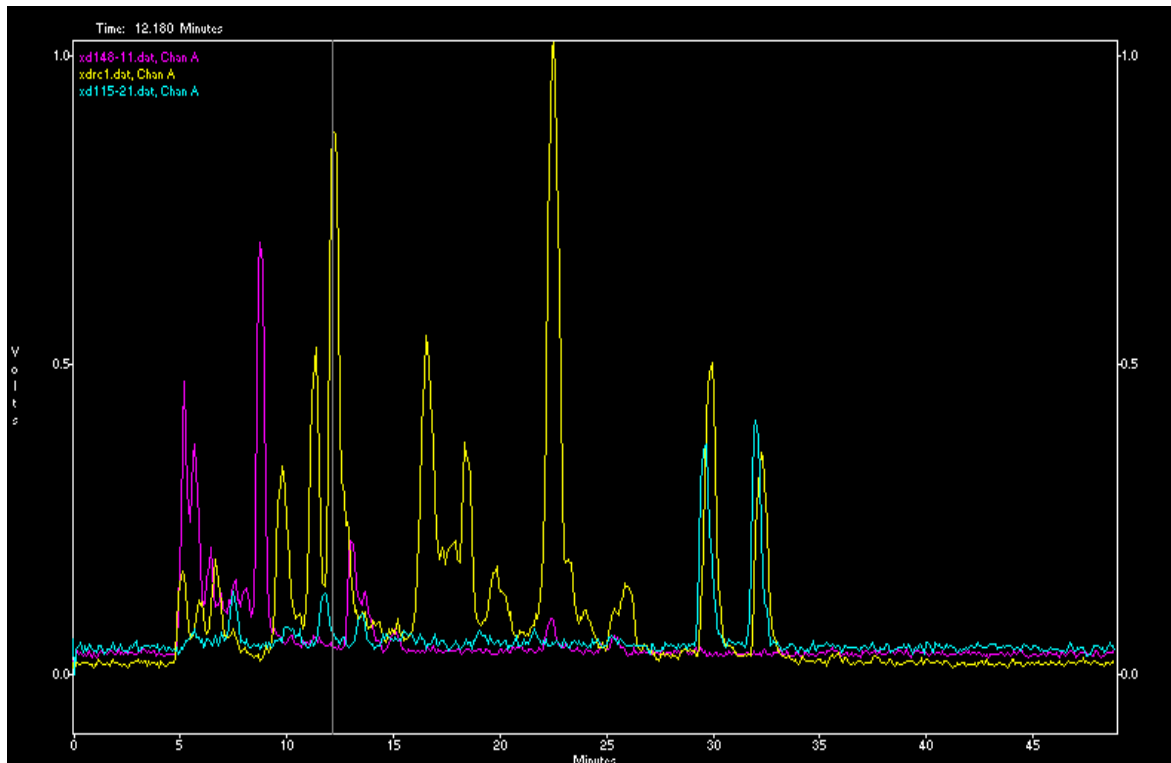
Figure 3-13. HPLC chromatograph of GA_{12} treated apple shoots from 806 dwarf apple seedlings at time periods: 15 min, 6 h and 48 h (Blue: 15 min; yellow: 6 h; purple: 48 h).

However, standard plants metabolized faster than dwarf plants. Comparing the metabolites profile of standard and dwarf plants at 3 h (Figure 3-14) and aligning feeding peaks to similar scale, the feeding peaks were still the most prominent compounds in dwarf seedlings, while in the standard plants they were swiftly metabolized into a group of compounds residing in the center of the profile with peak G (GA_{53}) the most abundant.
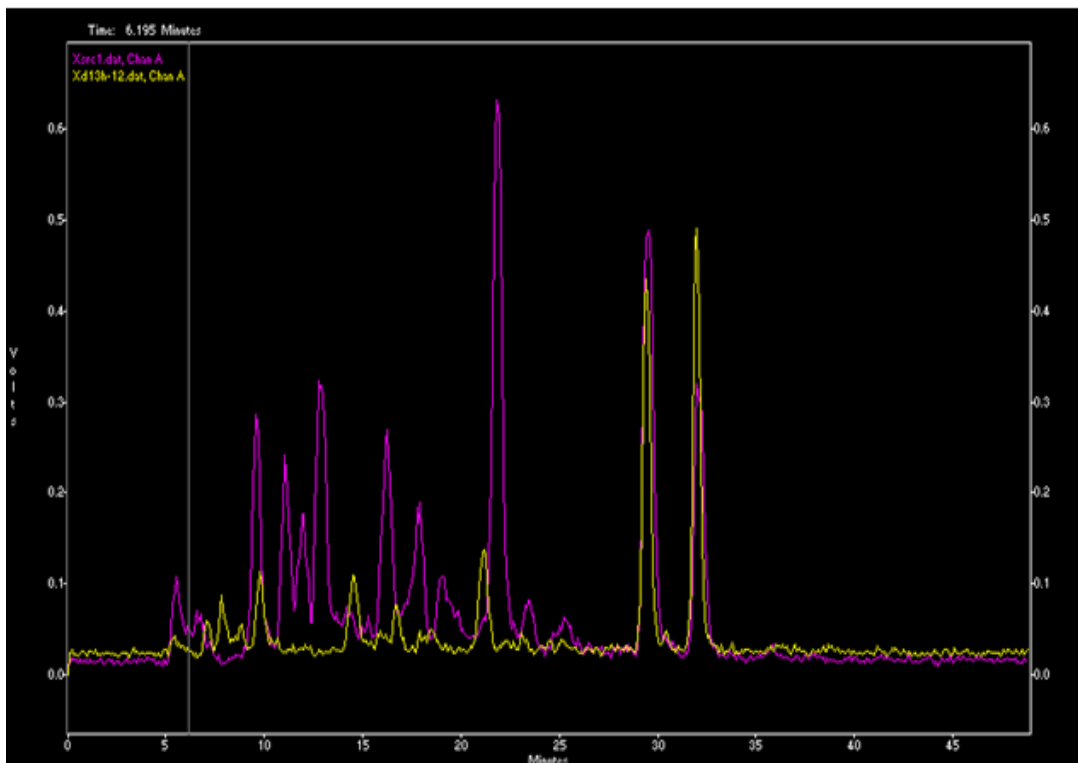
Figure 3-14. Comparison of HPLC chromatographs of 806 dwarf and standard apple seedlings at 3 h (red: standard; purple: dwarf).

Peak N, which transiently existed in the 3 h standard HPLC chromatograph as a principal metabolite, was not found at any time point in dwarf plants (Figure 3-15, Figure 3-16, and Figure 3-17).

Although most other metabolites existed in both dwarf and standard plants, they often followed different metabolic trends and different durations. Some examples are presented in Figure 3-18.
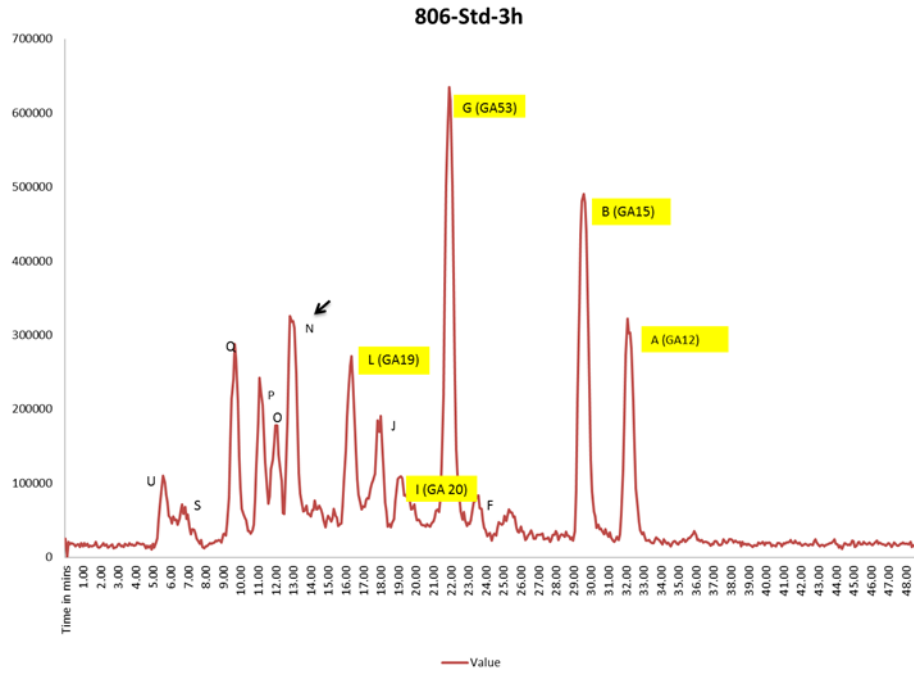
Figure 3-15. Metabolites of [^{14}C]GA$_{12}$ in 806 standard apple seedlings at 3 h.
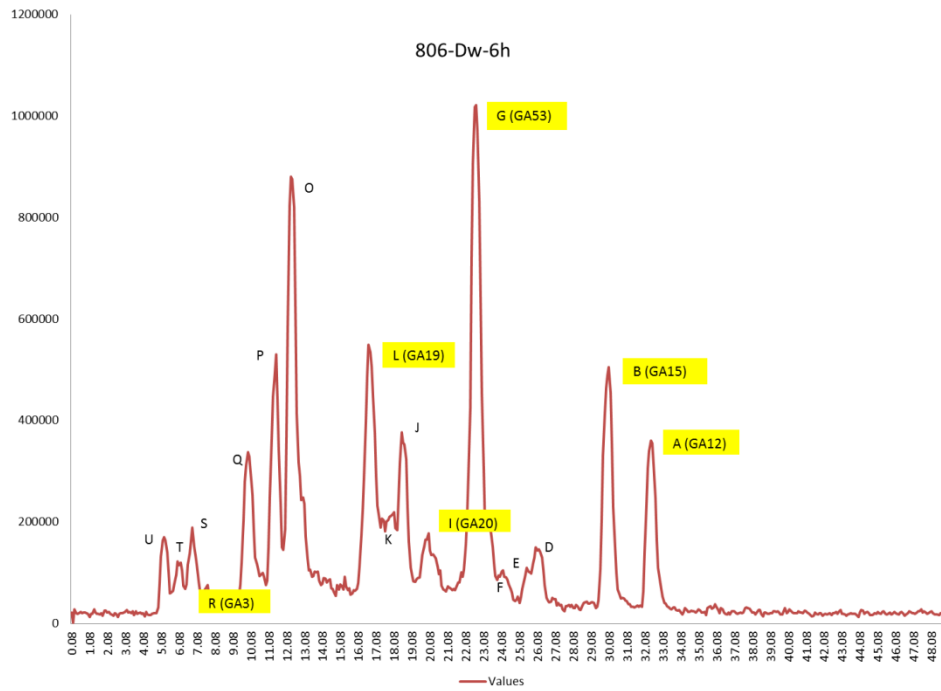
Figure 3-16. Metabolites of [^{14}C]GA$_{12}$ in 806 dwarf apple seedlings at 6 h.
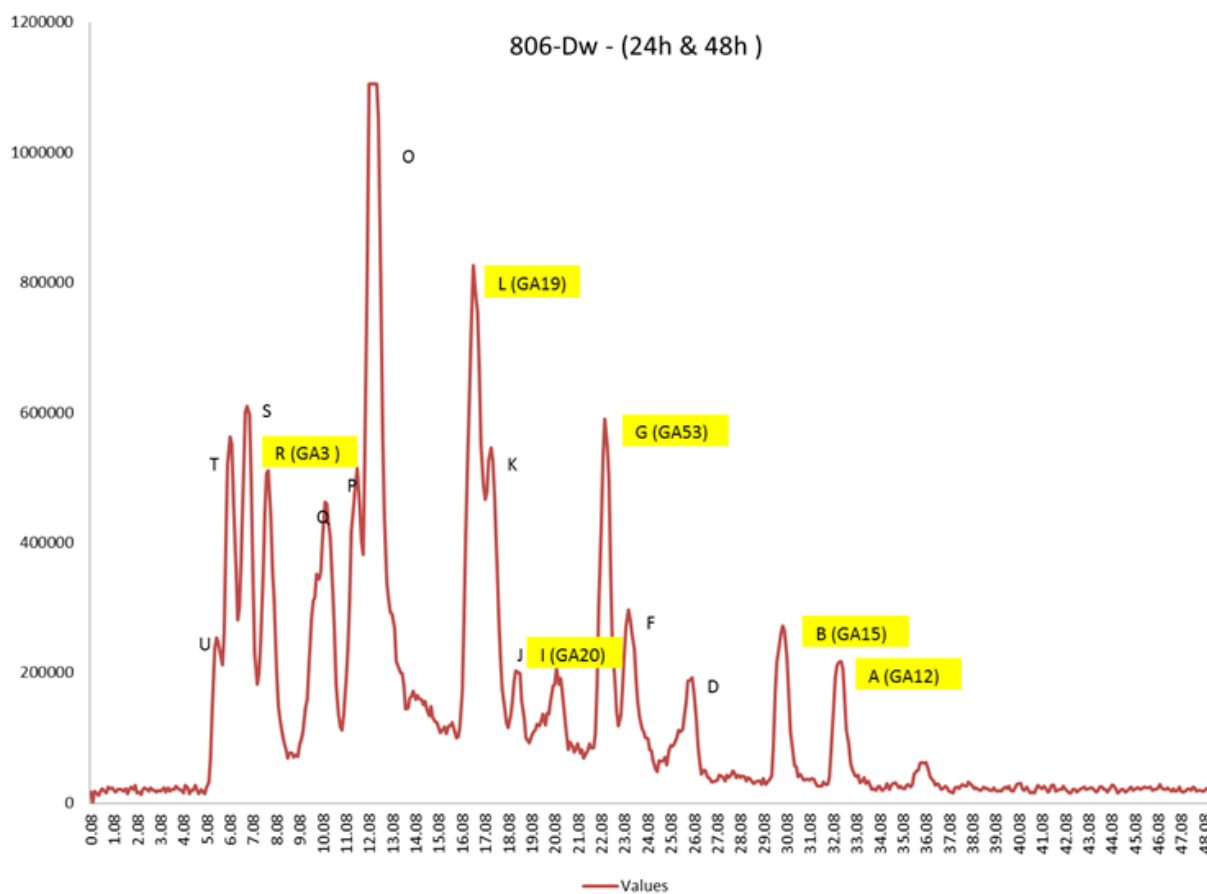
Figure 3-17. Metabolites of [^{14}C]GA$_{12}$ in 806 dwarf apple seedlings at 24 and 48 h.
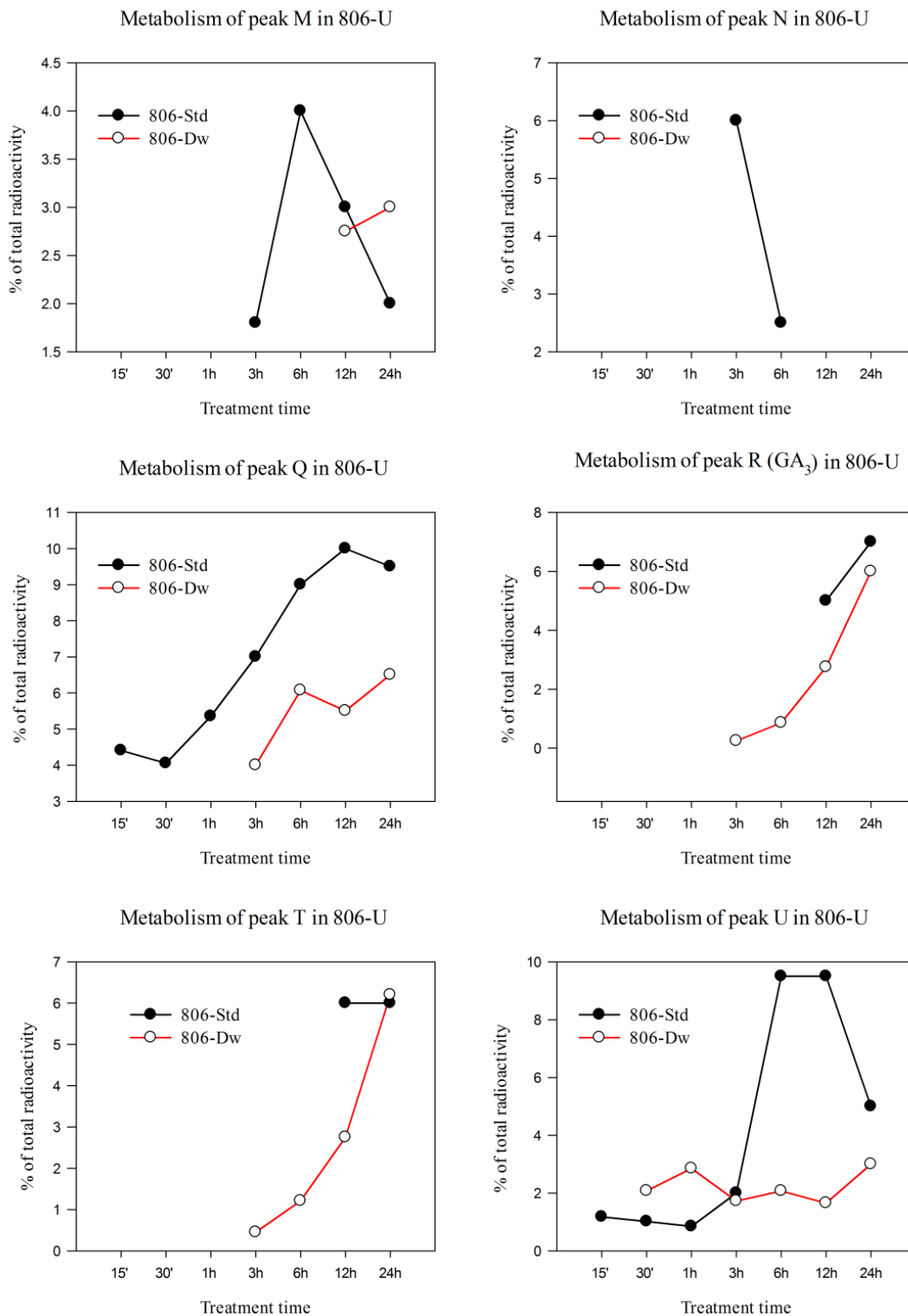
Figure 3-18. Comparison of metabolic trends of individual peaks between dwarf and standard apple seedlings in progeny 806.

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Compared to 'Redcort', 806 dwarf and standard plants had a much faster metabolic rate (Figure 3-19). For plants in 806, GA_{12} and GA_{15} were mostly metabolized between 1 and 6 h. However, in 'Redcort', these two compounds were consumed at a more gradual speed with downstream metabolites also appeared later (1 h versus 15 min in progeny 806). At the end of the study, 'Redcort' peak A and B still had significant amounts (20.5% and 14.5% respectively), yet <5% in both 806 dwarf and standard seedlings. It is possible that these differences result from the much larger size of the 'Redcort' shoots as they were taken from fully grown apple trees rather than seedlings. Redcort shoots use averaged 3.2 g whereas the seedling shoots averaged 2.0 g.
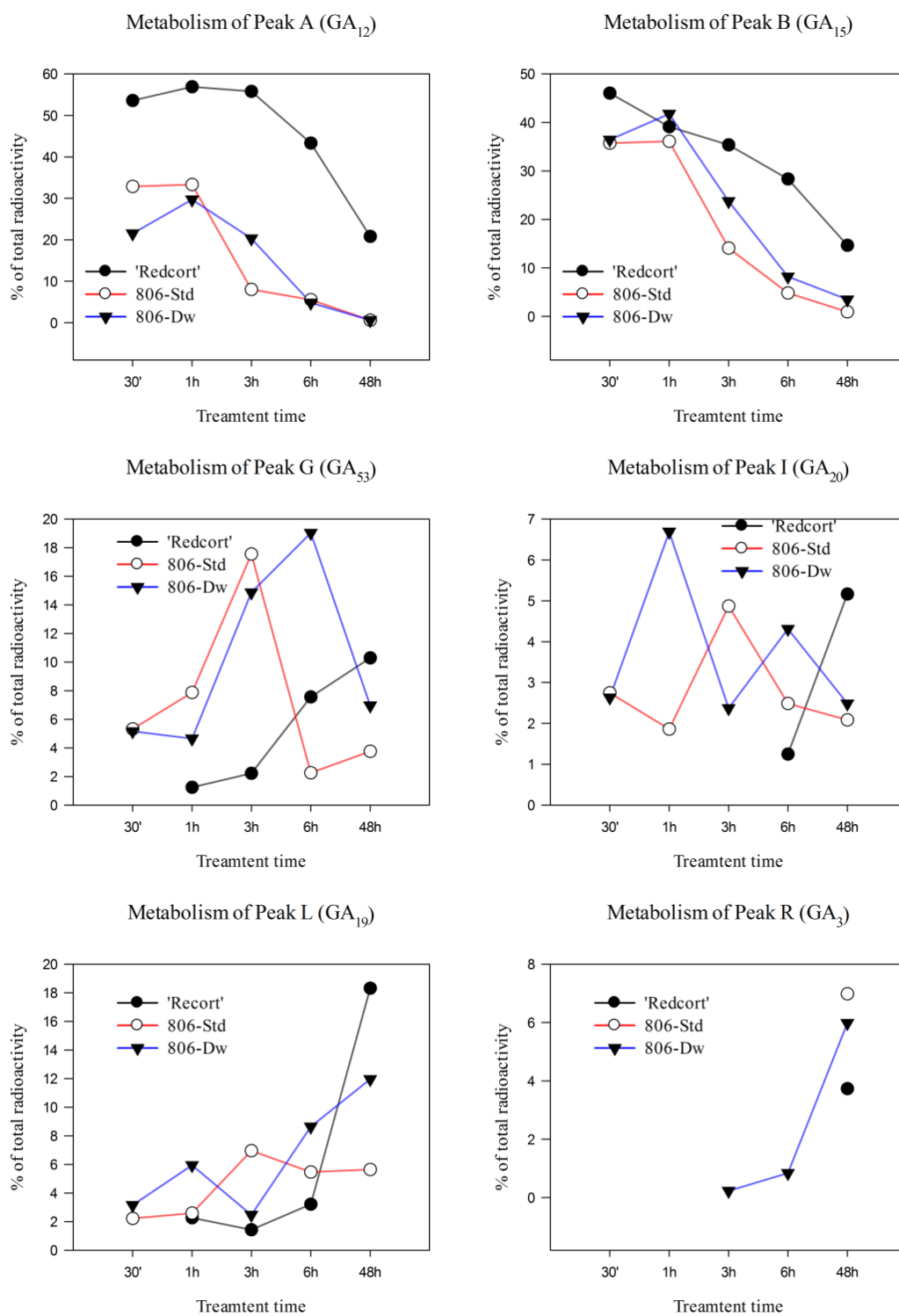
Figure 3-19. Comparison of metabolic trends of individual GAs between 'Redcort' apple and 806 standard and dwarf apple seedlings.

For 806-std, GA_{53} peaked at 3 h, where GA_{12} and GA_{15} were at the end of their sharp decrease. After 3 h, the decrease of GA_{12} and GA_{15} became slower, where GA_{53} was quickly metabolized. The same holds true for 806-dwarf plants, just in a slightly slower speed. However, in 'Redcort', the amount of GA_{53} rose from 1 h to 48 h, but never rose to a high percentage of the metabolites, suggesting that GA_{53} always was produced faster than it was consumed.

GA_{19} and GA_{20} appeared at the same time in both standard and dwarf apple progeny, which suggests GA_{19} was metabolized immediately to GA_{20}. However, there was more GA_{19} produced than consumed for GA_{20}, suggesting that the conversion from GA_{19} to GA_{20} is tightly regulated. In 'Redcort', GA_{20} first appeared 5 hours after GA_{19}'s initial appearance at 1 h. From 6 to 48 h, GA_{19} increased about 15% while GA_{20} increased only about 4%.

GA_3 appeared in 806-dwarf plants at 3 h and in 806-standard plants at 12 h. Samples were not collected between 6 h and 48 h in 'Redcort', so the time of first appearance of GA_3 could be earlier than the 48 h recorded.

DISCUSSION

Metabolism of $[^{14}C]GA_{12}$ in Apple Shoots

GA_{12} is the common precursor for all GAs in higher plants and is at a branch-point in the pathway, undergoing either oxidation at C-20, or hydroxylation at C-13 to produce GA_{53} prior to further oxidation at C-20. GA_{12} and GA_{53} are precursors of the non-13-hydroxylation and the 13-hydroxylation pathways, respectively (Sponsel and Hedden, 2004).

The identification of most of the members in 13-hydroxylation pathway (GA_{53}, GA_{44}, GA_{19}, GA_{20} and GA_3) strongly suggests that this is the major metabolic pathway in vigorously growing apple shoots, and is consistent with GA metabolism in vegetative tissues of potato (*Solanum tuberosum* spp. *Andigena*, Van den Berg et al., 1995) and most other plant species (Sponsel and Hedden, 2004), but different from the pathway and fate in apple seeds which favors the production of GA_4 and GA_7 by the non-13-hydroxylation pathway.

Zhao et al. (2010) speculated the existence of both pathways in apple, as *MdGA20ox1*, *MdGA3ox1* and *MdGA2ox1* were shown to be able to catalyze GAs of both the 13-hydroxyl and non-hydroxyl pathways *in vitro*. However, *in vitro* experiments cannot fully represent *in vivo* situations as the enzymes may not have access to certain substrates due to tissue-specificity. As the dioxygenases GA20ox, GA3ox, and GA2ox are often encoded by multi-gene families (Sponsel and Hedden, 2004), different family members probably function in different tissues and at developmental stages. As the cDNA of all these three genes are localized in seeds or flowers, they probably function in the non-hydroxyl pathway in reproductive tissues *in vivo*.

GA_{15} is upstream in the non-hydroxyl pathway, which leads to bioactive GA_4. However, GA_4 was not identified in this study which suggests that the consumption of GA_{15} was probably not

used in this pathway in apple vegetative tissues. GA_{15} could be metabolized into GA_{44} to produce GA_3. Putative GA_{15} was also detected by Motosugi et al. (1996) (though without MS identification) together with putative GA_{53}, GA_{44}, and GA_{19} in the xylem exudates of 'Fuji' apple on 'Marubakaido' and 'M.26' rootstocks. The authors suggested this was an indication that both the early hydroxylation and non-hydroxylation pathways functioned in rootstocks.

GA_{44} was only detected in sample 2 of 'Redcort', perhaps a result of an insufficient amount in each individual sample and/or different sample preparation methods necessitated by the presence of the phlorizin.

In shoots from pea (*Pisum sativum* L.) G2 line (a dwarf pea which lacks the dominant *Le* allele that controls the conversion from GA_{20} to GA_1), GA_{12}-aldehyde was quickly metabolized into a large variety of compounds including GAs 53, 44, 19, 20 and 17 under both LD (long day) and SD (short day) conditions (Davies et al., 1986). In potato shoots, twenty metabolites were detected from the metabolism of $[^{14}C]GA_{12}$ (Van den Berg et al., 1995), which include GAs 53, 44, 19, 20, 29, 1, and 8.

GA_3 is the Major Bioactive GA in Vigorous Growing Apple Shoots

GA_3 was the only bio-active GA detected in our study and has been identified in many other plant species (Davies, 2004). The GA_3 was not a contamination because it contained both the native and the $[^{14}C]GA_3$ MS ions, which can only have been produced from the applied $[^{14}C]GA_{12}$. While shoot vigor increased from 806-dwarf to 806-standard to 'Redcort', the initial appearance (time) of GA_3 appears to be negatively correlated with shoot vigor. The late appearance of GA_3 in 806-standard plants and in 'Redcort' can be justified on the basis of

feedback (Ross et al, 1999), as it could be hypothesized that there are already sufficient bioactive GA in the shoots, whereas 806-dwarf plants were in a GA-limited situation, so that GA_3 was generated soon after the availability of its upstream precursors, GA_{19} and GA_{20}.

Other bioactive GAs detected by others in apples include GA_1, GA_4 and GA_7. GA_4 and GA_7, which were the major compounds detected in seeds ten weeks after full bloom in 'McIntosh' (Oyama et al., 1996) are also believed to be involved in seed dormancy removal and floral initiation (Sinska and Lewak, 1970; Halińska et al., 1987; Oyama et al., 1996; Lewak, 2011). They were also detected in fruit exudates from different apple cultivars: 'Elstar', 'Golden Delicious', 'Jonica' and 'Spencer Seedless' (Stephan et al., 1999a). However, they were not detected in xylem exudates by Motosugi (1996), nor in our study, suggesting that GA_4 and GA_7 may function primarily in apple reproductive tissues (Faust, 1989).

GA_1 (together with GA_9) was identified as the minor component in apple leaves and buds from 'McIntosh' and 'Jonathan' harvested during periods of rapid growth, while GA_{19} and GA_{20} were the major GAs characterized (Koshioka et al., 1985). Steffens's group (1992a; 1992b) also reported considerably lower levels of GA_1 and GA_3 as compared to the predominant GA_{19} and GA_{20} in the shoo tips of both standard and dwarf phenotypes. GA_1 has also been identified in fruit exudates (Stephan et al., 1999a) and immature seeds (10 weeks after full bloom) from cultivars 'Cox's Orange Pippin', 'Dabinett' and 'Tremlett's Bitter'(Hedden et al., 1993).

[^{14}C]GA$_{12}$ Metabolism Differed in Own Rooted Apples Shoots and Shoots on Rootstocks

[^{14}C]GA$_{12}$ metabolism differed in different plant materials used in this study both in compounds produced and metabolic rates. Although they were stronger shoots to start with, the plant

materials in 'Redcort' metabolized $[^{14}C]GA_{12}$ much slower than shoots in 806. At the end of the study there was still about one fifth feeding material that was not metabolized in shoots from 'Redcort' while this figure was smaller than 5% both in 806 dwarf and standard shoots.

The conversion from GA_{19} to GA_{20} seems to be a rate limiting step in 'Redcort': as GA_{19} and GA_{53} both were detected at 1 h, but GA_{20} was not detected until 6 h. This slow conversion of GA_{19} also led to the continuous accumulation of GA_{53}, although initially it was a quick step from GA_{53} to GA_{19}. In shoots from progeny 806, both GA_{19} and GA_{20} were detected at 30 min and GA_{53} started to decline after 3 h for standard plants and 6 h for dwarf plants.

These differences could possibly be attributed to the greater amount of radioactive material applied to the 'Redcort' shoots ('Redcort': 0.2 μCi/shoot = 560 pmol, 190 ng/shoot; 806: 0.1 μCi/shoot = 280 pmol, 95 ng/shoot). While earlier discussion suggested that the 3β-hydroxylation was regulated by the bioactive GAs present in the plant materials, the different rates of GA_{19} metabolism and accumulation of GA_{53} in 'Redcort' demonstrated that the 3-step 20-oxidation was under the feedback control as well. The fact that GA_{19} increased for 15% of the total radioactivity while GA_{20} only increased for 4% between 6 and 48 h suggested that the last step of 20-oxidation was more strongly feedback-regulated than 3β-hydroxylation.

Steffens et al. (1992b) suggested that one reason the shoot elongation of dwarf plants were sensitive to temperature was because the conversion rate of GA_{19} to GA_{20} were different under different temperature regimes, which also suggested this was a critical step for GA biosynthesis regulation in apple.

In pea, it was shown that both the 3β-hydroxylation and the last step of 20-oxidattion (from GA_{19} to GA_{20}) were feedback-regulated by GA_1 (Ross et al, 1993) and the difference in GA

metabolism between GA_1-deficient lines and their wild-type lines were more obvious after short-term feeds (3.5 or 4 h).

Another difference for the plant materials used in the study was that 'Redcort' was on a vigorous rootstock, while plants from 806 were on their own roots. Many articles have discussed the transport of GAs between scions and rootstocks. Lochard and Schneider et al. (1981) argued that shoot-produced GAs might be transported to the roots and converted to other GAs and re-circulated back to the shoots. Motosugi et al. (1996) believed that rootstocks produced less-active GAs translocated into xylem, and then converted into bio-active GAs in shoot tips or other growing organs. The latter point was further confirmed by Bulley's results (2005), where the dwarfing effects of scion variety 'Greensleeves' were not corrected by grafting onto invigorating rootstocks. If root-produced GAs can be transported to scions, the dwarfing effects should be corrected by the invigorating rootstocks, unless the mobile GAs are not biologically active and are supposed to be converted to active form at the action site, namely the vigorous growing shoots.

In both pea and potato plants, GA_{20} is the major transported GA (Proebsting et al., 1992; Prat, 2010). If the major transported GA in grafted apples is also GA_{20} that will further explain the slow conversion from $[^{14}C]GA_{19}$ to $[^{14}C]GA_{20}$ in shoots from 'Redcort'. However, GA_{20} was not identified in the xylem exudates from apple, whereas GA_{19} was so identified (Motosugi et al., 1996). The concentration of GA_{19} was also shown to increase with increasing rootstock vigor in the xylem sap of grafted apples in the growing season (van Hooijdonk et al., 2011). Therefore, it is possible that mobility of GAs is different in different species and GA_{19} is the major transported GA in apples. A study of the transport of radio-labeled GAs in grafted apples would aid in this clarification.

The differences in metabolism in the study can also be attributed to cultivar differences, shoot size, age and physiological status of the plant materials used, e.g. flowering vs. non-flowering plants, and possibly in the amount of $[^{14}C]GA$ applied.

The Dwarf Phenotype in 806 is Unlikely to Be Caused by GA Biosynthesis Defects Downstream of GA_{12}

In our study, dwarf and standard plants in 806 shared similar GA metabolic patterns despite different GA metabolic rates. The metabolite N only was detected in standard plants, not in the dwarf plants. N elutes earlier than GA_{19}, which means it is more polar and may be a bioactive GA downstream of GA_{19}. However, its transient presence (3 to 6 h) suggests it is more likely to be a rapidly metabolized intermediate.

The faster metabolic rate in standard plants may be a result of low bioactivity of certain enzymes in the GA biosynthesis pathway of dwarf plants, or may be due to the dwarf plants' low vigor. Looney et al. (1988) also reported that the bioactivity of polar GAs in shoot tips from compact growth strain 'McIntosh' was significantly lower than from the normal strain.

The dioxygenases GA20ox, GA3ox, and GA2ox are often encoded by multi-gene families (Sponsel and Hedden, 2004), so defects of certain enzymes in dwarf plants may be covered by other gene family members, which may have overlapping expression or can be transported from other parts of the plant, and result in leaky mutants. Hence Selection 1 and dwarf plants in 806 are still able to bear fruits.

Although different genetic dwarfs can be caused by different reasons (e.g. lesions at different locations of the pathway), our results are consistent with Steffens et al.'s (1992a) findings where the same GAs (GA_{19}, GA_{20}, GA_1, GA_{29}, GA_8 and GA_3) existed in both dwarf and standard plants.

We conclude that dwarf phenotype in 806 is probably not caused by GA biosynthesis defects downstream of GA_{12}, but this does not exclude the possibility of impairment above GA_{12} in the biosynthesis pathway, which can be tested by applying exogenous GA to dwarf plants (Chapter 3). If the dwarf plants can be restored to wild type, that is an indication that no bioactive GAs or not sufficient GAs are produced in dwarf plants. Alternatively, dwarfism can be caused by a blockage in the GA signaling pathway. It will also be interesting to test whether metabolite N is a sugar conjugate. If that is the case, dwarf and stand plants from progeny 806 could be involved in different GA conjugation processes.

Other plant hormones that can be possible causes of dwarfism include brassinosteroids (BRs) and strigolactone (Pereira-Lorenzo et al., 2009; Rameau, 2010). Their possible involvement in the dwarfism of plants in 806 is discussed in Chapter 3.

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APPENDIX

A: Diazomethane Synthesis (toxic and explosive, wear gloves and facial mask)

- Prepare the ice bucket and start the pump and the condenser for cooling.
- Solution B: measure 0.4g potassium hydroxide (KOH) and dissolve in 10 mL 95% ethanol (put on a hot plate and with a magnet inside), put on ice right after.
- Put 30ml ethyl ether into a round bottom flask and place on ice.
- Solution A: measure 2.14g diazald and put into 30ml ether (can sonicate to help dissolve), place on ice.
- Carefully put solution B into solution A (wipe the beaker of solution B to avoid ice going into the mix).
- Secure flask into the distillation head and adjust the height of the heater to hold the flask.
- Put the collection vial on ice below the head of the condenser (support with the adjustable stair).
- Start the heater and closely watch the reagent.
- When it loses its yellow color and become pale lower the heater (turn off the heater).
- Take off the adjustable stair and lower the ice bucket. Still keep the collection vial facing the condenser head to collect the last several drops of sample
- Remove reacting bottle form distillation head and let it evaporate (wash with ethanol).

CHAPTER 4

EXOGENOUS APPLICATION OF PLANT HORMONES TO PLANTS IN 806-U

ABSTRACT

Exogenous GA_3 and GR24 (a strigolactone analog) were applied to plants in progeny 806 to explore the mechanism of reduced stature and enhanced branching. Branches treated with GA_3 had significantly more new growth due to increased number of nodes as compared to control branches in dwarf plants. Decapitation was conducted before the application of GR24 to induce outgrowth of axillary buds. Different degrees of dominance were observed for shoots induced by decapitation in the greenhouse in population 28 (derived from open pollinated Selection 1), whereas for plants in progeny 806, shoots from bud 1 and bud 2 always became dominant. GR24 did not effectively inhibit of branching in our study, perhaps due to the application method or the different branching mechanisms in herbaceous and woody plants.

INTRODUCTION

The objective of this section of research was an attempt to determine the causes of the reduced stature and enhanced branching of the dwarf plants in progeny 806-U (refer to Chapter 2). We examined these plants' responses to exogenously applied GA_3 and GR24. Branches treated with GA_3 had significantly more new growth due to increased number of nodes as compared to control branches in dwarf plants. Decapitation was conducted both in the greenhouse on 1-year-old seedlings from population 28 and the 3-year-old plants from progeny 806-U in the field.

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Whereas different degrees of dominance were observed for shoots induced by decapitation in the greenhouse, in the field experiment, shoots from bud 1 and bud 2 always became dominant. GR24 did not effectively inhibit branching in our study, perhaps due to the application method.

Shoot Branching

Shoot branching is a two-step process: the formation of the axillary bud by the secondary shoot apical meristem (at the axil of each leaf) and the outgrowth of the buds. Thus the final branching of the plants is decided by the number of axillary meristems and the activity of the buds. The process by which a dormant bud activates and becomes an actively growing branch is complex and very finely tuned. It is regulated by the interaction of environmental and endogenous signals (e.g.: plant hormones) thus giving the most plasticity to the branching process of a plant (Domagalska and Leyser, 2011). Therefore, this will be our only focus of discussion in this chapter. Interaction of hormones in shoot and root meristems was reviewed by Durbak et al. (2012).

Hormone Network

Plants control their number of branches partially by selective activation of dormant axillary buds. The dominance of the main shoot over lateral shoots with the help of plant hormones is called apical dominance.

Auxin was the first plant hormone to be linked with this concept (Leyser, 2003; 2005), as applying auxin to the tip of decapitated shoots mimics the effect of the removed apex, preventing

bud outgrowth. However, auxin does not function on buds directly as radioactively-labeled auxin applied apically does not enter the axillary bud in any quantity, and direct auxin application onto an axillary bud does not inhibit outgrowth (Ongaro and Leyser, 2007).

Auxin is produced mostly in young expanding leaves at the shoot apex and distributed throughout the plant. It is transported basipetally down the stem in a polar auxin transport (PAT) stream via a cell-to-cell transport system (Domagalska and Leyser, 2011). PIN formed proteins which belong to a family of auxin efflux carriers, facilitate efficient and directional auxin export out of cells. In the primary stem, *PIN1* is basally localized in the files of xylem parenchema cells that are required for the efficient basipetal transport of auxin from the shoot to the root. Polar auxin transport and the establishment of an auxin gradient resulting from cellular efflux are very important in determining plant growth and morphological patterning (Aloni, 2010; Benková et al., 2003).

However, while decapitation is able to promote bud outgrowth, preventing auxin's transport down the stem, either by application of auxin transport inhibitors or girdling the stems, is not sufficient to induce bud outgrowth (Dun et al., 2009). These observations support the idea that auxin needs to act together with other signals to regulate bud outgrowth.

Different from auxin, **cytokinins (CKs)** work on buds directly, so CKs have long been thought to be the second messenger of auxin in the inhibition of lateral bud growth. CKs are mostly synthesized in the roots, but there is some synthesis in shoots as well (local synthesis), and it is transported acropetally in the xylem. Various lines of evidence suggest apically derived auxin affects cytokinin synthesis both at the node and in the root, and that the levels of CK from these sources correlate with bud activity (Ongaro and Leyser, 2007). One mechanism for auxin-

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mediated bud inhibition can be through the down-regulation of cytokinin synthesis, limiting CK supply to the bud and reducing bud outgrowth. Axillary buds may rely on locally synthesized cytokinin for stimulation of branching rather than on cytokinin produced at a distance, as shown in rice (*Oryza sativa*) (Kurakawa et al., 2007) and *Arabidopsis* (Dun et al., 2011).

The discovery of a class of highly branched mutants in *Arabidopsis*, *Pisum*, *Oryza* and *Petunia* led to the proposal of an acropetally transported inhibiting signal, presumably in the xylem, that represses bud activity (Mouchel and Leyser, 2007). Auxin cannot provide this signal, as transport of auxin in the stem was strictly basipetal in the polar auxin transport (PAT) stream.

The signal was later characterized as **strigolactone** (SL) by two research groups simultaneously (Gomez-Roldan et al., 2008; Umehara et al., 2008). Strigolactones are a group of terpenoid lactones. They were originally shown to be the triggers for germination of parasitic weeds witchweeds, *Striga* spp., and broomrapes, *Orobanche* spp., (Gomez-Roldan et al., 2008; Umehara et al., 2008). Later they were found to serve as a signal that is exuded from nutrient-deprived plant roots to promote hyphal branching of symbiotic arbuscular mycorrhizal fungi (Domagalska and Leyser, 2011). The fungi facilitate uptake of soil nutrients by plants. This symbiosis is observed in more than 80% of terrestrial plants, coinciding with the wide distribution of this class of terpenes. Strigolactones may have other functions as well because they induce seed germination of non-parasitic plants and are also produced by non-hosts of arbuscular mycorrhizal fungi such as *Arabidopsis* (Umehara et al., 2008). Strigolactones affect leaf morphology in *max* mutants of *Arabidopsis* (Stirnberg et al., 2002) and delay senescence and reduce root growth in *Petunia dad1*/*ccd8* mutants (Snowden et al., 2005).

The currently proposed pathway for SL is that *CCD7* (*Carotenoid Cleavage Dioxygenases*) and *CCD8* cleave a carotenoid substrate in the chloroplast with *MAX1*, a cytochrome P450, working on more mobile substrates downstream of *CCD7* and *CCD8* (Yamaguchi and Kyozuka, 2010). Gene *D27* of rice, an iron-containing protein, is also required for SL biosynthesis. The newly discovered *D14* of rice (also known as *D88* and *HTD2*) is member of a hydrolase family, it functions either in the late SL biosynthesis pathway or after strigolactone is produced, suggesting the hormone is a SL derivative (Domagalska and Leyser, 2011). *D14 also* may work as a strigo-receptor (Arite et al., 2009). *MAX2/RMS/D3* encodes an F-box protein, suggesting that ubiquitin mediated degradation of negative regulators may be used in SL signaling regulation (Yamaguchi and Kyozuka, 2010). The recent characterization of *BRC1* (*BRANCHED1*), a gene specifically expressed in the bud and essential for bud growth suppression, supports the idea that SLs may act directly in axillary buds. *BRC1*'s expression level is tightly regulated by SL and CK (Dun et al., 2011).

After SL has been placed in the plant-branching map, many have reviewed its function in branching control (Ferguson and Beveridge, 2009; Goulet and Klee, 2010; Ongaro and Leyser, 2007; Rameau, 2010), although mostly in herbaceous plants (Waldie et al., 2010). Two models have been proposed to explain SL's interaction with other plant hormones in regulating bud outgrowth.

The auxin transport-canalization model proposes that auxin transport, rather than the concentration, out of the bud is crucial for bud activation (Li and Bangerth, 1999). A strong auxin source (intact apex or auxin applied to the decapitation site) prevents auxin export and bud outgrowth. It describes a process where the initial outflow of auxin from the bud is further up-regulated and polarized by its own flux to establish the canalization between the source (shoot

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apex, auxin is synthesized in young and expanding leaves) and the sink (the primary stem whose sink strength is determined by auxin concentration) where cells form files to direct auxin polar transportation. These files can later differentiate into vascular strands. This is controlled by auxin-efflux carriers: *PIN*, whose expression can be up-regulated by auxin, a demonstration of a positive feedback loop between auxin and its transport (Aloni, 2010; Vieten et al., 2005; Sauer et al., 2006). In the *max* mutants, the *PIN* level and the amount of auxin moving in PAT (polar auxin transport) is elevated when measured in isolated stems (Bennett et al., 2006; Prusinkiewicz et al., 2009).

If the initial auxin outflow from a certain bud is high, the connection between this bud and the primary stem can be established, and then the bud is activated. This means that all the buds are competing for the PAT, which is limited. Assuming the auxin content in the buds or the auxin content that is exported from each bud is comparable, timing becomes crucial, because the bud that is activated first will quickly establish connection with the primary stem and prevent the activation of other buds. However, this can be altered when the sink strength increases due to a decrease of auxin level in the stem (e.g. decapitation) or if the source strength of an individual bud increases (Domagalska and Leyser, 2011).

Assessing the strigolactone biosynthesis and signaling mutants by using *PIN* reporters demonstrated that strigolactone regulates *PIN* accumulation on cell membrane (regulating the expression location of *PIN* protein rather than their capability of transporting auxin), therefore auxin transport in the stem (Lazar and Goodman, 2006; Lin et al., 2009; Crawford et al., 2010). Indeed, strigolactone mutants in *Arabidopsis* and rice have elevated levels of auxin transport (Lazar and Goodman, 2006; Lin et al., 2009; Crawford et al., 2010). However, it is also argued that SL's regulation of auxin transport may not be crucial for shoot branching but for other

aspects of plant development (Agusti et al., 2011; Waters et al., 2011), as SL can directly acts in buds via *BRC1* without requiring a change in auxin status (Dun et al., 2011).

Strigolactone enhances competition between the buds on isolated stem with two buds: instead of inhibiting both buds, basal application of SL allows one bud to become dominant while the other remains dormant. This inhibition has no effect when there is only one bud on a stem segment, but comes into play when a second auxin source is added, such as application to the decapitation point (Domagalska and Leyser, 2011). This allows the plant to establish a connection between global and local regulation: all the plant meristems are connected through an auxin transport network which can be affected globally by strigolactone that is transported in the vascular system. When overall strigolactone content is low, many buds can be activated; when strigolactone level is high, buds that are more competitive, which is probably determined by local environment and bud developmental stages, take dominance. Buds vary in inherent activation potential, affecting their ability to respond to regulatory signals.

The second messenger model proposes that auxin regulates the production of a second messenger that moves acropetally and acts in the bud directly. Two hormones can fulfill this role: cytokinin and strigolactone.

Cytokinin has long been thought to regulate correlative dominance together with auxin (Bangerth et al., 1989; 2000) and demonstrated to promote bud outgrowth. *SMS* mutants in pea all exhibit a large reduction in the export of xylem sap cytokinin (X-CK) from the roots and increased expression of *CCD7* and *CCD8* (Dun et al., 2009). However, it is proposed that X-CK stimulates the continued outgrowth of fully or partially released bud, whereas local CK enhancement stimulates initial bud release as although SL mutants have depleted levels in the xylem sap

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relative to wild type plants, no difference can be detected in shoots. In either scenario, SL and CK act antagonistically on bud outgrowth (Dun et al., 2011).

Direct application of cytokinin promotes bud outgrowth. Auxin regulates cytokinin biosynthesis by regulating members of IPT family which play key roles in CK biosynthesis (Nordström et al., 2004; Tanaka et al., 2006).

Hayward et al. (2009) found that apically derived auxin up regulated the biosynthesis of *MAX3* and *MAX4* in *Arabidopsis* and their orthologs in rice, petunia and pea; removal of auxin source by decapitation led to a decrease in the transcript levels of these biosynthesis genes. Therefore, SL can potentially be the second messenger. However, it is also shown that SL can act directly in buds via *BRC1* (Dun et al., 2011).

It is difficult to quantify strigolactones from shoot tissues, as it exists at extremely low level in plants, ~1000 fold less abundant than IAA (Dun et al., 2009). SLs are below the detectable level in putative hormone deficient mutants, whose defects can be rescued by application of GR24, a SL analog. As low as 10 nM of GR24 is enough to sufficiently inhibit the bud outgrowth, consistent with the idea the SL, or its derivative, works as a plant hormone (Yamaguchi and Kyozuka, 2010) .

It is not clear whether the active form/hormone is strigolactone itself or its derivatives. Important future experiments will include testing whether branching phenotypes are correlated with levels of particular strigolactones in shoots of various grafted plants (Beveridge and Kyozuka, 2010)

Environmental Cues

Shoot branching is a way for plants to express genetic information and respond to environment cues. Plasticity in branching determines a plant's competitiveness in relation to adjacent plants in regard to nutrients absorption, light capture and other resources (Evers et al., 2011). Therefore the ultimate branching of a plant is a result of the interaction of processes within and between plants.

It is tempting to speculate that SL may play an important role in communication between the above- and below-ground parts of a plant: where nutrient availability sensed by the roots will be used to adjust SL level (Umehara, 2011). This alteration will change plant branching patterns and also the symbiotic interaction with AM (arbuscular mycorrhizal) fungi that facilitate minerals uptake (Schachtschabel and Boland, 2009; Umehara et al., 2008, 2010).

Nitrogen and phosphorous in the soil influence auxin, cytokinin and strigolactone's biosynthesis and transport (Gomez-Roldan et al., 2008; Evers et al., 2011). Phosphorus starvation leads to an increase in auxin sensitivity (Dun et al., 2006, 2009) and elevation of SL level in rice, which inhibits tiller growth (Umehara et al., 2010).

Plant branching is also affected by light. Higher light intensity results in more branches (higher photosynthetic rates and more assimilates for the plants) and a reduction in light quality reduces shoot branching (Domagalska and Leyser, 2011). A low ratio of red light and far red light (R: FR) can be used by plants as a cue for the existence of surrounding vegetation, as red light mainly is absorbed by green plants, while far-red light is scattered. When future competition for light is anticipated to be intense, plants devote more resources to height growth than branching, a phenomenon known as "shade avoidance syndrome" (Evers et al., 2011).

Shoot Branching in Apple

Apple Branching Pattern

In herbaceous plants, the position or age of a bud along the stem determines its ability to grow out (Waldie et al., 2010). Plant species like *Pisum sativum* (pea), *Lycopersicon esculentum* (tomato) and *Arabidopsis* seem to have three branching zones with abrupt transitions in between: a basal zone, whose branches are often a reiteration of the main shoot; a middle zone, where buds are mostly repressed and an upper zone that is often associated with increased number of reproductive growing points as the outgrowth starts just before the emergence of the inflorescence (Waldie et al., 2010).

In woody plants (apple included), the branching pattern is more complex as the existence of proleptic and sylleptic growth brings timing as another dimension: whether the flushes, phase of vegetative elongation of the main axis is coincident with the outgrowth of the buds from previous season (proleptic), or the buds newly formed (sylleptic) (Turnbull, 2005).

Most cultivars in apple exhibit 6 successive branching zones (Figure 4-1) from the most distal end, zone 1 is a mixture of long proleptic shoots, latent buds and short shoots; zone 2 is mainly covered by lateral bourses and latent buds; the third zone corresponds exclusively to sylleptic shoots. The other three zones are located at the basal part of a shoot: a large branching zone with latent buds, spurs and long proleptic shoots flanked by two non-branching zones. The length of each zone and the relative proportion of each type of shoots/buds vary from cultivar to cultivar (Costes et al., 2006).
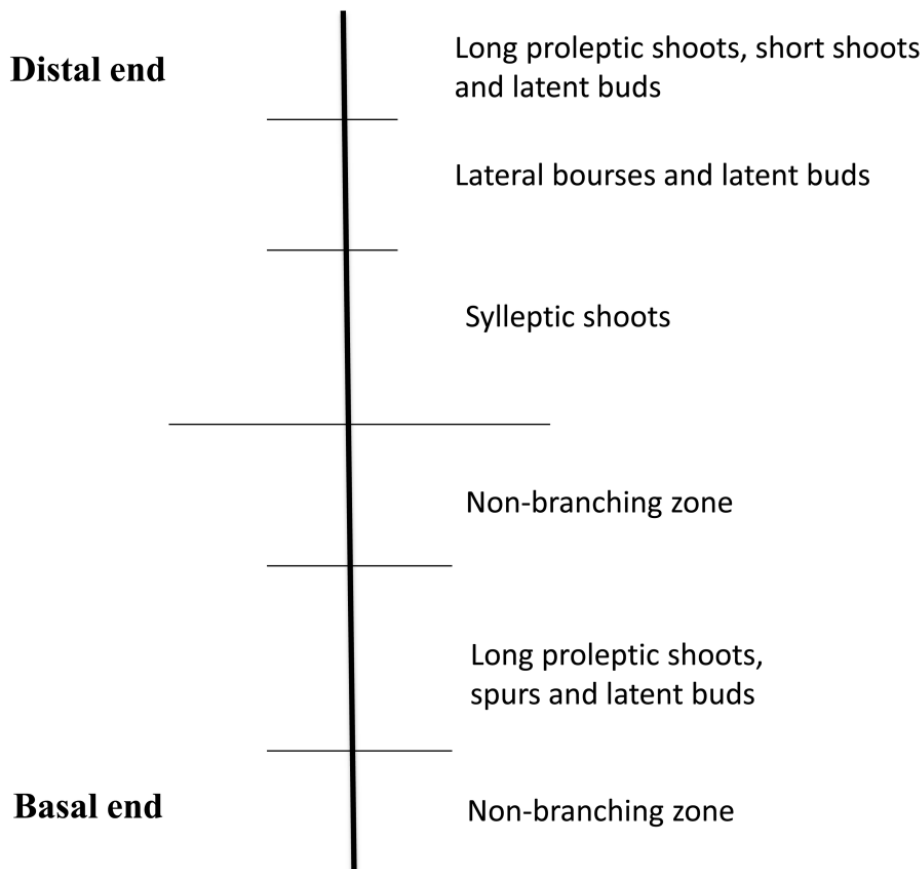
Figure 4-1. Branching zones in apple (*Malus ×domestica* Borkh.) at a branch scale.

In 'Fuji' tree ontogeny, as GU length decreased, most central zones disappeared and length of the floral zone reduced progressively, which together led to a progressively simplified branching pattern (Renton et al., 2006).

Sylleptic shoots are often seen in an early stage of tree development ("feathers" in the nursery) and the ability of axillary buds to grow into sylleptic shoots depends on the growth rate of the parent shoot and the maturity level of the whole tree (Costes et al., 2006).

In 'Braeburn', the influence of shoot growth on the pattern of axillary development was investigated (Lauri and Terouanne, 1998). The distributions of types of axillary structures were different between monopodial and sympodial shoots in the first growth flush but similar in the second. The parent metamer was important in determining the absence and presence of vegetative or generative branches. The authors concluded that shoot growth characteristics conditioned both the location of the vegetative side branches and distribution of blind nodes and inflorescences.

Bud development was also shown to be related to hydraulic conductance independent of acrotonic effect (proximal vs. distal) as illustrated by the heterogeneity of bud potential in the distal zone. This hydraulic force mediates competition between buds within the same branching zone (Lauri et al., 2008). By studying apple cultivars 'Fuji', 'Ariane', 'Braeburn', 'Gala' and 'Granny Smith', the authors concluded bud size and hydraulics conductance were cultivar-specific.

Apple Branching and Plant Hormones

In Tworkoski and Miller's 2007 study , apple scions with contrasting growth habits were selected from a cross of 'Goldspur Delicious' x 'Redspur Delicious' and budded to rootstocks with different levels of vigor ('M.9', 'M.7' and *Malus domestica* 'Antonovka' seedlings). Shoot tips from scions with many short branches and a more upright narrow canopy (UN) exhibited a statistically higher auxin to CK ratio (ACR), which may have led to stronger apical dominance. The UN growth habit, as contrasted to the spreading round (SR) canopy with fewer short branches, also had more increased bud break following BA (benzyladenine) application. Both

scion types had about twice the ACR on the seedling rootstocks than on 'M.9' and 'M.7'.The authors suggested that ACR may be involved in regulating bud-break and the development of growth habit; and rootstocks can affect the hormone concentration in shoot tips.

Columnar apple trees are characterized by reduced branching. When comparing endogenous IAA level between columnar and standard plants, contrasting results exist: Bendokas and Stanys, (2009) argued that columnar apple had high auxin content, higher auxin/cytokinin ratios and lower GA_3/IAA ratios whereas weeping trees had low abscisic acid and high IAA/ABA and GA_3/IAA ratios compared to other canopy architectures. For columnar cultivars 'Maypole' and 'Tuscan' and standard cultivar 'McIntosh', no significant difference in IAA concentration was found but CK concentration, especially zeatin riboside (ZR), was higher in shoots from columnar type trees than that of standard trees in June (Watanabe et al., 2004). The IAA concentration of columnar cultivar 'Trajan' was higher in apical shoots than in lateral shoots (Watanabe et al., 2006). When heading-back pruning was performed, terminal shoot growth was not inhibited in 'Trajan', while distal lateral shoot growth was promoted (Watanabe et al., 2006).

Seven partial clones encoding auxin efflux carriers (*MdPIN_A/B*, *MdPIN10_A/B*, *MdPIN4, MdPIN7_A/B*) and three encoding influx carriers (*MdLAX1/2/3*) were identified in eight-year-old apple trees 'Golden Delicious'/'M.9'. These genes were differentially expressed in diverse plant organs at different developmental states (Dal Cin et al., 2009).

To produce good quality feathered nursery trees (which give greater production earlier in the orchard life), products with CK (e.g.: 6-BA, 6-benzyladenine) with or without a mixture of GAs (usually GA_{4+7}) have proven to be most useful (Elfving and Visser, 2006), although the effectiveness varies from cultivar to cultivar. Promalin® is one of the commercially products

available. Type and concentration of CK strongly affects *in-vitro* organogenesis of apple, which is also genotype and explant dependent (Magyar-Tábori et al., 2010).

Cyclanilide® (CYC,) a putative auxin transport inhibitor, has emerged as a promised apple branching agent in recent years. Although it resulted in only a small reduction in shoot length, CYC seemed to be effective in inducing lateral branches from current-year's shoots and spurs from older wood (Elfving and Visser, 2005). One application of CYC increased lateral branching 5 to 6-fold in 'Cameo' (known as a cultivar that is difficult to branch).

Decapitation

Despite diverse branching patterns in different plant species, almost every plant reacts to shoot tip damage by initiating new branch growth (Stafstrom and Sussex, 1988). The vast majority of leaf axils in a plant possess at least one lateral bud; under normal circumstances, not all grow out to form branches. Decapitation can induce bud outgrowth at almost any node with the most common position being the node(s) directly below the damage (Turnbull, 2005). Decapitating young pea seedlings will lead to rapid activation of multiple buds in every axil; however, only one of these buds will maintain growth and become a replacement shoot while the rest return to dormancy (Stafstrom and Sussex, 1988).

   In the apple cultivar 'Anna', decapitation induced bud break and enhanced lateral bud growth. Inhibition of bud out growth was achieved by applying auxin immediately after terminal bud removal, whereas delayed application reduced the effect of IAA, suggesting that IAA inhibits the primary stage of later bud growth in apple. Application of auxin also inhibited the movement of water to lateral buds (an increase of free water is the first sign of bud break) and

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changed lipid composition of the membrane (which may be associated with the movement of water into cells and changes in cellular organizational processes). Thus, it is possible that IAA from terminal buds inhibits lateral bud growth by limiting the availability of the free water in the buds. However, the experiment was done in-vitro (on shoots collected in August) rather than on intact branches (Wang et al., 1994).

One horticultural practice that resembles decapitation is pruning, which is often performed in apple orchards for positioning scaffold branches or inducing secondary branching (Faust, 1989a). Removal of either the shoot tips or unexpanded leaves will release the axillary buds from the relative inhibition and allow them to grow into laterals; however, removal of fully expanded leaves will not, suggesting the substance that is effective in inducing branching exists only in the fast growing tissues (Mika, 1971).

Apples react to pruning differently based on the pruning techniques (thinning and heading cuts) as well as the position of the action/condition of the buds (Fumey et al., 2011; Miller and Tworkoski, 2003). Better-developed buds are often observed on the upper three-fourth of strong apple shoots as compared to the base of the shoot; also buds on top of the canopy have greater growth potential than those at the base. Therefore stronger re-growth is expected when pruning the upper part of the shoots or at the top canopy (Mika, 1986).

The timing of pruning relative to the growing season is also important. When heading is done early in the growing season on a second year shoot from a spur cultivar, the lower buds develop into strong laterals without changing the overall growth of the tree much; however, if done in the dormant season, both the number of strong laterals and the ration of long shoots compared to

spurs increase significantly. The crotch angle of newly grown laterals from headed shoots is much narrower than non-headed shoots (Faust, 1989b).

Further, the reactions of apples to pruning are shown to be highly cultivar-specific (Fumey et al., 2011). Spur type cultivars have stronger apical dominance and react differently to pruning from standard cultivars: shoots heading near the apex of spur cultivars give similar results as to heading the standard cultivars at the shoot base (Faust, 1989b).

A five-year study of contrasted pruning procedures on apple cultivars with different architectures ('Scarletspur Delicious', 'Golden Delicious' and 'Granny Smith') revealed that variability of responses to pruning partly depends on the genetically determined growth and flowering habit (Stephan et al., 2007).

Stem Elongation

Hormone Network

Auxin: Cleland (2010) argued there are three main lines of evidence for auxin's role in stem elongation: 1) exogenous auxin results in a rapid and prolonged growth in both dwarf and standard pea stems (Yang et al., 1996); 2) a ring coated with TIBA (an auxin transport inhibitor) can block the elongation of a pea stem from the growing region when placed on top of the segment (McKay et al., 1994); 3) gravitropic curvature due to the change of auxin concentration at both sides of the stem.

Gibberellin (GA): Stem elongation is caused by cell elongation and cell division. GA is involved in both processes by inducing gene transcription (Sun, 2010). GAs have opposite

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effects to light on photomorphogenesis during seed germination: they promote etiolated growth [17]whereas GA-deficiency induces a partially de-etiolated phenotype in the dark (De Lucas et al., 2008). GA can trigger stem elongation in light-grown *le* mutants but had little effects on isolated stem segments (Galston and Kaur, 1961)**.**

Brassinosteroid (BR): A series of pea BR mutants (*lk, lka, lkb, lkc* and *lkd*) responded poorly to GA_1 exogenous application and had similar GA_1 levels compared to wild type. Although they shared the features of reduced internode length and darkened leaf color as that of GA-deficient mutants, they also exhibited thick stems with banding (corrugated), smaller leaves and substantially shorter petioles and peduncles, a set of morphologically characteristics referred to as 'erectoides phenotype' collectively (Reid and Ross, 1993).

Examining the stem tissue of pea mutants *lk* and *lka*, reduction in both cell length and cell number can be observed in epidermal and outer cortical cells (Behringer et al., 1990; Reid and Ross, 1993), while the dwarfism in *lkb* is primarily caused by the reduction in cell length. The authors argued that BRs reduce stem elongation mainly by reducing cell length.

BRs are involved in cell-wall modification. *lka* and *lkb* have increased wall-yield threshold, which reduces cell-wall relaxation and therefore the ability of these cells to elongate, and increased osmotic and turgor pressures (caused by the decrease in cell extension) without a concurrent decrease in solute transport in the system (Behringer et al., 1990). In contrast, the turgor pressure in GA mutant *ls* is similar to that of wild type so the stem width is not affected here or in other GA-deficient mutants (Reid and Ross, 1993), suggesting that GA and BR function in stem elongation in different ways.

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[17] A phenotype characterized by long hypocotyls, small and closed cotyledons, undifferentiated chloroplasts and repression of light-regulated genes.

Brassinolide (BL) is believed to be the bioactive form of BR, however no conclusive evidence is available and different species may have different bioactive BRs. Current results suggest campesterol (CS) might be the main biologically active one in tobacco, rice and mung beans, or even in tomato. BL is probably the major bioactive form in *Arabidopsis* (Reid et al., 2010).

Interaction: Isolated stem segments from the elongation zone can grow at a comparable rate to an intact plant when auxin is applied, but to a lesser extent to GA or BR alone, or may not grow at all (Cleland, 2010).

While it is acknowledged that auxin, GA and BR all promote stem elongation in plants, it is less well known how much each of the hormones contribute or how they interact; how they act similarly or differently to environmental stimuli and whether they involve the same set of genes when functioning (Santner et al., 2009). However, possible hypothesis include those suggested by Cleland (2010):

1. The presence of GA and BR allow plant stems to react to auxin, and the elongation rate of the stem is not related to the concentration of GA or BR.

2. The function of auxin in stem elongation is to promote synthesis of GA, which controls the rate of cell elongation.

3. Auxin and GA promote stem elongation separately, so they can function at different locations or function at different times.

GA and Auxin: Yang et al. (1996) suggested that auxin and GA promote growth of different parts of the stem as "the effect of GA was mainly in internodes less than 25% elongated, whereas that of IAA was in the older, elongation internodes." They further argued that IAA stimulates

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growth by cell extension and GA stimulates growth both by cell numbers and cell expansion, as shown in mutants *le* and *lkb* (Yang et al., 1996).

Auxin is the main determinant of cell elongation, and GA can enhance this process. While GA cannot cause an increase in cell length without the presence of auxin, it can function alone to increase cell division. Therefore, the absence of either hormone will lead to dwarf phenotype (Yang et al., 1996).

GA and IAA co-treatment is able to induce a response greater than either treatment individually (Yang et al., 1996). The initial response (3 to 4 h) of this treatment is similar to that of IAA treatment alone. Then a growth rate increase is observed for 1 h, coinciding with the initial response by GA alone, and maintained for about 20 h (Yang et al., 1996). Stem elongation induced by auxin treatment has a lag time of about 20 min, as compared to GA, where the lag period is 3 h (Yang, 1996). These results suggest that the effects of GA and IAA are, at least partially, additive.

Auxin and GA have been found to positively regulate each other's biosynthesis (Law and Davies, 1990; O'Neill and Ross, 2002; Ross et al., 2000; Swarup et al., 2002). In *Arabidopsis*, mutants deficient in GA biosynthesis and signaling exhibited a reduced auxin transport, which led to impairment in two *PIN* dependent growth processes: cotyledon differentiation and root gravitropic responses. This reduction in auxin transport correlated with a reduction in *PIN* abundance that could be recovered to wild type after GA treatment. GA deficiency also promoted the targeting of *PIN2* for vacuolar degradation, proposing a GA-dependent modulation of *PIN* turnover (Willige et al., 2011).

GA and BR: Despite the fact that GA and BR-deficient mutants share many similarities, such as dwarfism, reduced seed germination and delayed flowering (Wang et al., 2009), it was believed that there was little interaction between GA and BR pathways (Reid et al., 2010). However, microarray analysis in rice (*Oryza sativa)* demonstrated that BR and GA regulate a certain groups of genes coordinately (Yang et al., 2004) and both positive (*OsGSR1)* and negative (*SPINDLY*) regulators of GA signaling pathway are involved in BR biosynthesis (Shimada et al., 2006; Wang et al., 2009).

BR and Auxin: Both auxin and BR regulate plant growth by stimulating cell elongation and cell division (Krizek, 2009). Genome-wide microarray expression analysis demonstrated that a large number of genes respond to both auxin and BR (Hardtke, 2007); auxin-induced or repressed genes are also induced or repressed by BR (Santner et al., 2009), suggesting a synergistic relationship between these two hormones. Further, BR regulated kinase *BIN2* (*BR insensitive 2*) can increase the expression of auxin-induced genes by inhibiting the binding ability of an organ growth repressor *ARF2* (*auxin response factor 2*, a transcriptional regulator) through phosphorylation (Vert et al., 2008).

Environmental Cues

The growth inhibition caused partly by a decrease in GA_1 content (the perception of light by phytochrome A leads to the up-regulation of GA_1 to GA_8) when pea seedlings are transferred from dark[18] to light is a good example of how environmental factors can affect stem elongation

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[18] Dark growing pea seedlings have longer stems and fewer internodes compared to pea seedlings grown in the light, a phenotype called "etiolated".

(Weller et al., 1994). Nitrogen levels, temperature and light intensity have all been shown to affect GA levels (Prat, 2010).

Transcription factors *PIF3* (phytochrome-interacting factor 3) and *PIF4* in *Arabidopsis thaliana* mediate the signaling between light and gene expression (De Lucas et al., 2008; Feng et al., 2008). They are negatively regulated by activated phyB and positively control genes regulating cell elongation. *DELLA*[19] proteins (GA signaling repressors, whose accumulation is promoted by light by reducing GA levels) negatively regulate *PIF3/4* in the control of hypocotyl elongation, leading to a convergent point with the light pathway (De Lucas et al., 2008; Feng et al., 2008).

Temperature is another contributing factor when it comes to stem elongation as demonstrated by Steffens et al. (1992a)'s research in apples. After investigation of the GA content of tissue cultured plants (derived from dwarf seedlings from 'Goldspur Delicious' x 'Redspur Delicious') growing under both constant and ramped temperature regimes, the authors suggested that shoot elongation of dwarf plants was sensitive to elevated temperatures both as a result of reduced responsiveness to GAs and a reduction in the concentration of GA_1.

Stem Elongation in Apple

Attempts have been made to identify important components of GA pathways in apple. KO (ent-kaurene oxidase), a critical enzyme in GA biosynthesis pathway was cloned from the apical tissue of 'Fuji' apple stem and designated as *MdKO*. Sequence analysis demonstrated that *MdKO*

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[19] Named for the conserved order of amino acids at N-terminus. D: aspartic acid (Asp); E: glutamic acid (Glu); L: leucine (Leu); A: alanine (Ala)

belonged to cytochrome P450 superfamily and was located in endoplasmic reticulum membrane (Tian et al., 2011).

Kusaba et al. (2001) detected GA 20-oxidase from 'Fuji' apple primarily in immature seeds 1 to 3 months after full bloom. Zhao et al. (2010) isolated GA-20-oxidase (*MdGA20ox1*), 3-oxidase (*MdGA3ox1*) and 2-oxidase (*MdGA2ox1*) from 'Fuji' apple (apical shoots with unfolded young leaves, terminal flower buds, and flowers at balloon stage and full bloom) and tested their activities with 17-17-[^{2}H$_2$]-labeled GAs. Expression analysis indicated that these genes are tissue-specific.

Six endogenous *DELLA* proteins were identified from an apple EST database by Foster et al. (2007). The *MdDELLAs* clustered into 3 pairs (*MdRGL1a/b, MdRGL2a/b and MdRGL3a/b*) and their mRNA was highest in summer-arrested shoots tips and in autumn vegetative buds. Research suggests that there is a significant conservation of gene function between *DELLA* proteins from apple and *Arabidopsis*. Transgenic *Arabidopsis* expressing *MdRGL2a* had smaller leaves and shorter stems, took longer to flower under short days and exhibit a reduced response to exogenous GA$_3$. Over-expressing *Arabidopsis gai* gene in apple cultivars 'Gravenstein' and 'McIntosh' led to reduced growth in-vitro and in the greenhouse, with reduction in stem length, internode length and node number (Zhu et al., 2008).

Using PCR, Xu et al. (2010) investigated the transport of apple endogenous *GAI* mRNA between rootstock (*Malus xiaojinensis*) and scion ('Fuji'). The appearance of *GAI* mRNA in the graft partners led the authors to conclude that *GAI* mRNA moved both upward and downward in grafted trees. In situ hybridization detected *MdGAI* mRNA in the phloem but not the xylem.

How apples react to exogenous application of plant hormones/PGRs have also been explored in regard to stem elongation. When 'York Imperial' apple seedlings were treated with PAC (paclobutrazol) continuously for 66 days, 91% reduction in shoot length was observed and this could be reversed by application of GA_3 (Steffens et al., 1985).

Orchard grown dwarf plants (tissue cultured plants derived from dwarf seedlings from 'Goldspur Delicious' x 'Redspur Delicious') were not reduced in size by paclobutrazol treatment (a gibberellin biosynthesis inhibitor), nor rescued by exogenous GA_3 application (Steffens et al., 1989a, b).

Bulley et al. (2005) suppressed expression of GA biosynthetic enzyme GA20-oxidase in the scion variety 'Greensleeves', which led to the reduction of height, resulted from reduced bioactive GA levels. Application of GA_3 to the transgenic lines[20] restored internode length and number, but not leaf size and petiole length, to that of the wild type, indicating that GA biosynthesis had been perturbed in dwarf transgenic lines.

Effects of GA_{4+7} and/or benzylaminopurine (BAP) on apple scion cultivars and 1-N-naphthylphthalamic acid (NPA, an inhibitor of auxin transport) on rootstocks were explored on composite trees with 'Royal Gala' on rootstocks of different vigor. It was proposed by van Hooijdonk et al. 2011 that dwarfing rootstocks reduce the basipetal transport of indolyl-3-acetic acid (IAA), and therefore reduce the transport of GA and root-derived CK to the shoots. In the scion, reduced CK supply may lead to reduced branching whereas limited GA supply will shorten the duration of shoot extension growth.

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[20] To leaf disc, 200 mg/L Benelux (92% GA_3), weekly for 6 weeks.

5F-HCTS, a fluoro-derivative of 28-homocastasterone, stimulated shoot elongation and formation of new shoots in *Malus prunifolia* (the 'Marubakaido' apple rootstock) *in vitro* (Pereira-Netto et al., 2006), and these effects were under the control of changes in the endogenous BR pool (Pereira-Netto et al., 2009). Application of BL (brassinolide) to the same material resulted in reduced apical dominance, while Brz 200 (an inhibitor of BR biosynthesis) exhibited dose-dependent inhibition of main shoot elongation (Pereira-Netto et al., 2009).

MATERIALS AND METHODS

Grouping of Dwarf Plants in Progeny 806

The experiments were conducted at Cornell University's Gate East Research Farm in Geneva, NY in the summer of 2010 (the third growing season), when dwarf plants were further classified into subgroups based on their architecture (Figure 4-2, 4-3, 4-4, 4-5 and 4-6).

Due to forked branching, these plants had branches with similar vigor and comparable relative location on the plants, thus application of the control and treatment solutions was often conducted in the same plant on comparable branches to achieve homogeneity of the plant materials.
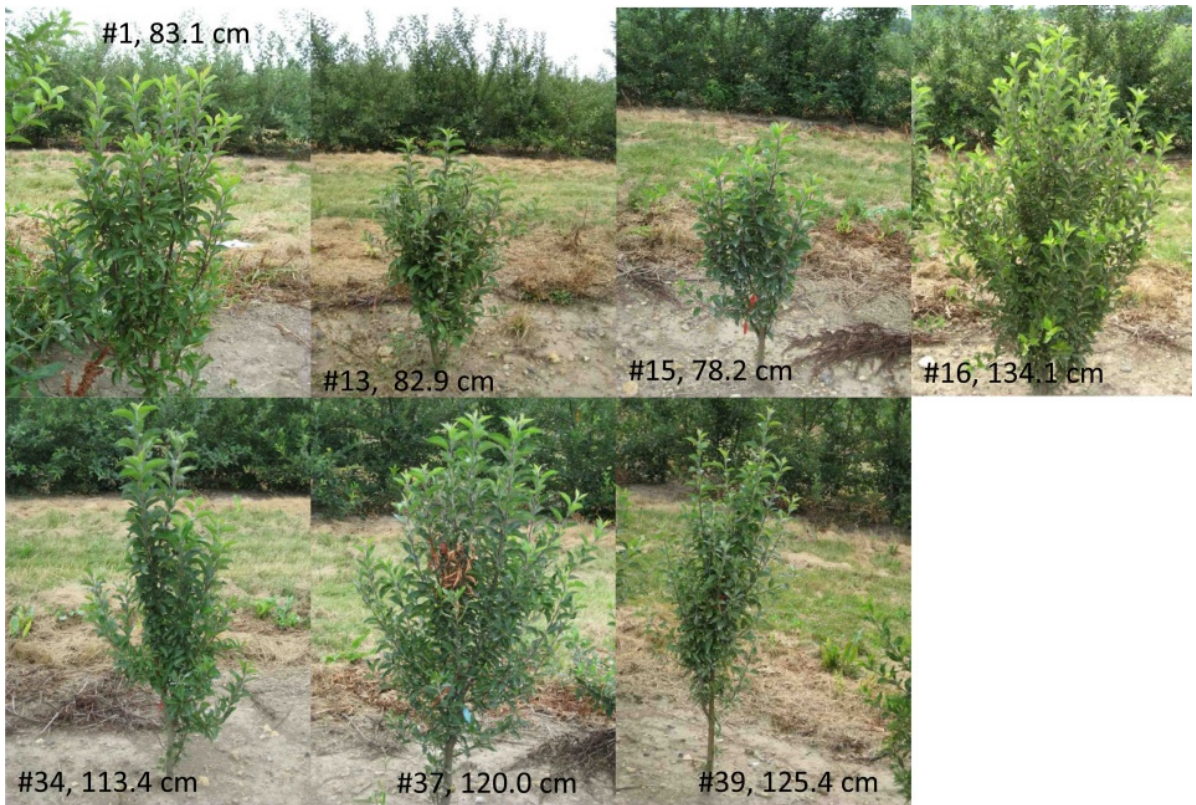
Figure 4-2. Dwarf trees in progeny 806 with a traditional tree habit (upright branching with many forks). Pictures were taken in the third growing season (2010); progeny number and height was labeled for each plant.
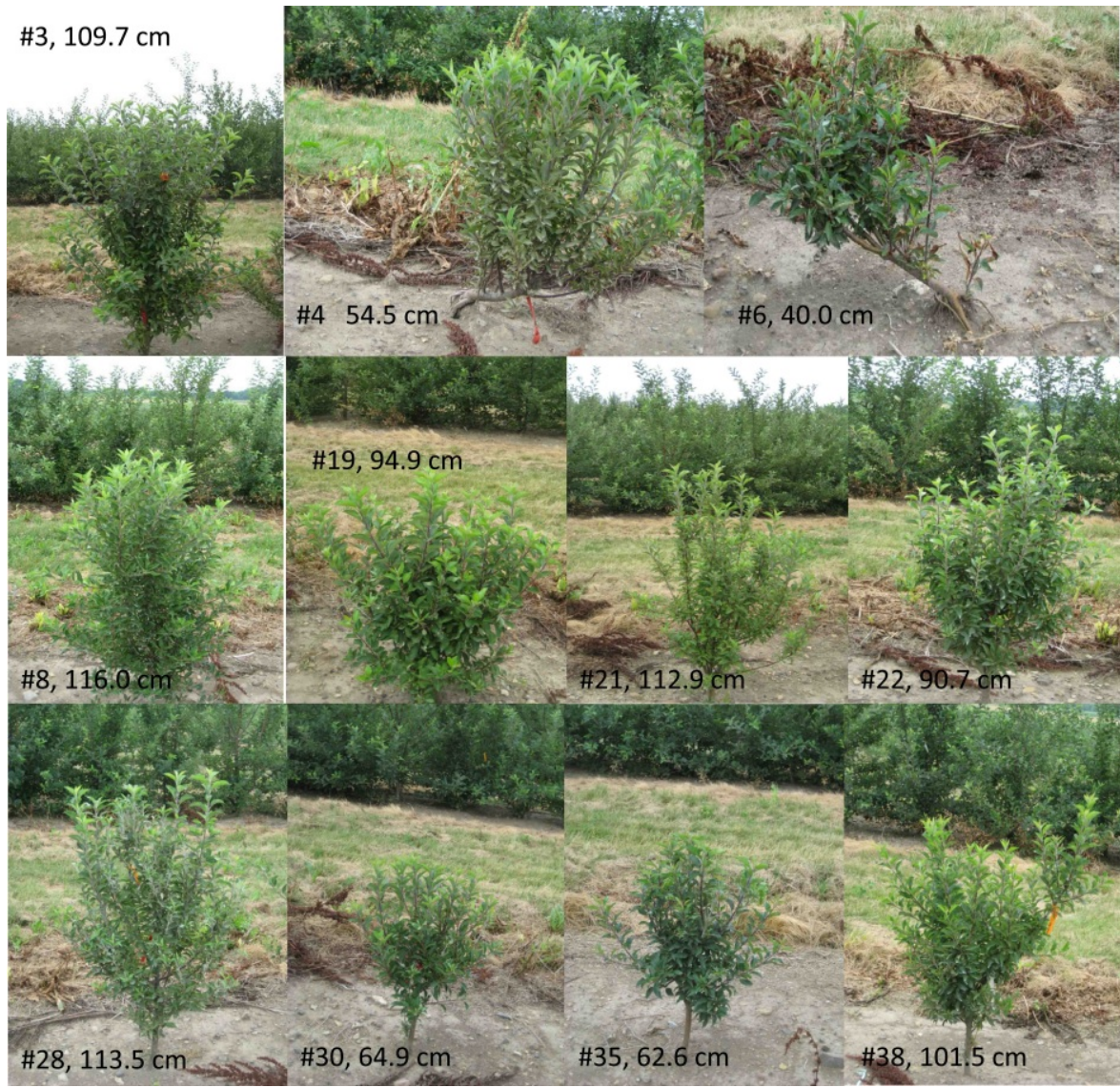
Figure 4-3. Dwarf trees in progeny 806 with a traditional tree habit but a more open canopy. Progeny #3, 19 & 38, #21 & 28, #30 & 35, #8 & 22 were further grouped together based on shape and height. Pictures were taken in the third growing season (2010); progeny

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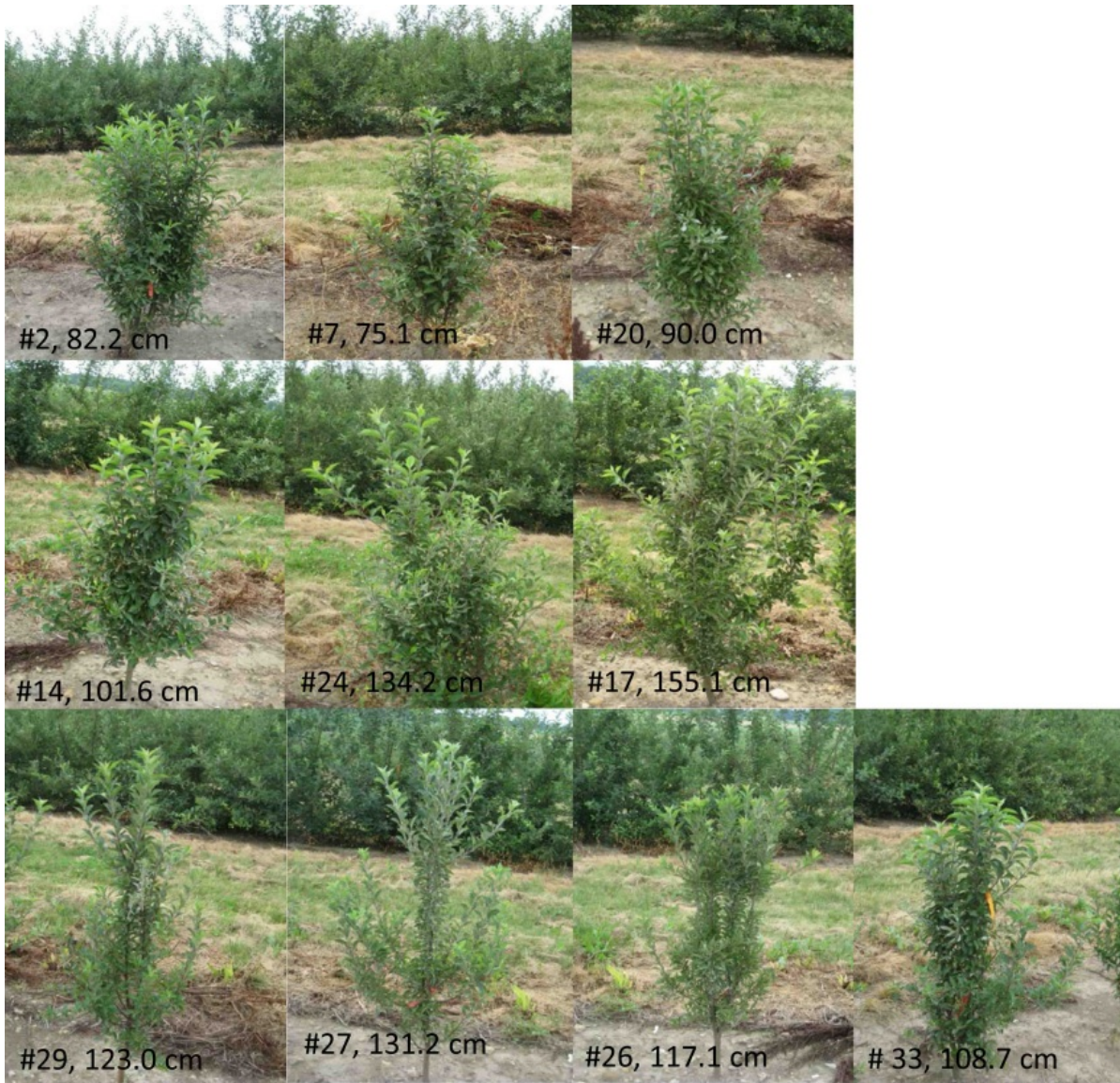
Figure 4-4. Dwarf trees in progeny 806 with two growth segments. Plants in the first row of the panel had a compact canopy with a bushy bottom; plants in the second row had a more open canopy; plants in the last row had two growth segments further apart and curved bottom branches. Pictures were taken in the third growing season (2010) and progeny number and height was labeled for each plant.

Figure 4-5. Dwarf trees in progeny 806 with chaotic architecture. It was difficult to dissect architectural components in this group of plants. Pictures were taken in the third growing season (2010) and progeny number and height was labeled for each plant.

Figure 4-6. Dwarf trees in progeny 806 with architecture that was difficult to define. Plants in this group had distinctive architecture but did not belong to any of the groups mentioned above. Pictures were taken in the third growing season (2010); progeny number and height was labeled for each plant.

**Traditional**: Plants in this group were used both for control and treatment. Within each plant, shoots that resembled each other were grouped together based on location and vigor. Within each group, an equal number of shoots served as control and treatment branches. Smaller seedlings #1, 13, 15 and 34 were used for GA treatment (elongating effects); larger and bushier plants #16, 37 and 39 were used for SL treatment (branching inhibiting effects).

Traditional with more open-canopy: 11 plants in this group were further divided into 5 groups (4 pairs and one group with 3 plants) based on height and architecture. Within each pair/group, the one with the smaller/smallest progeny number served as control, with the other plant(s) the treated plants. Plants with smaller stature were used for GA treatment (#4 & 6, #21 & 28, # 30 & 35) and those with bushy canopy were used for SL treatment (#3 & 19, 28; #8 & 22).

Two-growth-segments: Due to the undefined branching of plants in this group, they were not selected for GA or SL treatment.

Difficult to define: Progeny #10 was used for GA treatment both as a control and treatment plant; #31 was used for SL application both as a control and treatment plant.

Application of GA_3

Standard plants (progeny number 40, 45, and 47) were chosen as standard controls for GA application. Detailed assignments for branches as treatment and control are listed in Table 4-1.

GA$_3$ (Sigma-Aldrich, St. Louis, MO) was dissolved in a small amount of ethanol (less than 1% final concentration) and then added with distilled water. Tween-20 (used as a surfactant) was

weighed and pipetted into the mix to achieve a final concentration of 1 mg/mL GA_3 and 0.1% (v/v) Tween-20. A control solution was made using the same procedure without adding GA_3.

Solutions were brushed weekly to the back and front of newly grown leaves on the uppermost shoots till they were fully covered with a thin film for 5 consecutive weeks. Different brushes were used for each solution. The node right below the terminal bud of each branch was marked. Leaves and stems formed above the mark were designated as "new growth" and were measured/counted on the day of the 3rd application, 5th application and 2 weeks after the 5th application. Students' T-test (JMP9[®], SAS, Cary, NC) was used to compare growth parameters between control and treated branches.

Table 4-1. Control and GA treatment branches in 806-U progeny.

Experiment No.	Population	Progeny No.	Architecture Type	C/T[z]	Fork No.	Branch No.
1	806-U	40	Standard	2	f1[y]	C-1[x]
2					f1	T-1
3	806-U	45	Standard	2	f1	C-1
4					f1	T-1
5					f2	C-2
6					f2	T-2
7					f3	C-3
8					f3	T-3
9	806-U	47	Standard	2	f1	C-1
10					f1	T-1
11					f2	C-2
12					f2	T-2
13	806-U	1	Dwarf/Trad[w]	2	f1	C-1
14					f1	T-1
15	806-U	4	Dwarf/Open	0	f1	C-1
16					f1	C-2
17					f2	C-3
18	806-U	6	Dwarf/Open	1	f1	T-1
19	806-U	13	Dwarf/Trad	2	f1	C-1
20					f1	T-1
21	806-U	15	Dwarf/Trad	2	f1	C-1
22					f1	C-2
23					f1	T-1
24	806-U	21	Dwarf/Open	0	f1	C-1
25					f1	C-2
26	806-U	28	Dwarf/Open	1	f1	T-1
27					f2	T-2
28	806-U	30	Dwarf/Open	0	f1	C-1
29					f1	C-2
30	806-U	31	Dwarf/Trad	2	f1	C-1
31					f1	T-1
32					f2	C-2
33					f2	T-2
34	806-U	33	Dwarf/Trad	2	f1	C-1
35					f1	T-1
36					f2	C-2
37					f2	T-2
38	806-U	35	Dwarf/Open	1	f1	T-1

[z]: 0, plants are used as control; 1, plants are used for treatment; plants are used for both; [y]: branches with the same fork number belong to the same fork; [x]: a branch is used as control when the number starts with 'C', treatment when starts with 'T'; [w]: Trad: traditional; Open: open canopy.

Decapitation

Ten 3-month old plants from population 28 (derived from open pollinated Selection 1) were chosen for the decapitation experiment. Shears were used to cut right below the most vigorously growing shoot tips (1 shoot per plant). The plants were grown in the greenhouse with a day temperature of 21°C and night temperature of 17°C.

In the field, decapitation was conducted on plants from 806-U on July 29th, 2012. Shoots selected as control and treatment were decapitated as described previously to induce the growth of axillary buds.

Application of GR24

Detailed assignments for branches as treatment and control are listed in Table 4-2.

First buds below the site of decapitation were treated daily with 10 µL of 0 or 10 µM GR24 solution, with 50% ethanol and 2% poly ethylene glycol (PEG) 1450 for 4 consecutive days. Measurements were taken for 4 buds immediately below the decapitation site with a digital caliper (accurate to +/- 0.1 mm) on the day of decapitation and 10th, 25th, and 36th days after decapitation.

Table 4-2. Control and GR24 treatment branches in 806-U progeny.

Experiment No.	Population	Progeny No.	Architecture Type	C/T^z	Fork No.	Branch No.
1	806-U	3	Dwarf/Open	0	f1^y	C-1^x
2					f1	C-2
3	806-U	8	Dwarf/Open	0	f1	C-1
4	806-U	10	Dwarf/Difficult	2	f1	C-1
5					f2	T-1
6	806-U	16	Dwarf/Trad	2	f1	C-1
7					f1	T-1
8	806-U	19	Dwarf/Open	1	f1	T-1
9	806-U	22	Dwarf/Open	1	f1	T-1
10	806-U	37	Dwarf/Trad	2	f1	C-1
11					f2	T-1
12	806-U	38	Dwarf/Open	1	f1	T-1
13	806-U	39	Dwarf/Trad	2	f1	C-1
14					f2	T-1

^{z}**:** 0, plants are used as control; 1, plants are used for treatment; plants are used for both; ^{y}**:** branches with the same fork number belong to the same fork; ^{x}**:** a branch is used as control when the number starts with 'C', treatment when starts with 'T'; ^{w}**:** Trad: traditional; Open: open canopy.

RESULTS

Preliminary experiments of exogenous application of auxin, brassinosteroid and cytokinin on open-pollinated dwarf plants from Selection 1 didn't give any convincing results (data not shown). Hence these three hormones were not included in the field treatments.

Response to GA$_3$

Three control and three treated branches had measurable growth in the standard plants. The rest (3 control and 3 treated branches) either didn't elongate or were damaged. Due to the small sample size, statistical test were not performed. Nonetheless, treated branches had more growth and greater node numbers in the new growth segments compared to the control branch from the same plant, same fork.

For dwarf plants, control branch #13, 17 and 22 didn't have measureable growth for the duration of the experiment; hence they were excluded from the analysis. Treatment branch #23 stopped growing after Aug 26th and was also excluded. Data from 12 control and 8 treatment branches were analyzed with Student's T-test ($\alpha = 0.05$). Treatment branches were significantly longer than control branches (Prob $> t = 0.0442$), with more nodes (probe $> t = 0.0082$), but similar internode length.

Response to Decapitation

Ten plants in population 28 were decapitated in the greenhouse to study response to shoot tip decapitation.

Induced buds ranged from 2 to 5 mm in size. Bud break was observed as early as day 4. On day 10, four fully expanded leaves and early shoot elongation were often observed although the elongation response varied from plant to plant. Within the same plant, multiple shoots tended to break together but the degree of dominance varied. Examples of varying degree of dominance are presented in (Figure 4-7). Out of the 10 plants examined, two plants had one branch that became dominant (Figure 4-7, C); three plants had all branches properly developed, but certain branches were more vigorous than others (Figure 4-7. B and D); half of the plants had branches with similar vigor (Figure 4-7. A).
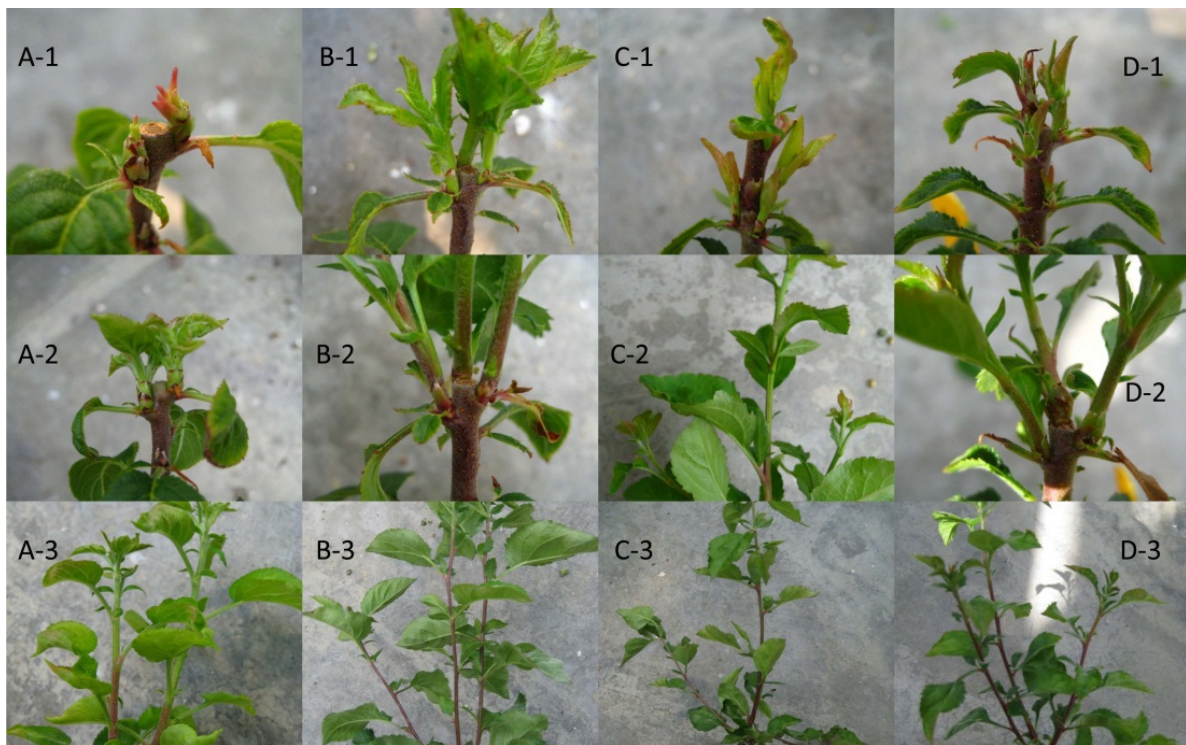

Figure 4-7. Varying degrees of dominance after decapitation in apple. Each column (A, B, C and D) records a single plant's growth on the 4th, 10th and 20th day after the decapitation.

At the end of the experiment, five plants (# 9, 11, 16, 19 and 20) were infected by powdery mildew, which may have stunted their growth.

In the field, decapitation induced axillary buds ranged between 2 to 3 mm. In contrast to the greenhouse study, where different degrees of dominance were observed, in the field, bud 1 (the first bud below the decapitation site) always became dominant in growth while bud 3 and bud 4 often stayed dormant, as was observed in the control branches.

Response to GR24

For the GR24 application experiment, decapitation was conducted first to induce the outgrowth of axillary buds so the inhibiting effects of GR24 on branching could be tested.

Since the major differences after decapitation were observed in bud 1 and bud 2 while bud 3 and bud 4/5 did not break most of the time, the comparison was focused on the dates of bud break and the length of bud/shoot size for bud 1 and bud 2. The numbers of bud breaks in control and treatment branches are listed in Table 4-3.

Table 4-3. Number of apple lateral buds that broke after 37 days of decapitation (33 days after the last application of control solution or GR24).

Type	No. of branches	No. of released buds per branch				
		0	1	2	3	4
Control	11	2	3	5	1	0
Treatment	11	1	3	6	1	0

For the control branches, if only one bud broke, it was always bud 1; when both bud 1 and bud 2 broke, five of the controls had these two buds break at a similar time, while one had bud 1 breaking earlier. For treatment branches, bud 1 broke when there was only one bud break; for the 7 branches where bud 1 and bud 2 both broke, three were at a similar time, three had an earlier bud 1 break and 1 branch (progeny #16) had bud 2 breaking earlier (14 days), which was also the only case, among control and treated branches, where bud 2 broke earlier than bud 1. On September 9th, 2012, the shoot from bud 1 measured 25.9 mm, while that from bud 2 was 42.7 mm. The control branch, on the same plant and same fork (progeny #16, f1, C-1) had bud 1 and bud 2 breaking at comparable times although bud 2 had a more vigorous growth: on September 9th, 2012, the shoot from bud 1 was 47.1 mm versus bud 2 at 60.1 mm (Figure 4-8).

Figure 4-8. Control and treatment branches of progeny #16 in population 806-U. Both branches belonged to the same fork and bud 2 broke earlier than bud 1. The picture was taken 10 days after decapitation.

When bud/shoot size was compared for bud 1 and bud 2 for both control and treated branches, no significant difference was detected. However, shoots from bud 2 had more elongation compared to those from bud 1 for treated branches, which was unexpected. It was possible that strigolactone exerted a certain level of apical control in apple, although this has not been reported in other, mostly herbaceous, species.

In 806-U progeny #39, bud 3 and 4 on branch T-1 already burst when shoot tip was removed. In the following several weeks these two buds rarely grew, while bud 1 and bud 2 broke and grew 116 and 107 mm, respectively. This was another example of apical control, where the

suppression exerted by buds with a comparative advantage inhibited shoot elongation rather than outgrowth of the dominated buds.

Size difference between bud 1 and bud 2 continued to increase in the duration of the experiment and this was not affected by the application of GR24, as the difference was comparable for control and treated branches at different time points (Table 4-4).

Table 4-4. Mean difference in size between bud 1 and bud 2 (bud 1-bud 2)^z.

	T1^y	T2	T3	T4
Control	-0.20	0.09	8.52	23.63
Treatment	0.03	1.05	8.16	28.50

^{z}: It was treated equal whether it was a bud or a shoot; ^{y}: T1, Aug. 3rd; T2, Aug. 12th; T3, Aug 26th; T4, Sept. 9th; year 2011.

To understand whether plant architecture has effects on bud behavior in the study, plants with similar shape and stature were grouped together. However, no specific trend was discovered (Table 4-5).

Table 4-5. Bud behavior after decapitation for apple seedlings with similar shape and stature in exogenous strigolactone (SL) experiment.

Group	Progeny	Control/Treatment	Response (Sept. 9th, 2012)
1	3[z]	C-1	Bud 1 broke
		C-2	Bud 1 & 2 broke
	19	T	Bud 1 & 2 broke
	38	T	Bud 1, 2 & 3 broke
2	8	C	Bud 1 broke
	22	T	Bud 1 & 2 broke

[z] : All five progenies belonged to the sub-group of "traditional with more open canopy".

DISCUSSION

Experimental System

A unique system was used in the study where individual branches rather than the intact plants were used as experimental units. Therefore, certain plants were used both as a control and treatment plant. To make this a robust system, the application of GA_3 or GR24 to one branch should not affect the rest of the plant.

In our study, difference was observed in growth between control and GA-treated branches in dwarf plants. However, due to the small sample size, no further comparison was made between the control branches located on the same plant as the treatment branches and those that didn't.

Therefore no conclusion can be drawn regarding whether being on the same plant as the treatment branch elevated the growth of the control branches.

It was suggested that the endogenous GAs that move in the xylem in apples were not the bioactive GAs themselves but rather the precursors (Motosugi et al., 1996; Bulley et al., 2005); it was also shown in potato that GA_{20} could be transported from shoots to stolon while GA_1 remained in the vicinity of the cells where it was produced (Prat, 2010); GA_{20} was the major GA transported in pea (Proebsting et al., 1992), although GA_1 was shown to be transferrable as well, the authors believed that the detectable level of GA_1 in the expanding leaf tissues "would be unlikely to exert a significant effect on growth".

However, this may not apply to exogenous GAs as leaf applied GAs can have effects on stem elongation (light dependent). It is indicated that applied gibberellins can be transported both by an active mechanism which appears to be independent of the vascular system, and by movement with the assimilate stream in the phloem. In symmetrically two-branched plants, no response was observed in the untreated branch even when GA was applied up to 20 times more than the saturation rate to the other branch; however, if the untreated branch was defoliated first, a considerable amount of GA translocation would occur (McCready, 1966). It seems that the cross branch movement of applied GAs correlates with the vigor and ability to photosynthesis of the untreated branch. In our experiment, treatments were towards the tip of vigorously growing branches; it is likely that there was no cross branch movement. Application of radio-labeled GA_3 will aid the confirmation or refutation of the speculation.

No significant effects of SL/GR24 were observed in our study. However, strigolactone was shown to move only upwards (Dun et al., 2009) and acted locally. Feeding GR24 solution into

pea's vascular system only led to bud inhibition in the buds above the feeding site (Dun et al., 2009); treating pea plants with a ring of lanolin containing strigolactone also only inhibited buds above the site of treatment (personal communication with Elizabeth Dun). More specifically, as demonstrated in wild type *Arabidopsis,* branching can be differentially affected on two-sides of the stem by unilateral *MAX* induction, suggesting that the competition between the primary shoot apex the buds can happen specifically on the bud's side of the shoot (Ongaroa et al., 2008). Therefore, GR24 applied to buds on one branch should not affect the bud growth on the other branches on the same plant.

GA$_3$

In the Introduction section, GA promotion of stem elongation by stimulating both cell elongation and cell division was reviewed. GA$_3$ applied during internode development increased both cell number and cell shape in pea (Daykin et al. 1997). We want to understand whether GA stimulates shoot elongation by promoting growth between nodes or by increasing node numbers.

In our study, GA$_3$ treated branches from dwarf plants had significantly more growth compared to control branches due only to an increased number of nodes, whereas in Bulley et al.'s (2005) research, GA$_3$ was able to restore both internode length and node number in the transgenic lines of 'Greensleeves' apple. The difference could be a result of different dwarfing mechanisms involved or due to cultivar differences.

Decapitation

Despite the diverse branching patterns in different plant species, almost every plant reacts to shoot tip damage by initiation new branch growth (Turnbull, 2005). Decapitation removes the source of auxin that leads to a decrease of the transcript levels of strigolactone biosynthesis genes therefore the production of strigolactones (Domagalska and Leyser, 2011). This auxin regulated SL depletion is probably a major cause of branching after decapitation (Brewer et al., 2009), which can be inhibited by application of GR24 to axillary buds in pea plants (Beveridge and Kyozuka, 2010).

Bud behavior after decapitation needs to be understood to determine the inhibiting effects of GR24 on apple buds, hence a preliminary experiment was performed in the greenhouse with population 28 before decapitation was conducted in the field with progeny 806-U to release axillary buds from apical dominance and synchronize their growth.

Based on the results from the greenhouse, after decapitation, multiple apple axillary buds tended to break at similar time. Although different degrees of dominance were observed, 50% of the plants had newly grown branches with similar vigor. However, in the field, it was obvious that bud 1 and 2 had a higher tendency of bud break and usually broke much earlier. In many occasions, bud 3-5 stayed dormant. Orientation of the branch should not be the difference here since the dwarf progeny in 806 is characterized as having upright branches, which would be similar to seedlings in the greenhouse, especially since most of the treated branches are one part of the fork, which by its nature is more upright.

One explanation for this difference could be in the greenhouse, the physiological status of the axillary buds at different locations did not differ enough for certain buds to gain dominance over

others (plants were young and short, so buds were in close proximity with each other), whereas in the field, the buds differed more both in location on the plants and developmental stages, which gave the first buds a stronger comparative advantage that allowed them to quickly establish auxin export to the mainstream and prevent other buds from, or delay their activation (Domagalska and Leyser, 2011).

It may be fair to consider the seedlings in the greenhouse as analogs of herbaceous plants and those in the field as woody plants. Models of apical dominance in herbaceous plants are often employed to explain apical dominance in woody plants as well, despite the recognition that with increased complexity in morphology and physiology and factors like perennial growth habit, predominance of woody vascular tissue and endodormancy, woody species may function differently in regard to apical dominance (Cline, 2000). It is suggested that the concept of 'apical dominance' as used for herbaceous plants should only be applied to the current year's growth in woody plants rather than a whole tree scale (Cline, 2000).

One thing that differs dramatically between herbaceous and woody plants is the scale of the organism and the distance plant hormones need to travel in mature apple trees were they assume the same mechanism in regulating plant branching, as strigolactone is shown to transport from root to shoot in herbaceous plants. Polar auxin transports at a speed of 1 cm/h and it could take days for an auxin molecule to transport from shoot tip to the root or *vice versa* in woody species (Cline, 2000). Although now many lines of evidence revealed that the bioactive form of hormones (e.g., GA, CK) may well be synthesized in the vicinity of the site of action (Benková et al., 2003; Zhao, 2008).

Another explanation could be genotypic (cultivar) differences. The strength of apical dominance varies among apple cultivars (Maguylo et al., 2012) as laterals rarely break earlier than the terminals in 'Golden Delicious', but laterals do break earlier in 'Granny Smith'. The degree of apical control also varies among different cultivars (Lauri, 2007).

The "forking" phenotype in progeny 806-U would make this population good plant material to study apical dominance in woody species. Follow-up experiments can be conducted once the trees are further into their maturity and compared with the current results to learn about the change of apical dominance as woody plants gain complexity in morphology.

GR24

Due to the low concentration of strigolactone in plants, there is no routine procedure to obtain pure strigolactone. Therefore GR24, a strigolactone chemical analog, is often used to test a mutant's reaction to exogenous application.

In our study, the first buds below the decapitation sites were not inhibited by GR24. If the application method was effective, the dwarf mutants were not responsive to GR24, hence strigolactone. The reason could be defects in the signaling pathway of SL similar to mutants *d3/rms4/max2* (Gomez-Roldan et al., 2008; Umehara et al., 2008) or a different branching mechanism in apples in contrast to herbaceous plants (see discussion in previous section).

Alternatively the application method could be ineffective because: 1) the treatment solution may not have been able to penetrate the apple buds' thick surface. Several methods exist to treat plants with strigolactone or GR24: direct application to buds; application through the vascular

stream; the lanolin ring method and growth of plants hydroponically (this requires a large amount of SL/GR24). GR24 was applied directly to apple buds in our experiments because it required less GR24 and was easy to apply. The vascular stream method could be used in subsequent studies to verify the effectiveness of applying solutions to the buds.

2) The concentration of GR24 was not sufficient to induce a reaction in apple seedlings. SL inhibition of branching in rice and pea was concentration dependent (Gomez-Roldan et al., 2008; Umehara et al., 2008) and as low as 10 nM GR24 was able to elicit a response. In pea plants, bud outgrowth in strigolactone-deficient mutants was successfully inhibited with one application of 1 µM GR24 solution directly to the bud in a volume of 2-10 µL (Beveridge and Kyozuka, 2010). If strigolactone functions in a similar manner in apples as in the species studied so far, multiple applications of 10 µM GR24 (in 10 µL volume) should be a concentration sufficient to induce an action, even considering that the outgrowth of axillary buds in apple is a much slower process.

3) The timing was off. Unlike herbaceous plants, seasonality is very important in the apical dominance of woody species. As demonstrated in white ash (*Fraxinus americana* var. *americana* L.), green ash [*Fraxinus pennsylvanica* var. *subintegerrima* (Vahl.) Fern], and red oak (*Quercus rubra* L.), the success of auxin replacement experiments were restricted to spring flush (Cline, 2000). Repression of bud outgrowth cannot be done if they are not growing out. Therefore, spring flush, during which buds are at their optimal growth, provides the ideal condition to test the repression effect of auxin, which is mediated by strigolactone. In our study, application of GR24 was conducted in the field in August, and it is possible that the responsiveness of the plants to strigolactone declined as the growing season came to an end. However, dwarf plants in

our study exhibited a longer growing period than standard plants, with new growth still evident in August when the plants were treated.

4) The decapitation site was not appropriate. For white ash, green ash and red oak, the auxin replacement experiment only worked for one-year-old proleptic buds (of branches or older trees) and current buds of seedlings during spring flush (Cline, 2000). Apical dominance (paradormancy) is just one of the mechanisms[21] that regulate bud dormancy and it is thought unfruitful to test auxin or strigolactone's inhibitory effects on irrepressible[22] buds (Cline, 2000).

Given the wide spectrum of morphology and physiology status in woody plants, experimental results in ash and oak may not represent the situation in apples, nonetheless, similarities are expected.

In our field study, bud outgrowth was observed within a week after decapitation, a result comparable to that of Cline (2000), so it is reasonable to assume that these buds were auxin-repressible buds. The diagram in Figure 4-1 can be used as a reference for conducting similar experiments in the future but plant to plant variation may exist.

The bioactive form and ultimate function of strigolactone is still not fully understood (Umehara et al., 2008), so that exogenous application combined with microarray studies could be useful to explore its distribution, movement and perception (Gomez-Roldan et al., 2008).

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[21] 1) paradormancy, the inhibition of growth by distal organ; 2) endodormancy, the inhibition of growth internal bud signals; 3) ecodormancy, the inhibition of growth by temporary unfavorable environment conditions (Lang et al., 1987).

[22] Repressible and irrepressible buds in woody species are distinguished by whether there is inhibition for their outgrowth during the spring flush (Cline, 2000).

In horticulture, the study of strigolactone should be of special interest as these natural compounds could be used to alter plant architecture without the need of transgenic technology and molecular breeding (Schachtschabel and Boland, 2009). The regulation can be specific without the side effects of auxin or cytokinin (Gomez-Roldan et al., 2008). However, so far, most strigolactone research has been on herbaceous plants (*Pisum*, *Arabidopsis* and *Oryza*) (Waldie, 2010). Whether this novel hormone functions similarly in woody plants needs further verification.

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GLOSSARY

acropetal   developing or maturing from the base toward the apex, as in those plant organs in which the younger tissues are nearer the apex

acrotony   increased vigor of the vegetative proleptic branches (from dormant buds), from the proximal to the distal part of the parent growth unit

basipetal   of or relating to the development or maturation of tissues or organs or the movement of substances, such as hormones, from the apex downward toward the base

benzylaminopurine (BAP)   a first-generation synthetic cytokinin that elicits plant growth and development responses, setting blossoms and stimulating fruit richness by stimulating cell division

biennial bearing   irregular fruiting across consecutive years characterized by large yields of small sized fruit in 'on' years and low yields, sometimes even no fruit, on 'off' years; a widely spread phenomenon and occur in both deciduous and evergreen trees

bourse   flowering growing unit

bourse bud   vegetative buds are formed in the axils of leaves below flower primordia, of which two are usually initiated at the time of flowering; the following spring, one of the two vegetative buds will usually become active and, if conditions are favorable, will be the site of flower bud formation for that spur during late summer; following dormancy in the spring but before the time of flower formation, the apex of the vegetative bud (bourse bud) will initiate leaf-like primordia (appendages) that mature and develop over the season to form the budscales

CYC®   cyclanilide, a plant growth regulator, acts as an auxin transport inhibitor; it temporarily interrupts apical dominance and allows latent buds to mature and elongate (also see **Tiberon®**)

Ethephon®   2-chloroethylphosphonic acid, a plant growth regulator, releases ethylene when applied

ecodormancy   the inhibition of growth by temporary unfavorable environment conditions

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endodormancy	the inhibition of growth by internal bud signals
etiolated growth	characterized by long hypocotyls, small and closed cotyledons, undifferentiated chloroplasts and repression of light-regulated genes
flush	a phase of vegetative elongation
GR24	a synthetic analog of strigolactone
growth unit (GU)	when a bud produces a sequence of meristems simultaneously, also called extension unit
gametophytic self-incompatibility (GSI)	a S-RNase-based self-incompatibility system that has been found in the families of *Solanaceae, Rosaceae* and *Plantaginaceae*
GRAS proteins	a plant-specific protein family, named after the first three members: *GIBBERELLIC-ACID INSENSITIVE* (*GAI*), *REPRESSOR of GAI* (*RGA*) and *SCARECROW* (*SCR*)
latent bud	an axillary bud whose development is inhibited, sometimes for many years, due to the influence of apical and other buds; also known as dormant bud
linkage group (LG)	genes that are inherited as a single unit are said to be linked, and are referred to as "linkage groups"
Maxcel®	a plant growth regulator that acts as fruit thinning agent with active ingredient 6-benzyladenine
metamer	the basic element of plant construction, composed of a node and its leaves and axillary bud(s) plus the subtending internode
1**-N-naphthylphthalamic acid (NPA)**	an inhibitor of auxin transport
progenitor	a direct ancestor
Promalin®	a plant growth regulator, a mixture of 6-BA (promotes cell division) and GA_{4+7} (promotes cell expansion)
paradormancy	the inhibition of growth by distal organs

proleptic shoot	shoots develop after a period of dormancy
paclobutrazol (PAC)	a plant growth retardant that inhibits gibberellin biosynthesis
ReTain®	a plant growth regulator, contains aminoethoxyvinylglycine hydrochloride (AVG, a naturally occurring inhibitor of ethylene biosynthesis in plants)
RNA-Seq	whole transcriptome shotgun sequencing
sylleptic shoot	shoots develop directly after meristem initiation
self-incompatibility (SI)	a genetic mechanism to prevent inbreeding and to promote outcrossing, found in many flowering plants
spur	short fruiting shoots
S-RNase	S locus encoded ribonuclease
Tiberon®	cyclanilide, a plant growth regulator that inhibits auxin transport activity, particularly in the meristematic tissue, thereby suppressing apical dominance and increasing branching
Thidiazuron (TDZ)	a cytokinin type growth regulator that activates bud outgrowth
2,3,5-triiodobenzoic acid (TIBA)	an auxin transport inhibitor
Unigene	a NCBI database of the trancriptome; each entry is a set of transcripts that appear to stem from the same transcription locus