Effect of roots on artemisinin and flavonoid production in shoots of *Artemisia annua.*

By

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A Thesis

Submitted to the Faculty of

WORCESTER POLYTECHNIC INSTITUTE

in partial fulfillment of the requirements for the degree of

Master of Science

In

Biology and Biotechnology

May 2015

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APPROVED

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Abstract

Artemisinin is a potent antimalarial sesquiterpene lactone produced and stored in the glandular trichomes (GLTs) of *Artemisia annua*. Although they produce no artemisinin, nor any of the precursor compounds, *A. annua* roots appear to have a regulatory effect on production of the terpene in leaves. However, more information is needed to define the role of the roots in artemisinin production in the plant. Grafting among three cultivars was used to measure phenotypic responses: SAM, and #15 cultivars both have GLTs, but produce artemisinin at 1.49% and 0.57% DW, respectively; GLS cultivar produces neither GLTs nor artemisinin. Compared to ungrafted plants, all self-grafts, e.g. SAM/SAM (scion/rootstock), increased scion artemisinin probably from grafting stress. SAM/#15 grafts yielded less artemisinin than SAM/SAM, but more than either #15/#15 or ungrafted #15 and SAM suggesting rootstock inhibition of the scion. SAM/SAM also had more artemisinin than #15/SAM, which was also greater than either #15/#15 or ungrafted #15 and SAM. The #15/SAM graft also produced more artemisinin than SAM/#15, and with the other grafting results suggested that SAM roots were stimulating artemisinin production in the #15 scion. There was no appearance of either GLTs or artemisinin when GLS scions were grafted to SAM indicating that GLTs had to be present to receive putative signals from SAM rootstocks. Furthermore, artemisinic acid and arteannuin B were only present in SAM scions and not scions of #15 suggesting a block in one of the side pathways of artemisinin biosynthesis. Other artemisinic metabolites, total flavonoids, and GLTs numbers were also measured. The various phenotypes were analyzed several months after grafting indicating a persistent change and suggesting a possible epigenetic alteration of the scion. This study will provide fundamental information regarding the role that roots play in the production of artemisinin in the shoots of *A. annua*.

Acknowledgements

I appreciate all the people who have ever helped me complete my MS project in the past two years. First of all, I would like to express sincere gratitude to my advisor, Dr. Pamela Weathers, for her guidance, support and encouragement. The most valuable thing she taught me is how to develop logical, independent and critical thinking in my research. Besides, her admirable personal qualities, firm and tenacious perseverance and assiduous attitude towards work and research, she deeply influenced on my attitude to life and work. I hope to be a person like her, to pursue self-dreams and never give up!

Secondly, I would like to deeply thank my committee members, Dr. Kristin Wobbe and Dr. Luis Vidali, for their helpful suggestions and constructive ideas to my project, especially on experimental design and figure pattern.

Thirdly, I would like to thank to the present and previous lab members, for your valuable help and suggestion for my project, I saved a lot of time and you inspired me with more innovative ideas. I sincerely appreciate Dr. Melissa Towler for all the huge help she provided for me. She was very nice and patient person. I want to thank her for those good ideas and teaching me all the related experimental skill and how to use experimental apparatus in my project. I would also like to thank Liwen Fei and Ying Yang. They were really like my elder sisters and gave me a lot of help and care on life and study. What's more, I would like to thank all the other lab members in our lab; your wonderful questions and ideas made me come up with new ideas for my project.

In addition, I wanted to thank WPI for giving me a valuable chance to study here and work with these excellent people! Also, I would like to thank the National Institutes of Health (NIH-2R15GM069562-03) for funding my project.

Lastly, huge and sincerely thanks must go to my family and close friends for their support and encouragement. Whatever difficulties I encountered, family love and friendships were always along with me to face and overcome challenges of during the past two years! Because I always believe that 99% difficulties are of our own imagination!

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Abbreviations Used

AA: artemisinic acid; AAA: artemisinic aldehyde; AB: arteannuin B; ABA: Abscisic acid; AACT: acetoacetyl-CoA thiolase; ACT: artemisinin combination therapy; DHAA: dihydroartemisinic acid; DHAAA: dihydroartemisinic aldehyde; DHOH: dihydroartemisinic alcohol; DeoxyAN: deoxyartemisinin; GLT: glandular trichomes; GLS: glandless *A. annua* cultivar; JA: Jasmonic acid; L10: leaf 10; MEP: plastidial Methyl-erythritol-4-phosphate; MVA: cytosolic acetate-mevalonate; nS: ungrafted SAM cultivar; n15: ungrafted #15 cultivar; nG: ungrafted glandless cultivar; ROS: reactive oxygen species; ShAM: shoot apical meristem; SA: Salicylic acid; S:SAM *A. annua* cultivar; TFs: transcription factors; pACT: whole plant therapy; 15: #15 *A. annua* cultivar.

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1. Introduction

1.1 Malaria

Malaria is a serious infectious and potentially fatal vector-born disease in many parts of the world. The World Health Organization (WHO) estimated there were 198 million cases of malaria and more than 584,000 deaths, mainly African children (WHO, 2014a). Even if children survive this disease, most of them often are inflicted with brain damage and learning impairments (Malaria 2011). In general, the parasite species, the vector, the human host, and the environment decide the infection intensity of malaria (WHO, 2014a).

Malaria caused by *Plasmodium falciparum* is the most deadly form of malaria found in sub-Saharan Africa (Malaria 2011). In recent years, however, some human malaria cases have also been caused by *Plasmodium knowlesi*, a species that previously only caused malaria among monkeys and occurs in certain forested areas of South-East Asia (WHO, 2014a). *Anopheles* mosquitoes carry the infective sporozoite stage in their salivary glands and inject sporozoites into the human blood stream during a blood meal. Currently, scientists have found about 20 different *Anopheles* species around the world. Infection is more intense in places where the lifespan of the mosquito is longer, which allows the parasite adequate time to complete its development inside the mosquito. Mosquito species, *Anopheles*, prefer to bite humans rather than other animals so this results in more intense transmission. For example, about 90% of the world's malaria deaths are caused by the long lifespan and strong human-biting habit of the African vector species (Okwa 2012).

Malaria epidemics generally happen in areas where people have little or no immunity to malaria. Partial immunity is developed over years of exposure, however, there is not complete protection, so infection can reoccur or become sever leading even to cerebral malaria. For this reason, Human malaria is mainly caused by several *Plasmodium* species: *P. vivax*, *P. malariae*, *P. ovale*, *P. falciparum*, and *P. vivax* are the most common (Perandin et al. 2004). Once parasites enter the blood stream, they will first migrate to and reproduce in the liver, after which they will continue infecting red blood cells (Fig.1). If there is no treatment, these infections will progress to the next stage referred to as the ‘blood stage’. Debilitating symptoms occur from

destruction of red blood cells, which disintegrate and clog capillaries that lead to the brain or other vital organs (Malaria 2011) (Fig.1). Symptoms including vomiting, headache, fever, as well as other flu-like symptoms of malaria usually appear 9-14 days after the individual has been bitten. If there is no treatment or if malarial drug resistance is present, the disease progresses to a severe level often leading to death if within 24 hours there is no therapy (Kim and Schneider 2013). Lethality of the disease results from developing one or more of the following symptoms: severe anemia, respiratory distress in relation to metabolic acidosis, or cerebral malaria (WHO, 2014b).

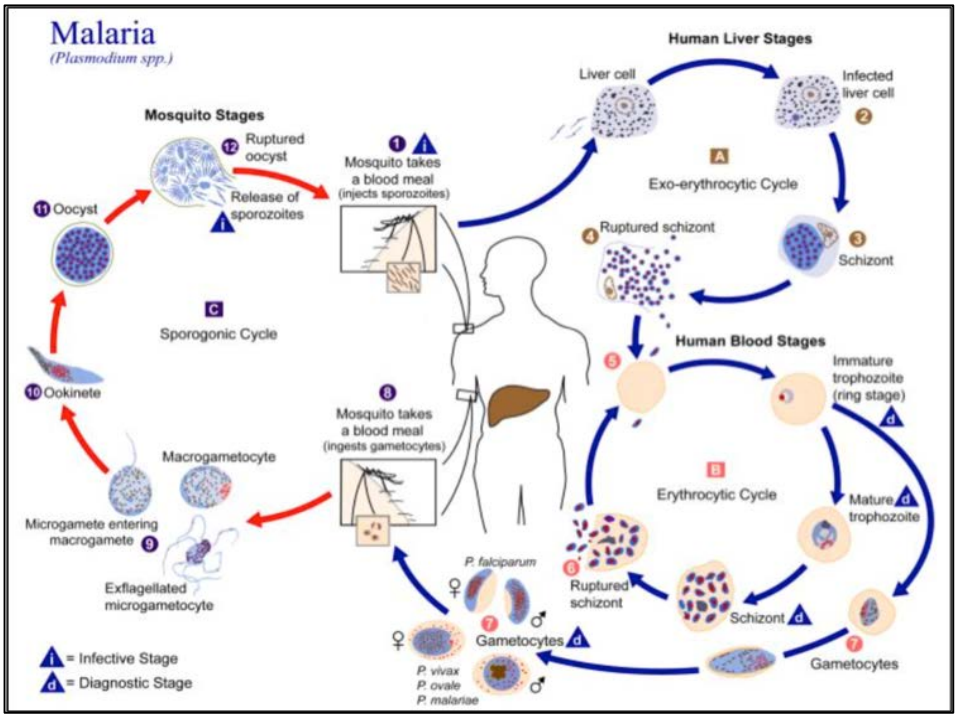


Figure 1 An illustration of the life cycle of the parasites that cause malaria.
(Taken from http://www.cdc.gov/malaria/about/biology/)

1.2 *Artemisia annua* L.

The herb *Artemisia annua* L. (Fig.2A) was initially used as a tea infusion in Chinese traditional medicine as a treatment for fever and malaria for more than 2,000 years (Klayman 1985). Artemisinin (AN) was originally extracted from *A. annua* by Chinese scientists more than 30 years ago in a search for new antimalarial drugs. AN is delivered in concert with other

antimalarial drug, for example artesunate or amodiaquine, as the current preferred treatment as artemisinin-based combination therapy (ACT). The use of ACTs is to avoid emergence of AN drug resistance, and to treat chloroquine resistant and cerebral malaria (Martensson et al. 2005).

A. annua is cultivated mainly in China and Vietnam, which produces about 80% of the global supply; the remaining 20% is produced in East Africa (Shretta and Yadav 2012). The plant is an annual herbaceous plant in the family Asteraceae. *A. annua* can grow to 2 meter in height and can be found in temperate regions of China, Vietnam, India, Russia, East Africa, the United States, and Brazil (Bhakuni et al. 1988).

1.3 Artemisinin, ACTs and synthetic Artemisinin

In the late 1950s, chloroquine was the main effective medicine used for malaria treatment by the WHO, but its long-term use drove development of drug-resistant parasites, and an apparent resurgence of malaria worldwide. In the 1970s, Chinese chemists isolated and identified the primary substance responsible for the anti-malarial action of *A. annua*, artemisinin (AN), and it soon replaced chloroquine. Although pure AN is very effective treatment and against malaria, its yield from *A. annua* is unfortunately low (0.5–1.2%) and it remains expensive for many people in developing countries (Kindermans et al. 2007).

Artemisinin is produced solely in shoots of glandular trichomes (GLTs) of this species. (Duke et al. 1994; Olsson et al. 2009) (Fig.2B). Besides its anti-malaria activity, AN may also be effective against other human diseases like schistosomiasis, hepatitis B, and leukemia, breast, colon and small-cell lung carcinomas (Firestone and Sundar 2009; Lai et al. 2013; Fox et al. 2014).

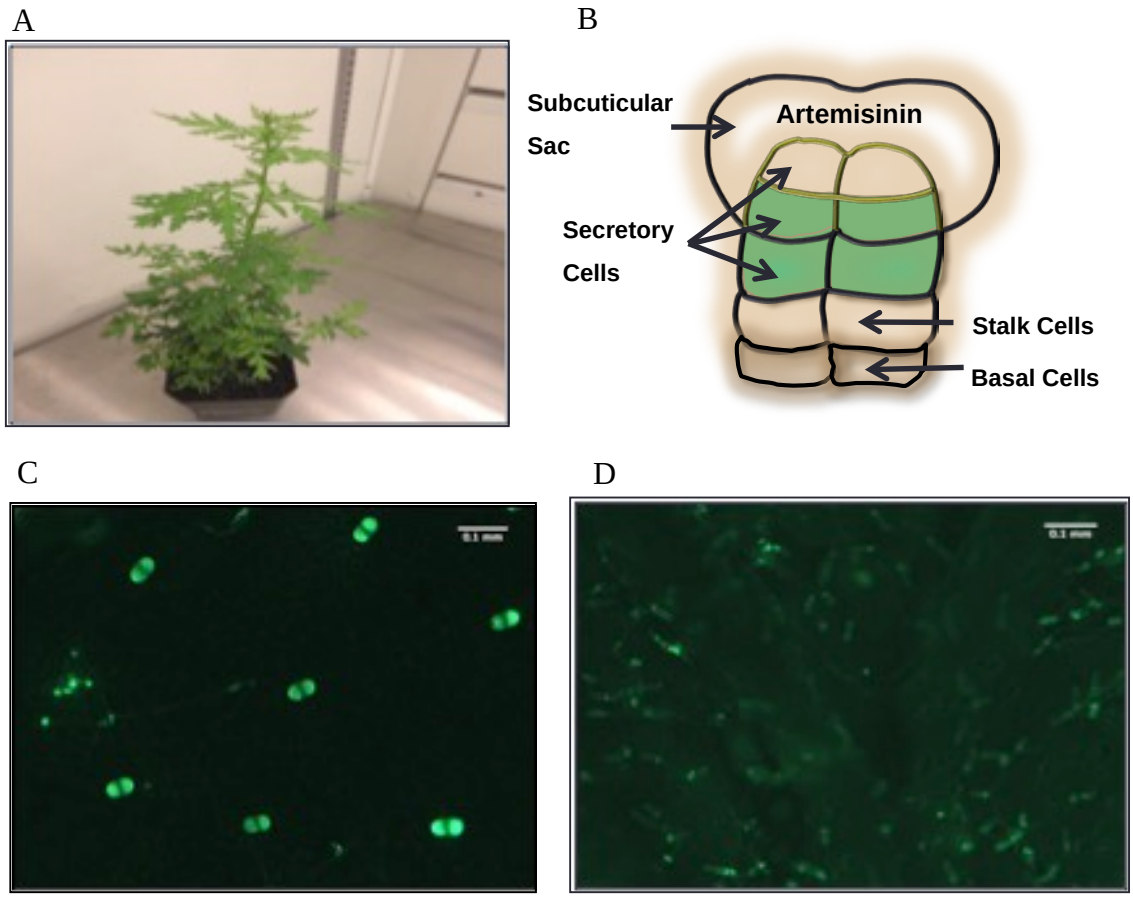


Figure 2 *Artemisia annua* plant and trichomes. A, *Artemisia annua* (SAM cultivar). B, an illustration showing localization of pathway enzymes in the glandular trichome cells. C, the glandular trichomes are visualized using fluorescence microscopy; D, filamentous trichomes, *bar* 0.1mm.

ACTs for anti-malarials have since been adopted by most countries, and are effective against both chloroquine-resistant and sensitive strains of *P. falciparum* as well as cerebral malaria with a high safety profile. In addition, ACTs are the first line of malaria treatment recommended by WHO to address the chloroquine resistance of *P. falciparum* (Klein 2013). Although ACTs are effective treatments, they cost $\geqslant$ 20 times more than monotherapies. Due to the high cost of production and low yield of the active drug, at best approximately 1.4% of the plant leaf mass (Kindermans et al. 2007; Towler and Weathers 2015), investigators have searched for a way to mass produce AN for a lower cost and to meet the market demand (Zeng et al. 2008; Lévesque and Seeberger 2012; Wang et al. 2014). Until recently, AN industrial production mainly depends on extraction from the plant and synthetic microbe-derived artemisinin.

Considering the increasing demand and low yields of AN, investigators are looking for various methods to increase the AN production, such as chemical synthesis (Zhu and Cook 2012) and use of genetically-modified microbes (Paddon and Keasling 2014). In 2014, Sanofi (Paris-based pharmaceutical company) began industrial-scale production of artemisinin-using a genetically engineered yeast strain that could produce artemisinic acid, a precursor in semi-synthesis of artemisinin. Initial cost is about $350-400 (£230 –260)/kg for producing AN, roughly the same as the botanical source (Peplow 2013).

1.4 Artemisinin delivered via oral consumption of *A. annua* dried leaves

Recently, some experimental data suggested that oral consumption of the dried leaves of *A. annua* might be more effective than treatment with the pure drug. It is hypothesized that the complex mixture of phytochemicals in the leaves may enhance AN efficacy (Weathers et al. 2014). This therapeutic approach is called plant artemisinin combination therapy (pACT), which may also be more effective than ACT.

Evidence from animal studies indicated that whole plant therapies (pACT) work as posited. When mice were fed AN, there was $\geqslant$ 40 fold more AN from oral ingested dried *A. annua* leaves found in the blood than in serum of mice fed equal amounts of pure AN (Weathers et al. 2011). Additionally, Elfawal et al. (2012) showed that orally ingested powdered dried leaves of *A. annua* could kill the malaria parasite more effectively than a comparable dose of the pure drug. Therefore, pACT has the potential to increase the number of patients treated each year with malaria by reducing the cost of drug production and by providing more doses per acre because the associated losses from extraction are eliminated. Compared to pure AN, Elfawal et al. (2015) also recently showed that pACT was three fold less likely to induce AN drug resistance, probably a result of the diversity of non-artemisinin anti-malarial phytochemicals in the plant (Weathers et al. 2014), Implementation of pACT can stimulate the economies of developing nations by locally cultivating the plant, processing the plant material, and distributing the pACT, thereby creating jobs (Elfawal et al. 2012).

About 60% of global AN yields still comes from the extraction from plants (Chemistryworld 2013). Hence, enhancement of the AN content in *A. annua* is considered important for reducing the cost of artemisinin and increasing its supply. To improve AN production, a better understanding of the regulation of AN biosynthetic pathway could provide new information for breeding *A. annua* with higher levels of AN.

1.5 Secondary metabolite biosynthesis in *A. annua* L

1.5.1 Artemisinin biosynthesis and its regulation

AN is bio-synthesized through the isoprenoid metabolic pathway from precursors produced in the cytosolic and plastid compartments of *A. annua* glandular secretory trichomes (Towler and Weathers 2007; Schramek et al. 2010). In general, three isopentenyl diphosphate (IPP) precursor molecules are condensed and oxidized to produce AN (Bouwmeester et al. 1999; Towler and Weathers 2007). IPP is produced through two pathways: the cytosolic mevalonate (MVA) pathway and/or the plastidic non-mevalonate (MEP) pathway (Fig.3). The MEP terpenoid pathway is regulated by 1-deoxyxylulose 5-phosphate synthase (DXS), and 1-deoxyxylulouse 5-phosphate reductoisomerase (DXR). The MVP pathway is mainly regulated by 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR). Farnesyl diphosphate is then produced via farnesyl diphosphate synthase (FPS) (Souret et al. 2003) (Fig.3).

In AN biosynthesis, farnesyl diphosphate (FPP) was catalyzed by amorpha-4,11-diene synthase (ADS) to form amorpha-4,11-diene, a sesquiterpene cyclase (Bouwmeester et al. 1999; Wallaart et al. 2001). This is the first step of AN biosynthesis (Bouwmeester et al. 1999; Mercer et al. 2007; Wallaart et al. 2001). The next, two reactions are the oxidation of amorpha-4,11-diene to artemisinic aldehyde and to artemisinic aldehyde (AAA) and are catalyzed by a cytochrome P450 dependent amorpha-4,11-diene 12-hydroxylase CYP71AV1 (CYP) (Teoh et al. 2006). From there, the biosynthetic pathway branches; one route leads to AN and the other to arteannuin B (AB).

For the route to AN, artemisinic aldehyde is converted to dihydroartemisinic aldehyde (DHAAA) by a double-bond reductase (Dbr2) and then oxidized to dihydroartemisinic acid

(DHAA) by aldehyde dehydrogenase 1 (ALDH1) (Teoh et al. 2009; Zhang et al. 2008b). DHAAA is also reduced to dihydroartemisinic alcohol (DHOH) via dihydroartemisinic aldehyde reductase RED1 (Rydén et al. 2010), which is not yet well studied *in planta*. Then, DHAA is converted to AN or deoxyartemisinin (deoxyAN) by a nonenzymatic photo-oxidation reaction. It has not been completely verified if this reaction is also nonenzymatic *in vivo.* Arsenault et al. (2010) showed that the transcription level of related genes, such as amorpha-4,11-diene synthase (ADS) and the cytochrome P450, CYP71AV1 (CYP), in the AN biosynthetic pathway closely correlated with precursors level of AN, DHAA. Trichome numbers also seemed to be correlated with the AN level but not its precursors. Feedback inhibition was also observed. When leaves of *A. annua* were sprayed with exogenous AN or AA, *CYP* transcription was inhibited; AA also inhibited *ADS* transcription. Together these results suggested a negative feedback loop of AN and AA on *ADS* and *CYP* genes, thereby regulating AN and its precursors (Arsenault et al. 2010).

For the route to AB, the intermediate DHAAA is oxidized by CYP or an aldehyde dehydrogenase (Aldh1) to form artemisinic acid (AA), then AA is converted to arteannuin B (AB) also in a non-enzymatic photo-oxygenic reaction (Brown 2010). So far, there is no evidence for conversion of AB to AN in *A. annua*. Brown and Sy (2007) investigated and also concluded that there is no evidence conversion of AA to AN. Thus, it is currently thought that AA → AB represents a separate branch in the AN biosynthetic pathway.

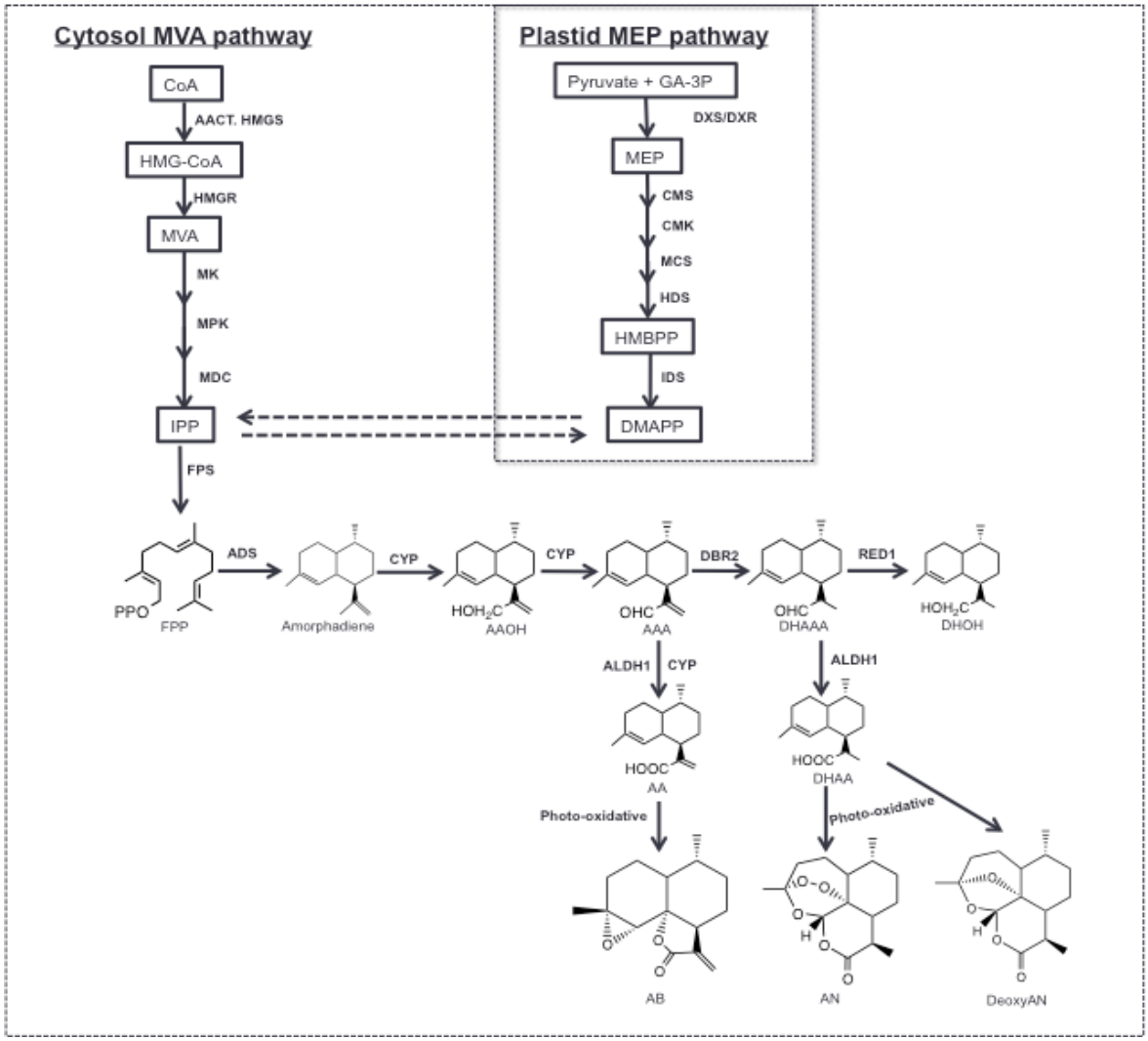


Figure 3 Artemisinin biosynthetic pathway. AA, artemisinic acid; AAA, artemisinic aldehyde; AACT, acetoacetyl-CoA thiolase; AAOH, artemisinic alcohol; AB, arteannuin B; ADS, amorpha-4, 11-diene synthase; AN, artemisinin; CYP, cytochrome P450; CYP71AV1, cytochrome P450-dependent hydroxylase;
CMK, 4-(cytidine-5'-diphospho)-2-C-methyl-d-Erythritol kinase;
CMS, 4-Diphosphocytidyl-2C-methyl-d-erythritol 4-phosphate synthase; DBR2 double bond reductase 2; DHAA, dihydroartemisinic acid; DHAAA, dihydroartemisinic aldehyde; DHOH, dihydroartemisinic alcohol; DeoxyAN, deoxyartemisinin; DXR, 1-deoxyxylulouse 5-phosphate reductoisomerase; DXS, 1-deoxyxylulose 5-phosphate synthase; FPS, farnesyl diphosphate synthase; FPP, farnesyl pyrophosphate; HBMPP, 4-hydroxy-3-methylbut-2-enyl diphosphate; HDS, 4-Hydroxy-3-methylbut2-en-yl-diphosphate synthase;
HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; HMGS, HMG-CoA synthase; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; IDS, IPP/DMAPP synthase; MCS, 2C-methyl-d-erythritol 2,4-cyclodiphosphate synthase; MDC, mevalonate 5-diphosphate decarboxylase; MK, mevalonate kinase; MPK, phosphomevalonate kinase;
MEP, plastidial Methyl-erythritol-4-phosphate (MEP) (plastid pathway); MVA, cytosolic acetate-mevalonate (MVA) pathways; RED1, dihydroartemisinic aldehyde reductase 1

1.5.2 Flavonoids and flavonoid biosynthesis

Flavonoids are general phenolic compounds based on a 15 carbon (C6-C3-C6) structure and they comprise pigments that color most flowers, fruits, seeds and leaves. Flavonoids are low molecular weight secondary metabolites that are not deemed essential for plant survival. They are classified into six main subgroups: chalcones, flavones, flavonols, flavandiols, anthocyanins, and proanthocyanidins or condensed tannins; in some species, a seventh group is found, the aurones (Winkel-Shirley 2001; Winkel 2006; Saito et al. 2013).

Currently, over 10,000 structural variants of flavonoids have been reported (Williams and Grayer, 2004). Their synthesis appears to be ubiquitous in plants and evolved early during land plant evolution, aiding in plant protection and signaling (Buer et al. 2010; Pollastri and Tattini 2011). Flavonoids have diverse biological functions *in planta*, including protection against ultraviolet (UV) radiation and phytopathogens, signaling during nodulation, male fertility, auxin transport, as well as pollinator attractants (Bohm 1998; Winkel-Shirley 2001; Koes et al. 2005; Wang et al. 2009). Flavonoids also have a wide spectrum of pharmacological uses in traditional Chinese medicine (Zhang and Ye 2009). Flavonols are probably the most important flavonoids participating in stress responses; they are the most ancient and widespread flavonoids, having a wide range of potent physiological activities (Stafford 1991; Pollastri and Tattini 2011). When ingested by animals and humans, flavonoids seem to have important antiviral, anti-allergic, anti-inflammatory, antitumor, and antioxidant activities (Miranda et al. 2000).

1.5.2.1 Flavonoid biosynthetic pathway and regulation

Flavonoid biosynthesis is one of the best-characterized secondary metabolic pathways in plants. Related enzymes in this biosynthetic pathway generate a super-molecular complex through protein-protein interactions and are localized in the membrane of the endoplasmic reticulum (Samanta et al. 2011). The key precursors for the synthesis of flavonoids are the amino acid phenylalanine (Phe) and malonyl-CoA produced from the shikimate and the TCA pathways (Samanta et al. 2011). Additionally, environmental factors, such as atmospheric CO_2,

ozone (O3) levels, UV light, and temperature to some extent influence the enzymatic activity of flavonoid biosynthesis (Cheng et al. 2012; Saito et al. 2013).

Flavonoid biosynthesis in plants generally involves the phenylpropanoid pathway (Fig. 4A), where first Phe is transformed into cinnamic acid by phenylalanine-ammonia lyase (PAL) a tetrameric, generally ubiquitous enzyme in the plant kingdom. In the second step, cinnamate 4-hydroxylase (C4H) catalyzes the hydroxylation of trans-cinnamic acid to generate *p*-coumaric acid (Samanta et al. 2011; López-Gresa et al. 2011; Ferreyra et al. 2012). Next, the hydroxycinnamic acid *p*-coumaric acid is converted to 4-coumaroyl-CoA mediated by *p*-coumarate CoA ligase (4CL). Then one molecule of *p*-coumaroyl-CoA condenses with three molecules of malonyl-CoA in the presence of chalcone synthase (CHS) resulting in the production of chalcone, the first true product of the flavonoid pathway. The enzyme chalcone isomerase (CHI) then catalyzes flavanone from chalcone. Flavanones undergo a variety of reactions including hydroxylation to yield dihydroflavonols catalyzed by flavanone hydroxylase (F3H), further leading to flavanols and anthocyanins, or to flavonols and their glucosides. Flavanones also can convert to other flavonoids molecules including flavones, isflavones or flavan-4-ols (Rangarajan et al. 2004; Samanta et al. 2011).

1.5.3 Flavonoids in *A. annua* and synergy with artemisinin

A.annua L. contains at least six main specific flavonoids, artemetin (ART), casticin (CAS), chrysoplenetin (CRY), chrysoplenol-D (CRYD), cirsilineol (CIR), and eupatorin (EUP) (Fig. 4B), that have been shown *in vitro* to work synergistically with AN to treat *P. falciparum* malaria (Elford et al. 1987; Liu et al. 1992). When each of these six methoxylated flavones was combined individually with AN, the IC50 of AN against *P. falciparum* decreased by 20–50%, demonstrating an apparent synergy with AN (Liu et al. 1992). Interestingly, Elford et al. (1987) also showed that casticin could synergistically aid with AN to kill *P. falciparum*, however, it did not keep the synergism with chloroquine, suggesting a different interactive mechanism between AN and chloroquine. Casticin and AN controlled the influx of

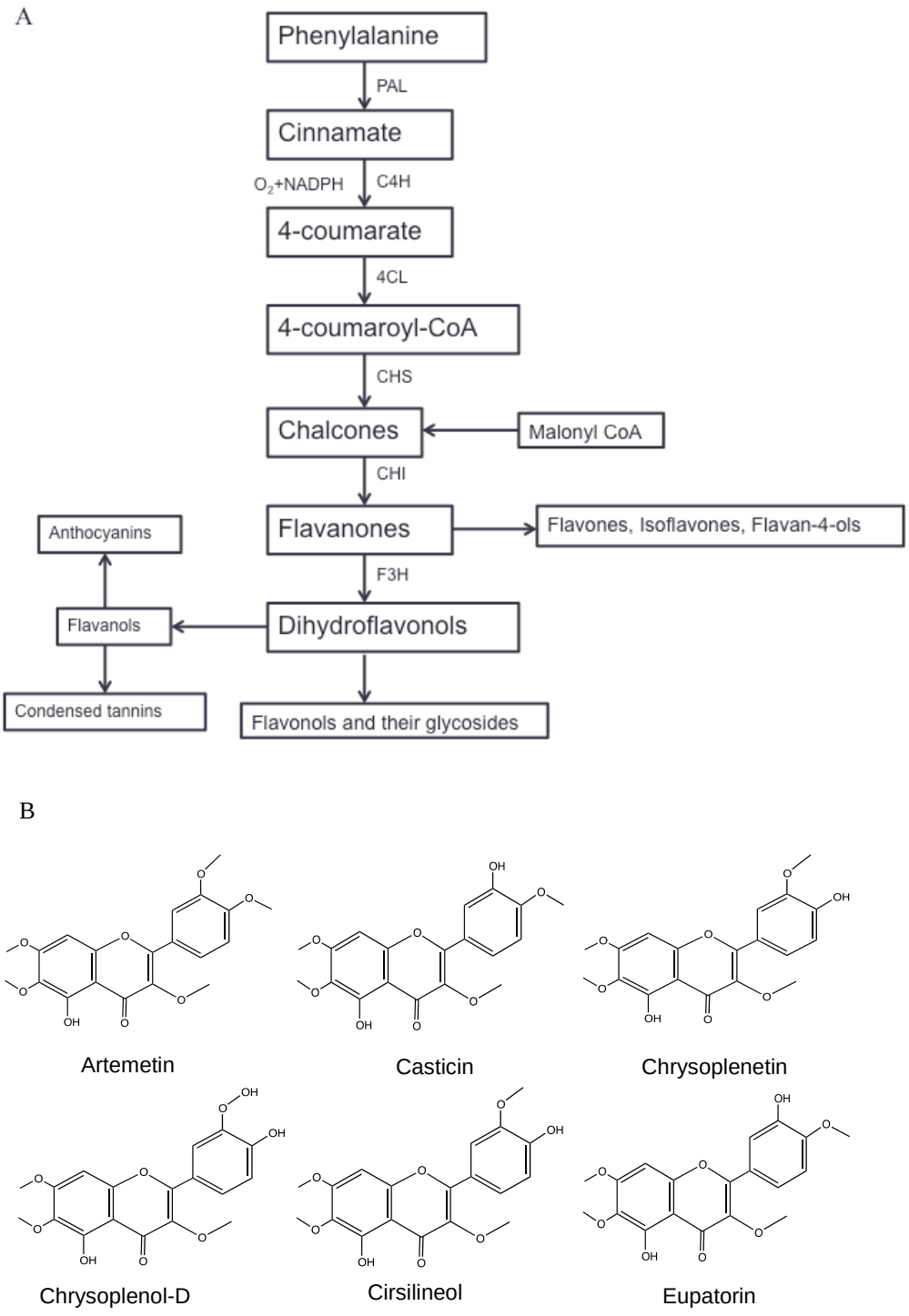


Figure 4 Flavonoids biosynthetic pathway. A, Simplified FLV biosynthetic pathway. PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate coenzyme A ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone hydroxylase; NAPDH, nicotinamide adenine dinucleotide phosphate. B, structure of six known synergistic flavonoids found in *A. annua*.

L-glutamine and myo-inositol lead to an inhibition of parasite-mediated transport systems by in erythrocytes infected with malaria parasites. This apparent synergistic action between AN and flavonoids suggested that flavonoids could be a component of the enhanced anti-malaria activity of in pACT (Weathers et al. 2014).

1.6 Trichome types, functions and development in *A. annua* L.

Trichomes on plants are small epidermal outgrowths of various kinds, which originate from epidermal cells of leaves, stems, and flowers. Trichomes range in size, shape, number of cells and morphology, as well as composition, from long cotton fibers, which are seed trichomes, to tiny bumps on the surface of a leaf. Trichomes have a variety of structural classifications including unicellular or multicellular, branched or unbranched, and glandular or non-glandular (Duke et al. 1994; Ferreira and Janick 1996). Trichomes provide many plants their distinctive look and smell, which aid in plant identification (Schilmiller et al. 2008).

Glandular secretory trichomes have the capacity to synthesize, store and sometimes secrete large amounts of specialized metabolites, including various classes of terpenes, phenylpropanoid, acyl sugars, methylketones, and flavonoids (Gang et al. 2001; Kroumova and Wagner 2003; Li and Steffens 2000; Voirin et al. 1993). Additionally, compounds in trichomes have enormous commercial value including as pharmaceuticals, fragrances, food additives, and natural pesticides (Duke et al. 2000; Wagner et al. 2004), and the prospect of exploiting glandular secretory trichomes as ‘chemical factories’ to produce high-value plant products has recently caught the attention of plant biotechnologists (Schilmiller et al. 2008).

In *A. annua*, two types of trichomes are present, biseriate peltate glandular trichomes (GLTs) and filamentous trichomes (FLTs) composed of stalk cells and an elongated T-shape cell (Fig.2C and D). However, artemisinin is detected only in the presence of GLTs. The biseriate GLTs of *A. annua* are comprised of two stalk cells, two basal cells, and three pairs of secretory cells (Duke and Paul 1993) (Fig.2B). AN and other mono and sesquiterpenoids are excreted into and stored in the subcuticular space of GLTs (Duke 1994; Tellez et al. 1999).

Since GLTs of *A. annua* are the sites of AN synthesis, it should be expected that the presence of more trichomes should result in more AN produced, and indeed, there is a strong correlation

between AN level and trichome density (Kapoor et al. 2007; Arsenault et al. 2010; Nguyen et al. 2011; Nguyen et al. 2013). Younger leaves also develop more trichomes and produce more AN than older leaves (Ferreira and Janick 1996; Nguyen et al. 2013). Lommen et al. (2006) further found that a higher GLT density and a higher capacity to produce AN per trichome in the upper leaves of the plant. Other leaf traits, such as perimeter, area and architecture, have also been targeted for increasing AN production (Graham et al. 2010).

1.7 Roots affect artemisinin production on shoots

AN is produced in shoots and sequestered to glandular trichomes (Duke and Paul 1993; Ferreira and Janick 1995). Despite the absence of AN and its precursor in the roots of *A. annua* (Duke and Paul 1993; Ferreira and Janick 1995, 1996), roots seem to play a pivotal role in regulating the production of this important secondary metabolite. Ferreira and Janick (1996) showed that when *in vitro*-cultured *A. annua* shoots developed roots, shoots produced more AN than shoots that did not develop roots; a result confirmed by Nguyen et al. (2013). Kapoor et al. (2007) provided further evidence that AN concentration in shoots significantly increased after inoculation of *A. annua* with mycorrhizal fungi, likely the result of increased jasmonic acid production and subsequent up regulation of related genes in AN biosynthesis (Mandal et al. 2014). Mannan et al. (2010) also investigated the role of roots in AN production and concluded that only rooted-shoots significantly increased their AN concentration in response to DMSO elicitation, while AN in unrooted-shoot did not display the enhancement of their AN level in response to DMSO. Similar results were concluded by Nguyen et al. (2013), who showed that roots were needed to drive the final ROS reaction from AN precursor DHAA to AN. When roots were present, DHAA was low in shoots and AN remained high. In contrast, when root growth was restricted, DHAA was high but AN was low in shoots (Nguyen et al. 2013). In spite of producing no AN themselves, these studies suggested that roots play a role in AN production, and so my study further investigated the effect of roots on AN production in *A, annua* shoots.

1.8 Signaling molecules that may affect artemisinin biosynthesis

1.8.1 Phytohormones

Phytohormones in plants can modulate both the onset of trichome initiation and secondary metabolism at the transcriptional level (Pauwels et al. 2009; Maes et al. 2011). Plants have developed structurally diverse and tightly regulated secondary metabolites to battle against herbivores and pathogens (Bednarek and Osbourn 2009), and accumulation of these metabolites in plants is often regulated directly or indirectly by phytohormones. It is well established that the phytohormones cytokinins, gibberellic acid, jasmonate (JA), salicylic acid (SA), and abscisic acid (ABA) aid plants in survival from biotic and abiotic stresses through synthesis and accumulation of protective metabolites and proteins (Tuteja 2007; Wolucka et al. 2005; Yuan and Liu 2008)

1.8.1.1 Gibberellic acid

Gibberellic acid (GA_3) is a diterpene found in various plants and fungi and generated from geranylgeranyl pyrophosphate (GGPP), where IPP and FPP are precursors of both GA_3 and AN (Banyai et al. 2011). In *Arabidopsis*, trichome development requires GA signaling and GA response positively correlates with trichome populations (Chien and Sussex 1996; Perazza et al. 1999).

In *A. annua*, sesquiterpenoids are synthesized in the cytosol from IPP/DMAPP originating from both the MVA and MEP pathway (Towler and Weathers 2007; Schramek et al. 2010) (Fig.3). Maes et al. (2011) soil drenched and sprayed with 100 μM GA_3 on low AN-producing and high AN-producing *A. annua* cultivars, and observed significant enhancement of filamentous trichome development, but only in the low AN-producing cultivar. Mansoori (2009) reported that GA_3 increased the activity of HMG-CoA reductase (HMGR), an important control point in the MVA pathway. Banyai et al. (2011) showed *FPS*, *ADS* and *CYP71AV1* transcripts increased 6 h after GA_3 treatment in *A. annua*, revealing the potential of exogenous GA_3 treatment for improvement of AN production as well as leaf biomass.

1.8.1.2 Cytokinins

The cytokinin, 6-benzylaminopurine (BAP), plays a positive role in regulating trichome development in *Arabidopsis* (Maes et al. 2008). In general, plants produce more trichomes per leaf after treatment with BAP, but they are shorter and their nuclear DNA content is less than in untreated plants, suggesting that BAP probably affects endoreduplication of nuclear DNA in trichome cells (Pattanaik et al. 2014). The expression of genes regulating trichome formation is also influenced by cytokinins (Gan and Mucke 2008; Zhou et al. 2013). Maes et al. (2011) showed that BAP increased both glandular and filamentous trichome development in both high and low AN-producing cultivars. Additionally, when Sa et al. (2001) constitutively expressed the *A. tumefaciens* cytokinin biosynthetic gene, *isopentenyl transferase (ipt)* in *A. annua*, the content of cytokinins, chlorophyll and artemisinin all increased *in vivo*. The above evidence strongly suggests that biosynthesis of AN is regulated by cytokinins.

1.8.1.3 Jasmonic acid

Jasmonic acid (JA) plays an important role in the regulation of gene and enzymes involved in biosynthesis, metabolism and signaling related to the wound response and some developmental processes. Additionally, JA also is a well-known secondary regulator that activates a plant's defense system against oxidative stress, inducing production of secondary metabolites (Van Nieuwerburgh and Veldkamp 2006). After spraying on plants, JA significantly increased newly produced leaves and trichome density in *Arabidopsis* (Traw and Bergelson 2003; An et al. 2011). Similarly, Mandal et al. (2014) observed that infections of mycorrhizal fungi increased JA leads, which subsequently significantly enhanced trichome number. JA seems to induce formation of glandular trichomes through the box protein CORONATINE INSENSITIVE 1 (COI 1), which is the central component of JA perception (Li et al. 2004; Sheard et al. 2010). In *Arabidopsis*, many transcription factors (TFs), such as MYB75, GL3 and EGL3 target JASMONATE ZIM-DOMAIN (JAZ) proteins that play a central role in signaling cascades triggered by jasmonates, and are also involved in anthocyanin biosynthesis and trichome initiation (Qi et al. 2011).

In *A. annua*, GLTs are the site of AN biosynthesis and sequestration, hence, the density and size of GLTs usually correlate with the AN level. For example, Liu et al. (2009) observed that exogenous JA positively up regulate trichome density and AN levels compared to their controls. Moreover, in soil-grown plants AN level significantly increased compared to controls 6 days after treatment with 300 μM methyl jasmonate (Wang et al. 2010a). JA also promoted expression of key genes of the AN biosynthetic pathway (Yu et al. 2011). Taken together the evidence indicates that JA is a likely signal element that regulates both glandular trichome formation and AN accumulation in GLTs (Maes et al. 2011).

1.8.1.4 Salicylic acid

Salicylic acid (SA) is one of the plant signals involved in defense (Durrant and Dong 2004). When exogenous SA was sprayed onto soil-grown *A. annua* plants, AN, AA, and DHAA all increased and the transcription levels of *ADS* and *HMGR* also showed significant enhancement; however, other related genes showed no response to the SA elicitation. After SA treatment, the ROS molecules, H_2O_2 and O_2^-, in these plants significantly increased 4 h after spraying, coinciding with increased in AN (Pu et al. 2009). ROS is a modulator of cytokinins activation and is involved in the regulation of the plant defense system and also affects the secondary metabolism. Hence, SA and JA may both act as signal elements activating the ROS and defense system in plants and subsquently secondary metabolites, such as AN. Evidence suggestes that SA is a key signaling molecule with functions similar to JA in the induction of the defense system in *Arabidopsis* (An et al. 2011). In contrast to JA, however, SA seems to inhibit trichome production and thus may reduce the positive effects of JA on GLTs (Traw and Bergelson 2003).

1.8.1.5 Abscisic acid

Abscisic acid (ABA) is an important plant hormone involved with a role in mediating whole plant responses to drought and salt stresses (Zhang et al. 2006b). ABA is generally produced in roots and then transports up to the leaves as a chemical signal to report on the

water status of the soil. Hence, ABA plays a dominant role in root to shoot signaling under drought and in the control of stomatal conductance (Schachtman and Goodger 2008). ABA may also mediate some secondary metabolism. For example, when *Hyoscyamus muticus* was elicited by exogenous application of ABA to hairy roots, hyoscyamine accumulation level decreased (Vanhala et al. 1998). More important, exogenous application of 1-100 μM ABA to soil-grown *A. annua* plants significantly increased in AN content compared to controls. At the same time, *HMGR*, *FPS*, and *CYP* transcripts also increased in ABA-treated *A. annua* plants (Jing et al. 2009).

1.8.2 Transcription factors

Although AN regulation of AN biosynthesis is not as well known as the pathway biosynthesis, a number of Transcription factors (TFs) have recently been identified in *A. annua.* Ma et al. (2009) isolated and charactered a TF (AaWRKY1) in *A. annua*. AaWRKY1 binds to the W-box cis-acting elements of the *ADS* promoter and regulates *ADS* gene expression and thus may affect AN biosynthesis (Ma et al. 2009). Han et al. (2014) found overexpressed AaWRKY1 in *A. annua* increased transcription of *CYP71AV1*. Trichome-specific overexpression of AaWRKY1 in GLTs was much more effective than that of constitutive overexpressing AaWRKY1 in whole plants. However, the transcription levels of *FDS*, *ADS*, and *DBR2* did not change with AaWRKY1 overexpression. On the other hand, AN level increased significantly with *CYP71AV1* up-regulation. Together those studies suggested that AaWRKY1 might be a signal factor involving in activating and promoting the transcription of *CYP71AV1*, thereby regulating AN biosynthesis.

In another report, Yu et al. (2011) showed that overexpression of the two AP2/ERF-type TFs, AaERF1 and AaERF2, belonging to the JA-responsive AP2 family of transcription factors, increased transcript levels of both *ADS* and *CYP71AV1*, resulting in increased accumulation of AN and AA in transgenic *A. annua* plants. Thus, the AP2/ERF TFs also may be signal elements in *A. annua*, possibly playing a role in up regulating AN biosynthesis. AaORA, another trichome-specific TF expressed in GLTs of *A. annua* (Lu et al. 2013). When the AaORA TF

overexpressed, *ADS*, *CYP71AV1* and *DBR2* genes in different tissues of *A. annua* showed similarly enhanced expression. RNA interference of AaORA decreased transcripts of *ADS*, *CYP71AV1*, *DBR2*, and *AaERF1* genes and also decreased the level of AN and DHAA (Lu et al. 2013). These results demonstrated that the AaORA TF is likely a positive regulator of AN biosynthesis.

Further, when the TF, AaPYL9, an ABA receptor ortholog involved in ABA regulation of AN biosynthesis was overexpressed, AN level increased (Cutler et al. 2010). AN was also increased in *A. annua*, when AabZIP1, a basic leucine zipper family transcription factor from *A. annua*, was overexpressed (Zhang et al. 2015), and transcripts of *ADS* and *CYP71AV1* in *A. annua* were also increased, suggesting that AabZIP TF also might be signal element regulating AN biosynthetic pathway.

1.8.3 Small RNAs

MicroRNAs (miRNAs), generated from folded stem-loop structures called precursor miRNAs (pre-miRNAs), are a class of small, non-protein-coding RNA sequences with an average of 21 nucleotides and some post-transcriptional regulatory functions (Breaker 2004). Although miRNAs have been identified and validated in many plant species, our knowledge of the transcriptional mechanisms of microRNA genes is limited.

MicroRNAs transcribed as parts of longer primary transcripts (pri-miRNAs), miRNAs are generated mainly by RNA polymerase II and III (Ha and Kim 2014) and have intronic and intergenic miRNA gene loci (Saini et al. 2007; Ying et al. 2010). Intronic miRNA is located within a host gene where it mainly resides in introns, so using the same promoter, intronic miRNA is transcribed in the same direction as the primary transcript. Conversely, intergenic miRNAs are transcribed as part of non-coding RNAs and are often clustered in the genome. Together this suggests that one pri-miRNA can be subsequently processed into several functional miRNAs (Saini et al. 2007; Ying et al. 2010). In plants, miRNA genes are initially transcribed into primary miRNAs by RNA polymerase II (pol II) (Zhou et al. 2007). In general, pri-miRNAs are processed into pre-miRNAs by DICER-

LIKE1, and are able to fold into a perfect or near-perfect secondary hairpin structure (Zhou et al. 2007; Voinnet 2009).

A mature miRNA has one single strand RNA that is later assembled into the RNA induced silencing complex (RISC) and directs the RISC complex to its complementary target sites in the mRNA. That miRNA then negatively regulates mRNA expression either by inhibiting the translation process or by causing its destruction (Bai et al. 2012). The activity of miRNA translation process or by causing its destruction (Bai et al. 2012). The activity of miRNA targeting mRNA is dependent on the degree of base pairing; different degrees of base pairing mediate target recognition (Tang et al. 2003). In animals, miRNAs are involved in regulating the expression of distinct genes by base-pairing with partially complementary sequences in mRNA and simply inhibiting their translation into protein (Novina and Sharp 2004). In contrast to animals, plant miRNAs generally interact with their targets through perfect or near-perfect complementarity and repress translation (Mallory et al. 2005; Nikovics et al. 2006; Meng et al. 2009). This property makes it easier to computationally predict miRNAs in plants. These predictions have been successfully applied to many model plant species. (Sunkar and Zhu 2004; Zhang et al. 2006a; Lu and Yang 2010), including *Arabidopsis* (Breakfield et al. 2012), rice (Campo et al. 2013), maize (Wang et al. 2011a), grape (Wang et al. 2012), soybean (Zhang et al. 2008a) and orange (Xu et al. 2010). The miRNAs are the second most abundant sRNAs in plants and act as powerful endogenous regulators, playing an important role in a wide range of developmental, biological, and metabolic processes in plants including metabolism, stress response, vegetative phase change, organogenesis, and signal transduction (Kasschau et al. 2003; Juarez et al. 2004; Mallory et al. 2005; Robert-Seilaniantz et al. 2010). To date, three common methods have been used to effectively identify miRNAs in various species: direct cloning after isolation of small RNAs via a computational strategy, express sequence tags (ESTs) analysis, and high throughput sequencing of small RNAs (Pantaleo et al. 2010; Song et al. 2010).

In *A.annua,* Pani et al. (2011) predicted six conserved miRNAs and eight potential target genes, these function in a variety of biological processes, including AN biosynthesis, signal

transduction and development. Most of the targets are unique to the *A. annua* genome, and encode the enzymes associated with AN biosynthesis pathway (Pani et al. 2011).

The miRNAs seems to remain conserved among various organisms, so this makes them valuable tools for the prediction of novel miRNAs by computational comparative genomics and has led to interesting new findings in plants (Barozai 2012, 2013). Currently, 16 novel miRNAs in *A. annua* have been identified from the available ESTs by using the computational genomic. These 16 new miRNAs in *A. annua* belong to 13 families (miR 156, 159, 160, 162, 166, 171, 172, 390, 395, 397, 535, 1310 and 4221) and their targets consist of transcription factors like; APETALA2, WRKY3, DELLA, MYB and other hypothetical proteins (Barozai 2013). With the aim of identifying possible miRNAs that may mediate regulation of AN biosynthetic pathway, Pérez-Quintero et al. (2012) identified *A. annua* miRNAs based on the available UniGene set. In their work, miR390 was found in libraries coming from young and mature leaf trichomes as well as meristems. Importantly, miR390 was also involved in trichome patterning and development, with mutants showing precocious production of trichomes. This makes miRNA390 an interesting candidate for *A. annua* transformation aiming to produce more AN in trichomes. Additionally, the miR166 and miR397 families were found in cotyledon tissues, the family miR167 in young leaf trichomes and the miR172 family in mature leaves of *A.annua* (Pérez-Quintero et al. 2012).

1.8.4 Other possible signal elements

1.8.4.1 Flavonoids

Buer et al. (2010) showed that flavonoids could move long distances in plants. Localized application of selective flavonoids, such as dihydrokaempferol and dihydroquercetin resulted in uptake by a variety of tissue locations: the root tip, mid-root, and cotyledons. This suggested that flavonoids could move long distances via cell-to-cell movement to distal tissues where they were then hydrolyzed to kaempferol and quercetin. In contrast, kaempferol and quercetin, which were located only at the root tip, did not move from their application position. The studies suggested that flavonoids also might be a signal element that moves from root to shoot.

1.8.4.2 Sugars

Sugars play an essential role in plant metabolism including intermediary and respiratory metabolism. Moreover, sugars are an important source for amino acid and fatty acid biosynthesis and essentially all other compounds present in plants. Thus, to our knowledge, sugars function as signal mainly to mediate plant growth, development, metabolism and alterations in gene expression and with controls similar power to hormones (Smeekens 2000). For example sugar produced in chloroplasts appeared to be a signal that was transmitted between the leaves and mediated the circadian clock in *Arabidopsis* roots (James et al. 2008). In *A. annua*, the type of carbon source influenced AN accumulation in hairy roots and seedlings; besides, *A. annua* seedlings showed that a twofold increase in AN was stimulated by glucose compared to sucrose, while fructose reduced AN levels. Taken together that study suggesting sugars could affect terpenoid production (Wang and Weathers 2007).

1.8.4.3 Protein

Additionally, protein may also be a signal candidate. For example, FLOWERING LOCUS T (FT) protein was shown to act as the long-distance florigenic signal in *Cucurbits*, *Arabidopsis* and other plant systems (Jaeger and Wigge 2007; Lin et al. 2007). *FT* transcription in leaf vascular tissue (phloem) and FT proteins moved through phloem to the apex as a long-distance signal that induced plant flowering (Corbesier et al. 2007). Tamaki et al. (2007) also showed Hd3a protein moved from the leaves to the shoot apical meristem and induces flowering in rice, suggesting that the Hd3a protein might be a signal factor involving in regulation of flowering in rice.

Table 1. Possible mobile elements in *A. annua.*

Category	Signal molecule	Long distance Movement	Function in *A. annua*	Reference
Phytohormone	GA	As yet unknown	Leaf treatment increased AN and related gene transcripts in AN biosynthetic pathway.	Banyai et al. 2011; Mansorri 2009
	BAP	As yet unknown	Increased trichome number and AN level in *A. annua.*	Sa et al. 2001; Maes et al. 2008; Maes et al. 2011
	SA	Leaf to leaf	As signal for systemic acquired resistance (SAR) and leafs sprays increased AN in *A. annua.*	Pu et al. 2009
	JA	Leaf to leaf	Leaf treatment JA increased AN and gene transcripts in AN biosynthetic pathway.	Wang et al. 2010; Yu et al. 2011; Maes et al 2011
	ABA	From root to shoot	Leaf treatment ABA increased AN and gene transcripts in AN biosynthetic pathway.	Jing et al. 2009
Transcription factor	AaWRKY1; AaERF1; AaERF2; AaORA *et al.*	Move between phloem cells	Bind to related gene promoters and increased AN in *A. annua.*	Han et al. 2014; Yu et al 2011; Lu et al. 2013.
Small RNA	miR167; miR390; miR397 *et al.*	Both root-to-shoot and shoot-to-root movement via phloem	Most of them involved in targeting trichome development genes and increased AN level.	Pani et al. 2011; Pérez-Quintero et al. 2012
Sugar	Glucose	Both shoot to root and root to shoot move	Glucose stimulated AN level compared to sucrose, while fructose reduced AN level.	Wang and Weathers 2007;
Flavonoid	Dihydrokaempferol; dihydroquercetin	From root to distal tissue	As yet unknown	Buer et al. 2010
Protein	FLOWERING LOCUS T (FT)	From leaf to shoot apex	As yet unknown	Corbesier et al. 2007

1.9 Grafting as tool to study root-shoot interactions

Plant grafting was first begun during the ‘Warring States’ period in China, about 400 BC. With the development of civilization, grafting techniques continuously improved (Wang 2011b). Nowadays, grafting has been commonly practiced for asexual plant propagation including vegetables, flowers and fruit trees. The three most commonly used methods include: tube grafting, pin grafting and cleft grafting (Lee et al. 2010) (Fig.5A-C). Grafts connect two plant segments, the shoot part known as the ‘scion’ and the root part called the ‘rootstock’ (Melnyk and Meyerowitz 2015). Grafted plants often have better stress resistance to cold, drought, salt and pests. The specific mechanism and reason for getting a better resistance or tolerance has not been intensely investigated (Rivero et al. 2003). Tolerance to environmental stress, such as to disease in grafted plants, may result from the resistance of the rootstocks. It is known that the root system synthesizes substances resistant to pathogen attack, and these are transported to the shoot through the pathway such as phloem or xylem (Biles et al. 1989). Additionally these useful traits often enhance yields and disease tolerance in commercial agriculture (Lee et al. 2010).

Grafting also has generally become a useful tool for studying long-distance signal transduction in plants (Omid et al. 2007; Kehr and Buhtz 2012; Spiegelman et al. 2013). For example, Corbesier et al. (2007) demonstrated via grafting that FT protein was a major, graft-transmissible component of the long-distance florigen. Using different combinations of mutants, transgenic plants, and wild-type plants as scion or rootstock, it is possible to analyze reciprocal long-distance signaling mechanisms. In recent years, long-distance transport of RNA and protein through the phloem were systematically studied. For example, Molnar et al. (2010) demonstrated the several sRNAs as transmissible signals, moving from root to shoot, directing epigenetic modifications in the genome of the recipient cells. Poethig (2009) suggested that juvenile-to-adult transitions were mediated by a marked decline in the expression of miR156 in annuals. Tzarfati et al., (2013) also observed miR156 (and the related miR157, which are same miRNAs were also recently found in *A. annua*, expression rates significantly decrease compared with ungrafted control seedlings in both heterograft and

homograft citrus scions. These studies suggested that miRNAs play an important role in grafting effects (Tzarfati et al. 2013).

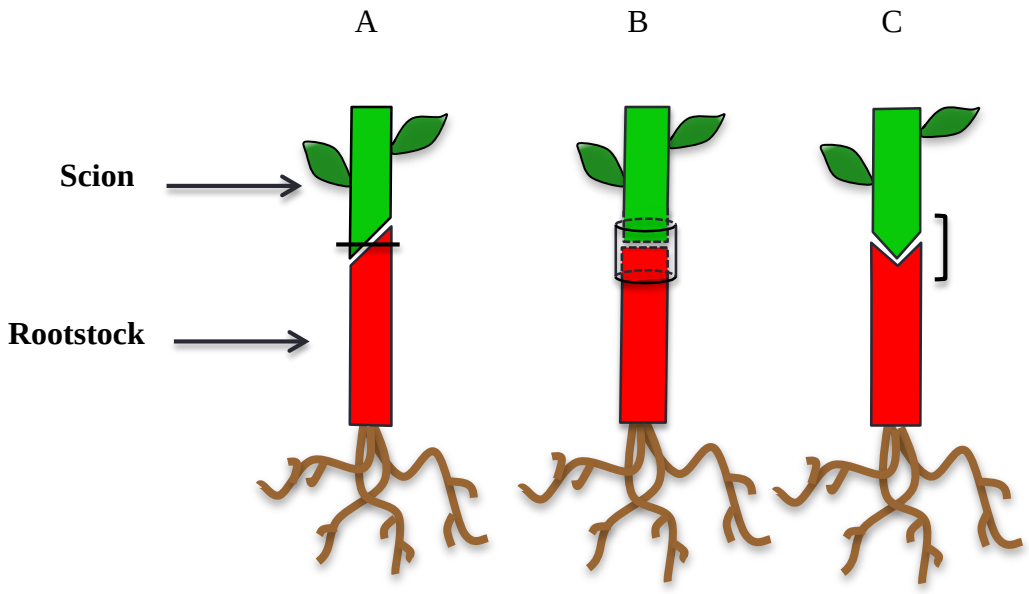


Figure 5 Schematic of main grafting methods. A, Pin grafting, the straight line represents the pin, the green region represents the scion, the red region represents the rootstock. B, tube grafting, the black line cylinder represents grafting tube. C, cleft grafting, the bracketed region represents grafting site.

2. Hypothesis and Objectives

2.1 Hypothesis

Early evidence is building to suggest that there is some signal factor that is moving from root to shoot to alter AN level. These putative mobile signals may regulate AN biosynthesis and the development of glandular trichomes where sesquiterpene biosynthesis occurs and AN is stored in *A. annua*.

2.2 Objectives

The overall goal was to measure and define phenotypic effects of the root on shoot production of AN. There were several specific objectives:

1.) Observe the phenotypic morphologies of all self and cross grafts from the three different *A. annua* cultivars including a high AN producing cultivar (SAM), a lower AN-producing cultivar (#15) and a null mutant cultivar that produces no AN or GLTs.

2.) Measure the AN level in the scions of ungrafted plants, self and cross grafts of the three different *A. annua* cultivars.

3.) Measure the development of trichomes in scions of ungrafted plants, self and cross grafts of the three different *A. annua* cultivars.

4.) Measure total flavonoid levels in scions of ungrafted plants, self and cross grafts of the three different *A. annua* cultivars.

3. Materials and methods

3.1 Plant material and cultivation conditions

The three clonal cultivars of *Artemisia annua* L. used in the study are described in Table 2. The clones were: SAM with about 1.49% (w/w) AN, clone #15 with about 0.57% (w/w)AN, and the Glandless (GLS) mutant that contained no glandular trichomes, nor AN. GLS was a gift from Stephen Duke (University of Mississippi, Oxford) (Duke et al. 1994). Plants were clonally propagated by cuttings dipped into rooting Hormodin 2 (Horticultural Tool and Supply Company) to induce rooting to insure that the outcrossing characteristics of *A. annua* L. did not result in genetic loss of secondary metabolite content. All rootstocks and grafted plants were grown in Metro Mix 360 soil (Sun Gro Inc.) at 25°C under a vegetative photoperiod of 16 h light, 8 h dark at approximately 80-120 (μmol m^{-2} sec^{-1}) in a Percival growth chamber. RH in the chamber was about 78%. All scions were clonally grown in glass-filtered natural light under a vegetative photoperiod in the lab.

Table 2. Three clonal cultivars of *A. annua* L. used in this study.

Clone	AN (% DW)	Tot. FLV (% DW)	GLTs (+/-)	Voucher
SAM	1.49[a]	6.4	+	MASS 00317314
#15	0.57[a]	6.1	+	MASS 00317313
GLS	0.0[b]	0.021[b]	0[b]	OR State University 171772 and 170353

[a] Weathers and Towler (2012).

[b] Tellez et al. (1999); Duke et al. (1994)

3.2 Grafting methods

When the plants used for rootstocks reached 4-5 cm and stem diameter was about 1mm, they were ready for grafting. Grafts used in this study are shown in Table 3. All leaves on the rootstock were removed before grafting to reduce interference of rootstock leaves on scion responses. Rootstock stems were cut horizontally at about 3.5-4 cm above the soil. Suitable scions were young, without much lignification and with a stem diameter equal to that of the

rootstock. Each scion length was about 1.5 cm and all fully expanded leaves and their axillary buds were removed except the ShAM as defined in Fig.6A. Leaf removal was to reduce water loss and enhance graft success and more importantly, to exclude the possibility that a signal might originate from rootstock leaves. In this study, there was no leaf regrowth once leaves and buds were removed from the rootstock (Fig. 6B). Rootstock and scion were quickly joined to give a tight connection inside a 0.5 cm (length), 1mm (i.d.) piece of Tubing (Tygon R-3603, Saint-Gobain, France) (Fig. 6A and B). The grafted junction was wetted with distilled water and the grafted cultivars were each set into a Magenta box GA-7 on which another magenta box GA-7 was inverted to maintain relative humidity (RH) until the graft was successful. About 6-7 days after grafting, the lid was partly opened to allow the grafted plant to adapt to the chamber environment for 2-3 days after which the lid of Magenta box was removed along with the graft tubing (Fig.6B).

Table 3. Grafts used in this study (scion/rootstock) with experimental rationale.

Crossed grafted group		**Self-grafted group (CONTROL 1)**		**Ungrafted group (CONTROL 2)**	
Graft	**Rationale**	**Graft**	**Rationale**	**Graft**	**Rationale**
#15/SAM	Stimulation test	#15/#15	Grafting stress test	#15	Baseline
SAM/#15	Inhibition test	SAM/SAM		SAM	
GLS/SAM	Stimulation test	GLS/GLS		GLS	
SAM/GLS	Inhibition test				

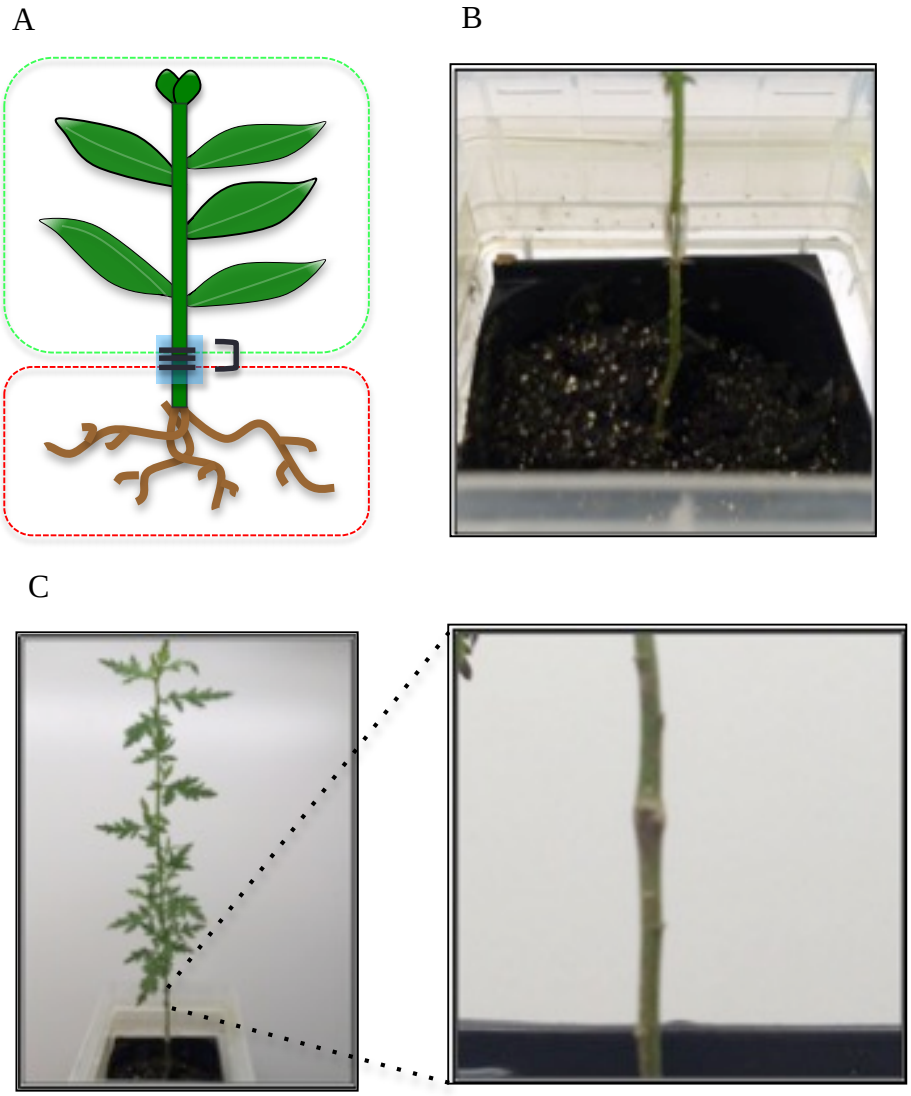


Figure 6 Grafting and experimental schematic. A, experimental schematic of a grafted plant, green dashed region is scion; the red dashed region is rootstock; the light blue square with black straight line and bracket stand for a plastic tubing at the grafting point, which secures the connection between the scion and rootstock. B, photos for tube grafting at the grafted point. C, grafted plant is on left, and the right is a magnified view of the graft junction.

3.3 Harvest, extraction, and analysis of metabolites

Grafted plants were grown in the growth chamber as previously described. They were watered daily as needed and fertilized once a week with Miracle-Gro (The Scotts Miracle-Gro, Inc., Marysville, CA). During growth, grafted plants that branched or had abnormal growth morphology, e.g., yellowing or wilted leaves were discarded and not used for further analysis.

Only grafts with a single stem and green leaves were used. Scion shoots were harvested only after they had grown to≥14 nodes at almost 30 cm height (Fig.7A). The ShAM and leaves 5, 7, 9, 12, and 14 were harvested for metabolite analysis. At the same time leaves 10, 11, and 13 were harvested for glandular trichome analysis (Fig. 7A).

3.4 Artemisinic metabolite analysis

Harvested tissues were weighed and then put into glass test tubes with 4 ml methylene chloride. Test tubes were sealed with Dura Seal stretch film (Diversified Biotech, Cat.No.DS2-500) to eliminate evaporation and then sonicated in a water bath (FS60, Fisher Scientific) for 30 minutes. Afterwards, tissues were removed with tweezers and extracts dried under nitrogen (N-EVAP 111, Organomation Associates, Inc., Berlin, MA). Dried extracts were stored at -10℃. For analysis, they were re-suspended in methylene chloride and a 10% aliquot was used for analysis of five artemisinic metabolites. Artemisinin (AN), arteannuin B (AB), deoxyartemisinin (deoxyAN), artemisinic acid (AA), were measured in the extracts using the following GC-MS method: GC, Agilent Technologies 7890B; MSD, Agilent Technologies 5977D; column, Agilent HP-5MS (30m×0.25mm×0.25 μm); carrier gas, He at 1 mL/min; injection volume, 1μL in splitless mode; ion source temperature, 280℃; inlet, 250℃; transfer line, 150℃. For AA and DHAA; oven temperature was 125℃ held for 1 min, and then increased to 300℃ at 5℃/min. Samples for AN, deoxyAN and AB analysis were re-suspended in 100 μL pentane prior to analysis. AA and DHAA were derivatized prior to injection by re-suspending dried sample aliquots in 20 μL of 1:1 pyridine: bis (trimethylsilyl) trifluoroacetamide (Sigma 270407 and Restek 35605, respectively) plus 50μL pentane. Metabolites were identified via their retention time and ion signatures compared to external standards, and some mass spectra of Zhang et al. (2010). AN was from Sigma-Aldrich Chemical; deoxyAN was from Toronto Research, Inc.; AB and AA, were a gift, from Dr. Nancy Acton of the Walter Reed Army Research Institute, Silver Spring, MD. DHAA, previously identified, is labile under long term storage making true standards unreliable so it was quantified based on an AA standard and expressed as AA equivalents.

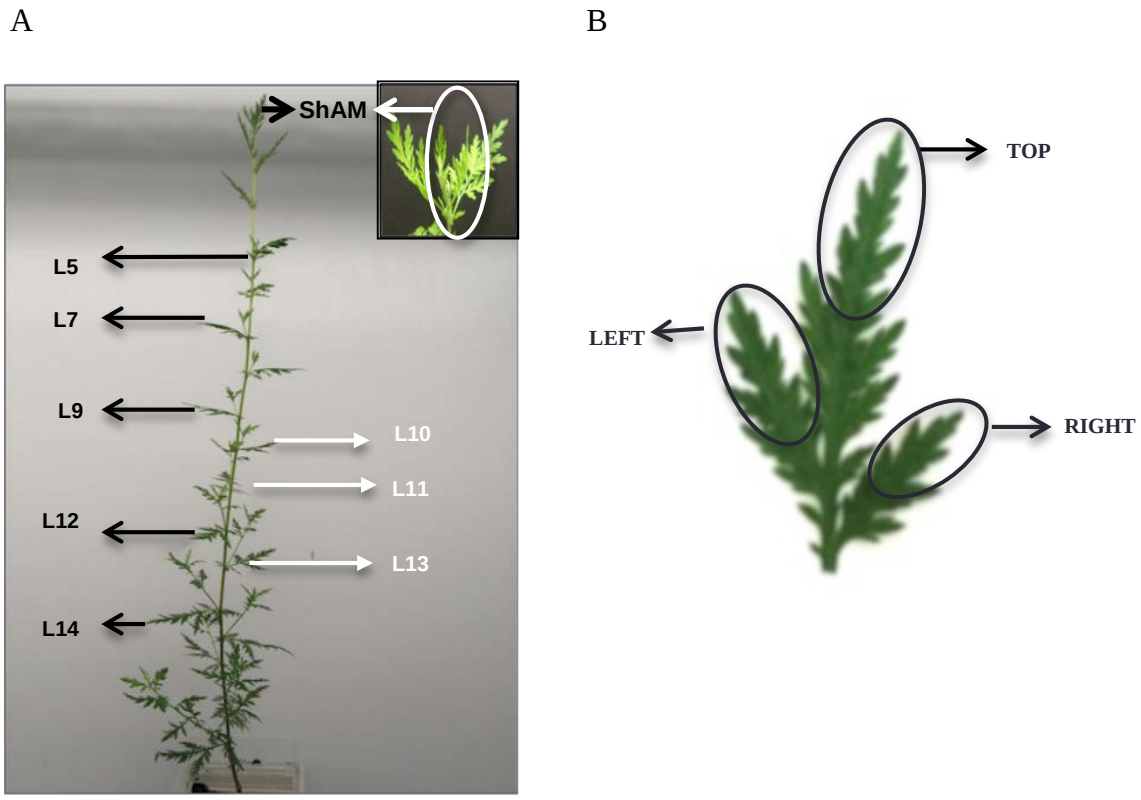


Figure 7 Grafted *Artemisia annua* plant with ShAM and leaf numbers as need in this experiment. A, ShAM and leaf numbers L5, 7, 9, 12 and 14 were used for secondary metabolite analysis; leaf numbers L10, 11 and 13 were used for GLT counts. The right side was the defined 'ShAM' region harvested for analysis as indicated by the white circle. B, three positions on leaf used for glandular trichome counts.

3.5 Total flavonoid analysis

Total flavonoid concentration was measured using the aluminum chloride ($AlCl_3$) assay described by Weathers and Towler (2014). Quercetin (5 to 25 μg total in 1ml 1% $AlCl_3$ MeOH solution) was used to generate a standard curve. The blank was 1 mL 1% $AlCl_3$ MeOH solution in a glass test tube, capped, and incubated for 25 minutes at room temperature. After incubation, OD at 415 nm was measured using a U-2800 UV-Vis spectrophotometer (Hitachi, Inc.). Quercetin concentration (μg/mL) was plotted against OD to generate a standard curve and a linear equation. Total flavonoid concentrations were expressed as quercetin equivalents.

3.6 Glandular trichome analysis

Leaves 10, 11, and 13 were used for comparing the number of glandular trichomes (GLTs) among all the grafted and ungrafted plants. Each leaf was placed on a glass slide with some drops of water and a cover slip to secure the sample and prevent evaporation. GLTs were observed, photographed and then counted in each of these three leaf regions: top, left and right as shown in Fig. 7B by using a Zeiss SteREO Discovery V12 fluorescence microscope under constant magnification (AxioCam MRc, GFP 470 filter, 3.5X objective，Mag Field 25X, 8mm). Three pictures of each region were randomly taken and all pictures exported to ImageJ (/http://rsbweb.nih.gov/ij/) for GLT counting. The averages of the three regions of each leaf were counted and the GLTs mm^2 calculated.

Total glandular trichomes per leaf were calculated based on total leaf area calculated by measuring the area of ≥10 leaves (for leaf 10) of each graft type using Image J. Calculations between leaf biomass and total leaf area for each graft are shown in Appendix Figure 16.

3.7 Statistical methods

There were at least 6 replicates of each grafted plant. Leaf biomass data were analyzed by one-way ANOVA. Metabolites and GLT data were averaged and analyzed using Student's *t* test.

4. Results

Previous studies suggested that the roots of *A. annua* played a key, but as yet unknown, role in the production of AN in the shoots (Ferreira and Janick 1996). Additionally, Kapoor et al. (2007), Mannan et al. (2010) and Nguyen et al. (2012) also provided evidence that roots were involved in the process of regulating the production of artemisinin in the shoots of *A. annua*. In this study, grafting was used to further study how the roots regulated the production of artemisinin and flavonoid biosynthesis and the response of glandular trichomes of shoots.

4.1 Vegetative phenotypes among different grafted *A. annua* cultivars.

Typical phenotypes of all the scions in the study are shown in Fig.8. Leaves were usually smaller in grafted scions. Glandless (GLS) scions in particular showed an unusual morphology in the shoot apical meristem (ShAM), where the youngest leaves clustered almost like a floral bud (Fig.9C). With leaf maturity, fresh mass declined in nS, but not in n15 or nG (Fig.10). Compared to ungrafted cultivars all self-grafts yielded significantly less fresh mass (Fig.10). Leaf mass increased slightly with maturity in 15 and G scions when S was the rootstock, but not in scions of S and G when 15 was the rootstock. When G was the rootstock, leaf mass was substantially less than all other un-grafted and grafted plants. Compared to their self-grafts, the biomass of G scions significantly increased when S was the rootstock (Fig.10). Conversely, the biomass of S was markedly reduced when G was the rootstock (Fig.10). Table 4 provides statistical comparisons among scion of the different grafted and ungrafted plants.

4.2 Glandular trichome development among grafted *A. annua* cultivars.

GLTs measured on L10, 11 and 13 all showed the same relative population patterns (Appendix), so only data from L10 are shown in detail (Fig.11). Likewise, the top, left and right positions yielded the same relative GLT populations for all mature leaves (Fig.11).

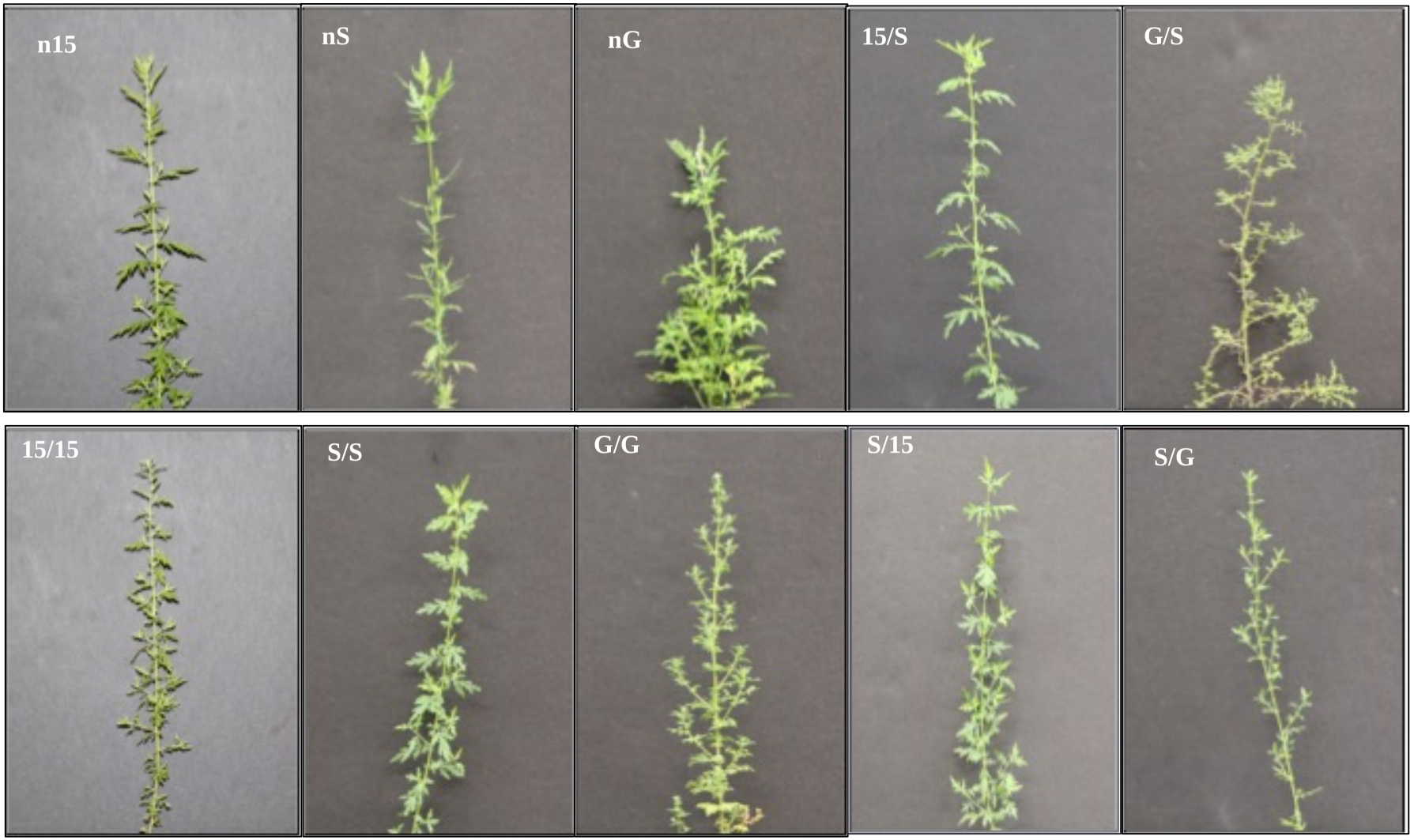


Figure 8 Representative examples of vegetative phenotypes among different grafted and ungrafted *A.annua* cultivars. S, Sam; 15, #15; G, Glandless cultivar; scion/rootstock.

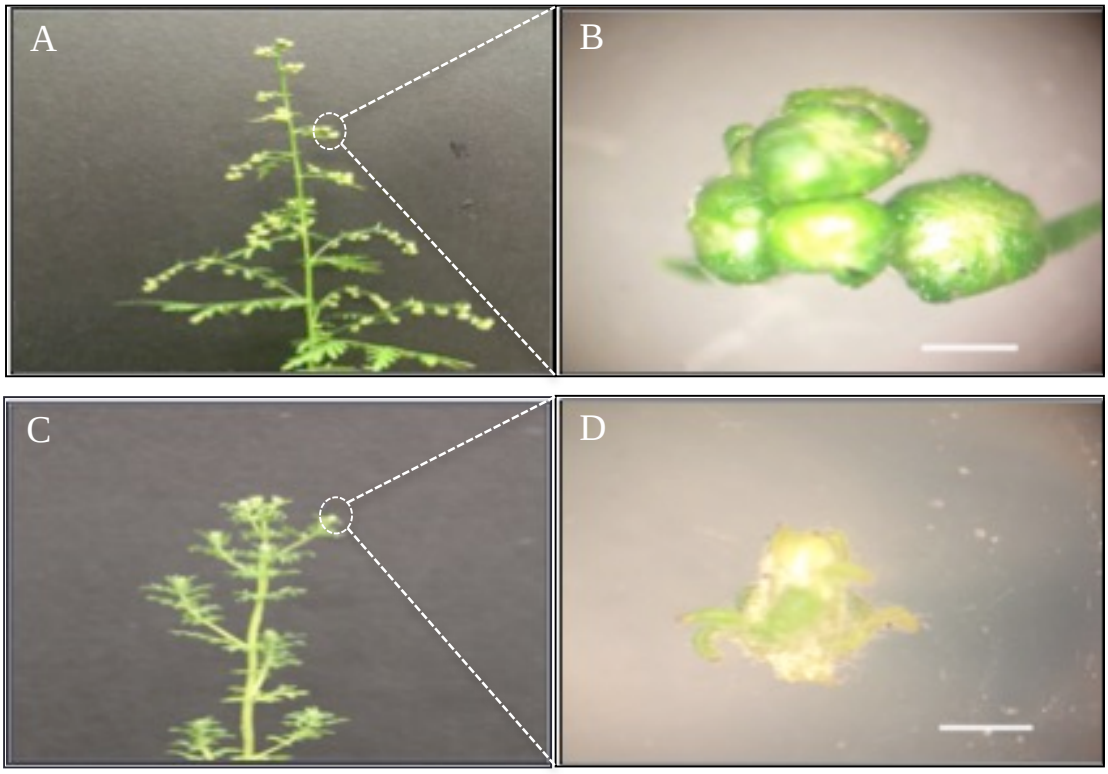


Figure 9 Comparative phenotypes of shoot and floral buds. A, vegetative morphology for flowering. B, magnification of normal floral buds of *A. annua*, *Bar* = 2mm. C. vegetative phenotype of G/G. D, unusual budding of leaves of G/G, *Bar* = 2mm.

No GLTs were found on ungrafted G (nG) or any G grafted scions. Although nS had the lowest overall GLT populations, when S was the rootstock, GLTs significantly increased on 15 and S scions compared to their ungrafted controls. In contrast, when 15 was the rootstock, the S scion produced about 75% fewer GLTs than the 15 scion with an S rootstock. GLT populations on leaves of self-grafted plants (S/S and 15/15) increased twofold respectively compared to their corresponding ungrafted controls (nS, n15) (Fig.11). Although there were no GLTs, GLT responded similarly for its filamentous trichomes (Fig. 12C). The 15 scion of the 15/S graft had the maximum GLTs, but they were not as filled out as those from any of the S scion and ungrafted plants (Fig.12A and B). GLTs on scions of S/S had a stronger florescence with more uniform distribution than GLTs on others grafts. Fluorescence was mainly concentrated in the center of GLTs on 15 scions, especially 15/S. In contrast, however, fluorescence of S/15 was mainly on both ends of the GLTs (Fig. 12B)

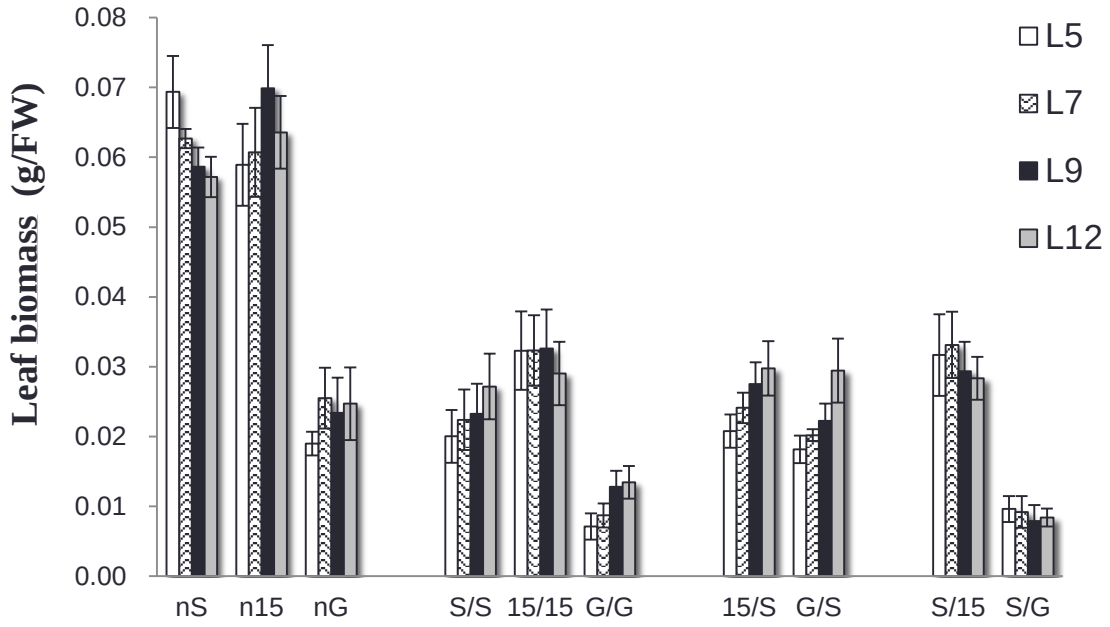


Figure 10 Comparison of leaves of biomass individual among different grafted and ungrafted cultivars. S, Sam; 15, #15; G, Glandless cultivar; nS, n15 and nG are ungrafted; scion/rootstock of respective cultivars. *Error bars* are ±SE and n = 4-9. Almost all leaf biomass among L5, L7, L9 and L12 in grafted groups significantly decreased ($p \leq 0.05$) when compared with corresponding scion of ungrafted cultivar, for example nS vs S/S or S/15. However, leaves of G/S did not decrease among L5, L7, L9 and L12 after grafting when compared with nG.

4.3 Artemisinic metabolite levels among different grafted and ungrafted *A. annua* cultivars

When artemisinic metabolites were measured, AN yield was highest in the ShAM and all leaves of the SAM self-graft S/S among all grafted and ungrafted plants. S rootstocks also significantly increased AN in the 15 scions compared to all plants with a 15 rootstock (Fig.13A). Interestingly, when grown in the Percival growth chamber, ungrafted S (nS) contained almost the same amount of AN as ungrafted 15 (n15). For both S and 15, self-grafts produced significantly more AN than their respective ungrafted controls (Fig.13A).

The precursor to AN biosynthesis is DHAA and in the ShAM, trends were similar to AN for self-grafted compared to their ungrafted controls (Fig.13B). In contrast to AN, however, there was no significant difference between the two cross-grafts 15/S and S/15 (Fig.13B). As leaves matured, DHAA decreased substantially with fewer comparisons to be made (Fig.13B). There was almost no detectable AN or DHAA in the G scion, even when S was the rootstock.

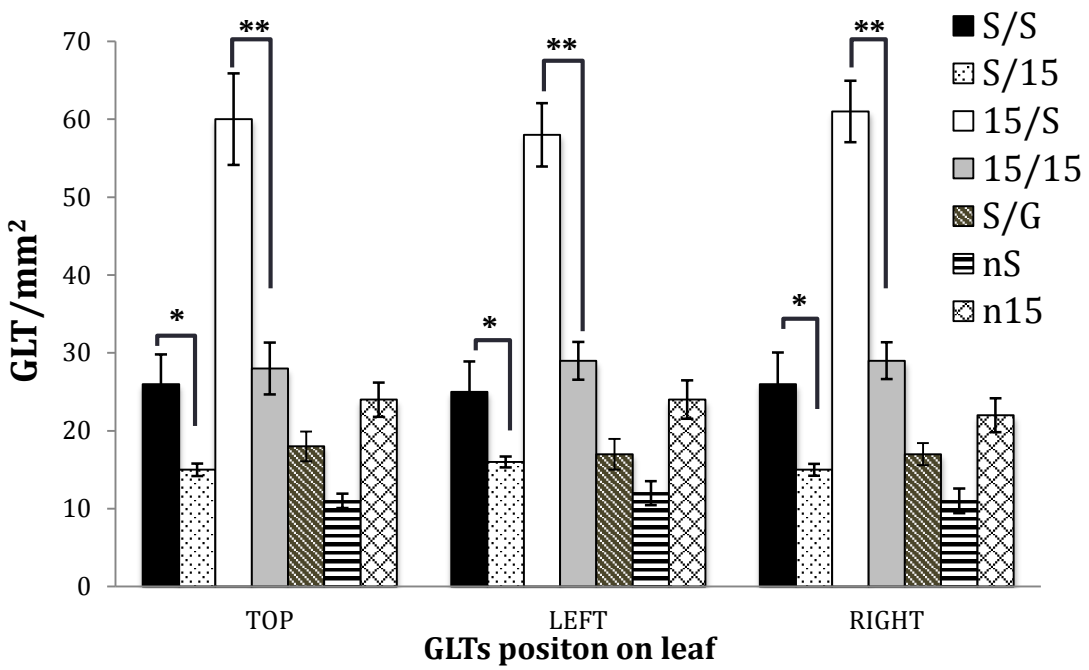


Figure 11 GLT population density. GLT population density of leaf 10 for all grafted and ungrafted plants in the three leaf regions. S, Sam; 15, #15; G, Glandless cultivar; nS, n15 and nG are ungrafted. Scion/rootstock of respective cultivars. *Error bars* are ±SE, n≥ 6, where * is $p \leq 0.05$ and ** is $p \leq 0.01$.

DHAA can also convert to dexoyAN (Fig.13C). In contrast to the AN production pattern, however, deoxyAN was mainly maximized in the 15/S crossed graft, but only in the leaves and not the ShAM. The S self-graft also produced more deoxyAN than the 15 self-graft (Fig.13C). Compared to the self-graft of 15, the S self-graft also produced considerably more deoxyAN than its ungrafted controls. As observed for AN, deoxyAN in ungrafted controls were not significantly different (Fig.13A, B and C).

The route from artemisinic aldehyde to AB is also an important branch in the AN biosynthetic pathway, so the level of AA and AB were also analyzed (Fig.13D and E). AA was only detected in S scions and mainly in the ShAM and grafted plants exhibited higher AA level than nS. Interestingly, in contrast with the AN data, the 15 rootstock significantly increased AA in the S scion compared to plants with an S rootstock when comparing S/15 with S/S. (Fig.13D).

A

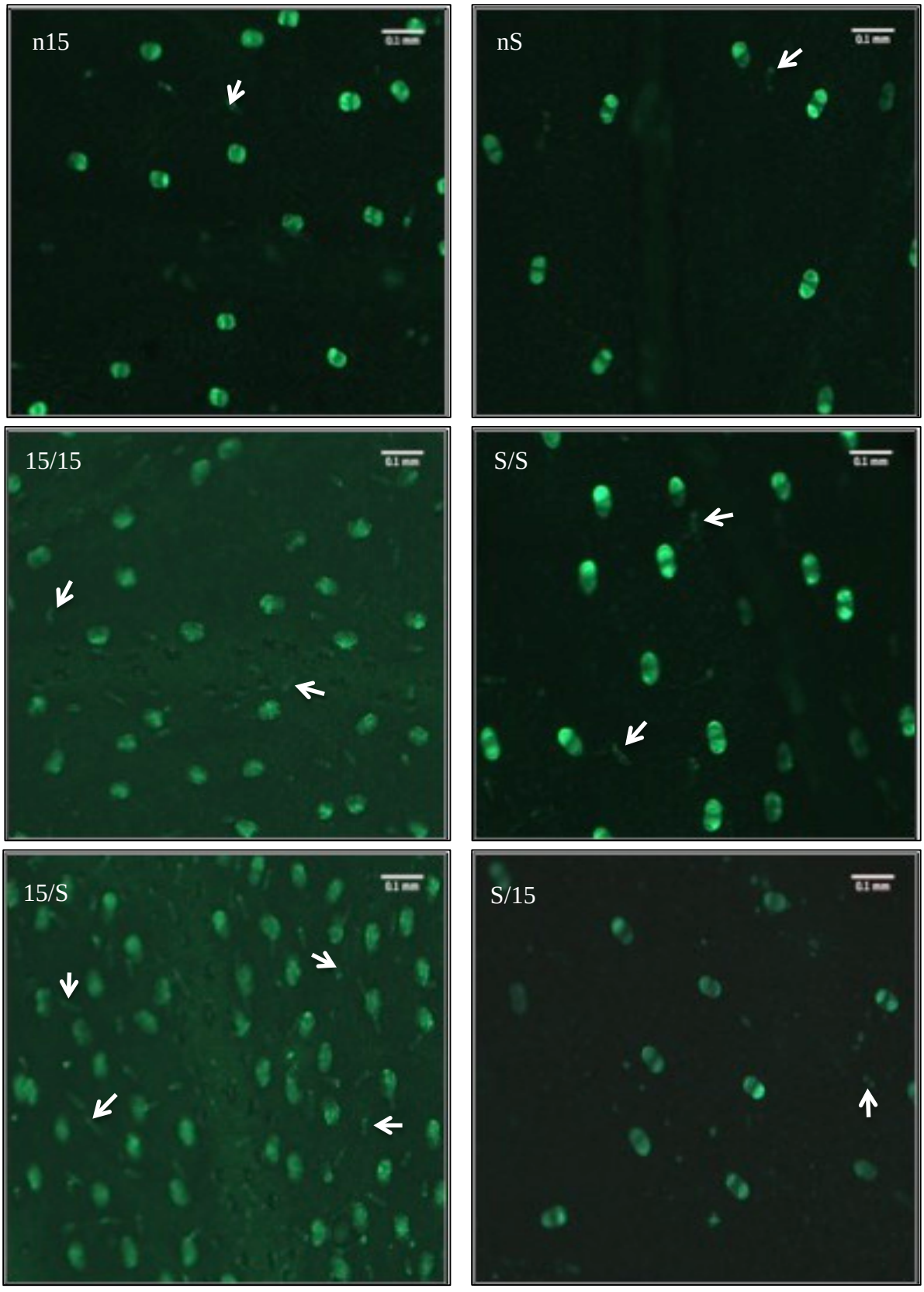


Figure 12 part 1

Figure 12 part 2

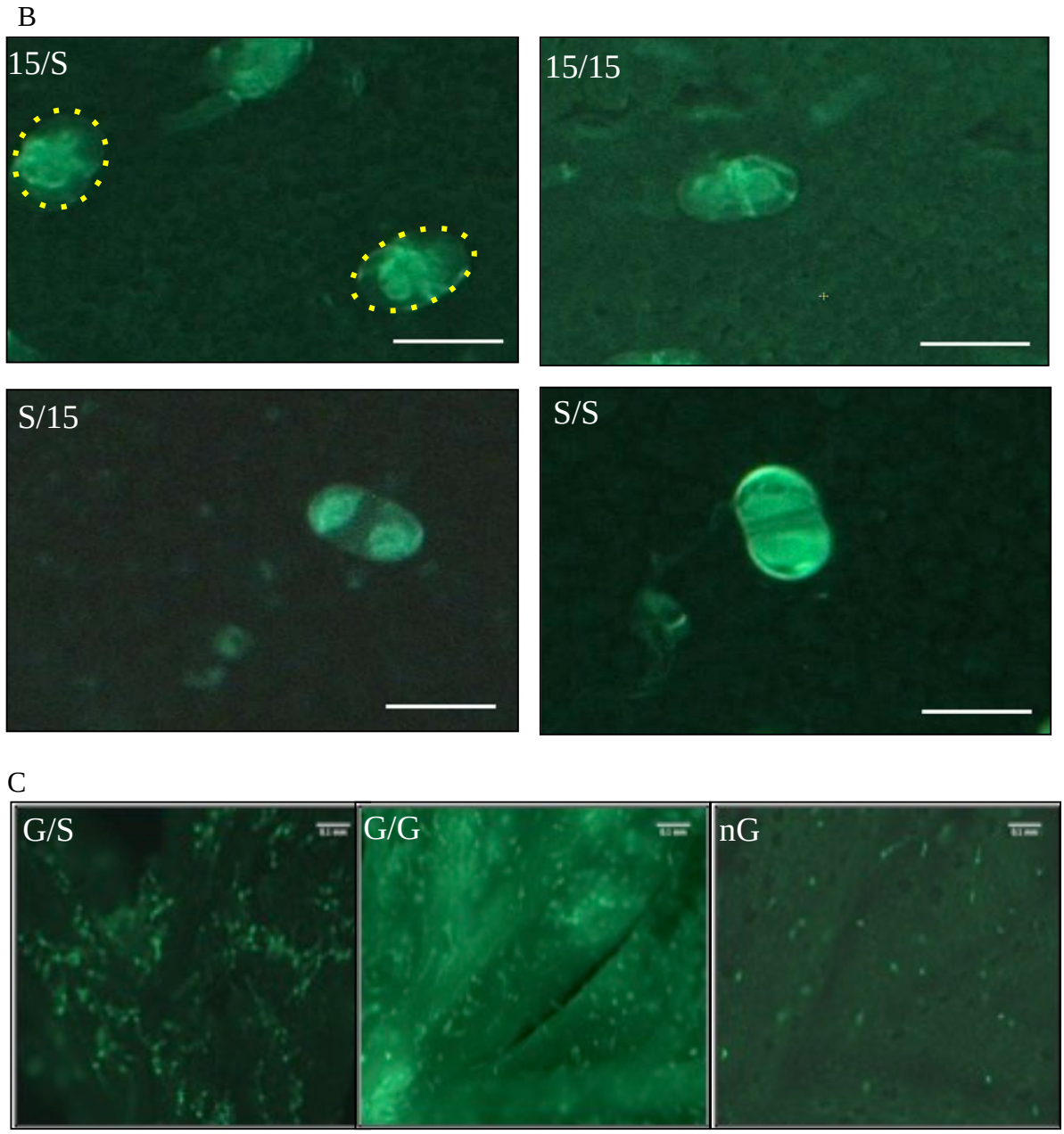
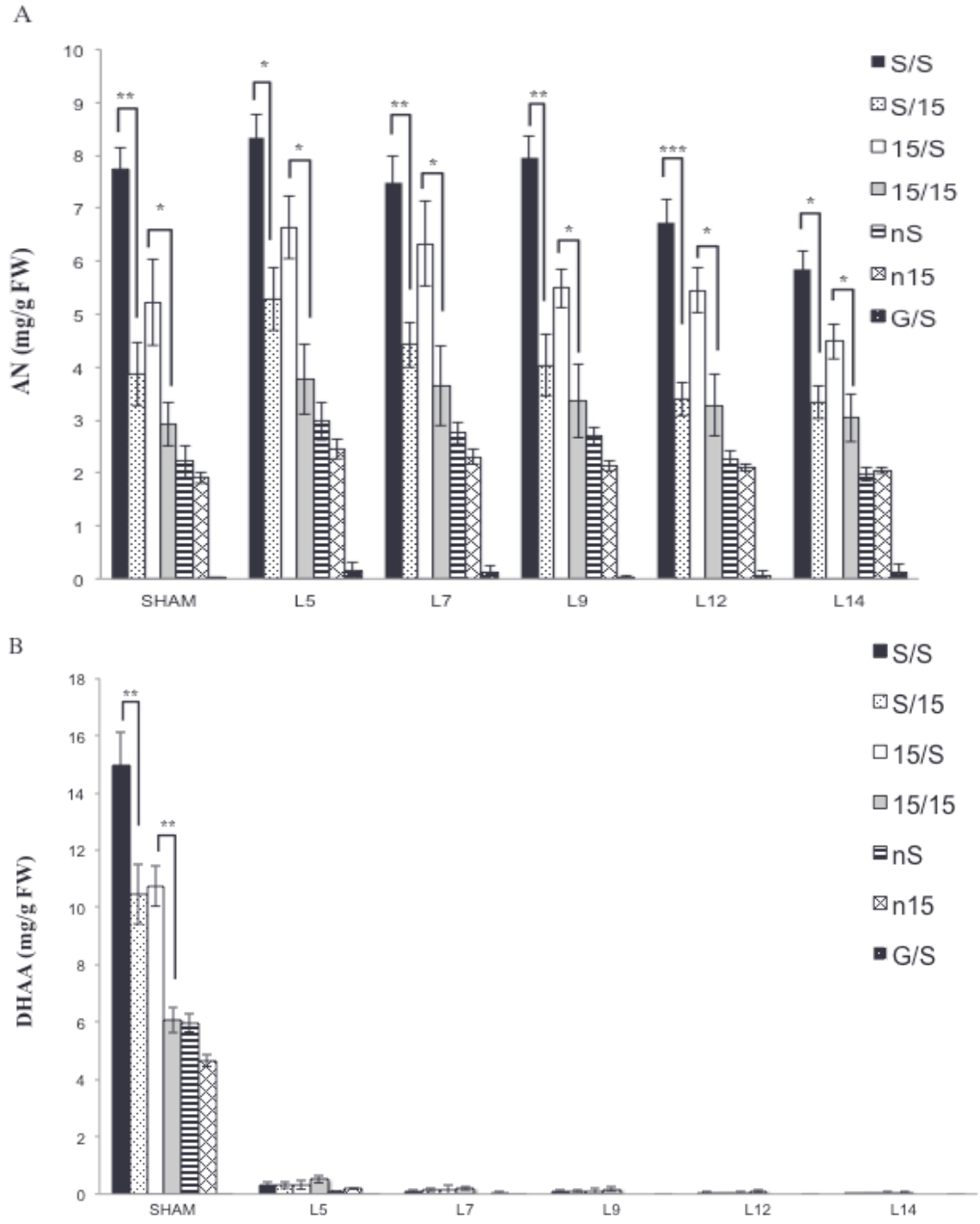
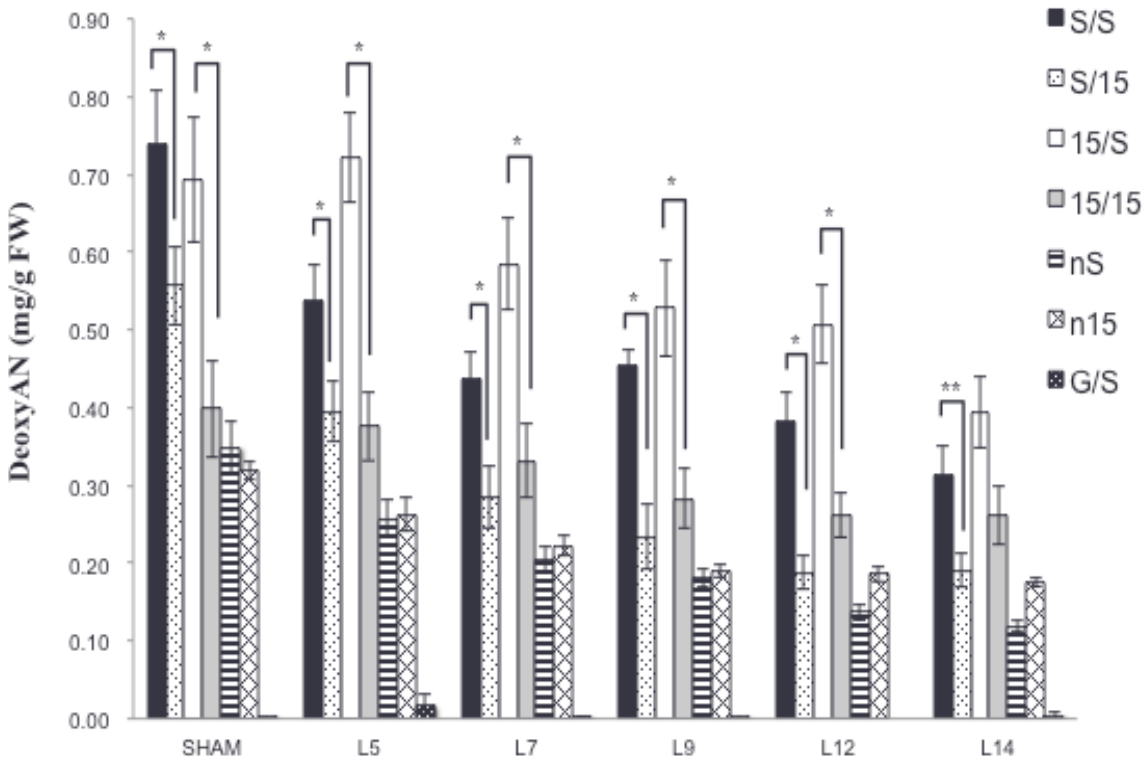


Figure 12 Comparison of glandular and filamentous trichomes among different kinds of grafted cultivars. A, representative glandular trichomes in the same leaf position from grafted and ungrafted cultivars viewed with fluorescence microscopy; White arrows show filamentous trichomes; *bar*=0.1mm. B, morphology of single glandular trichomes from the same numbered leaf and position for different grafted cultivars; yellow dotted outline shows the otherwise barely visible perimeter of an unfilled GLT; *bar*=0.05mm. C, variation in filamentous trichomes among grafted and ungrafted glandless cultivars; *bar*=0.1mm. All photos are from right position of leaf 10; S, Sam; 15, #15; G, Glandless cultivar; nS, n15 and nG are ungrafted; scion/rootstock of respective cultivars.

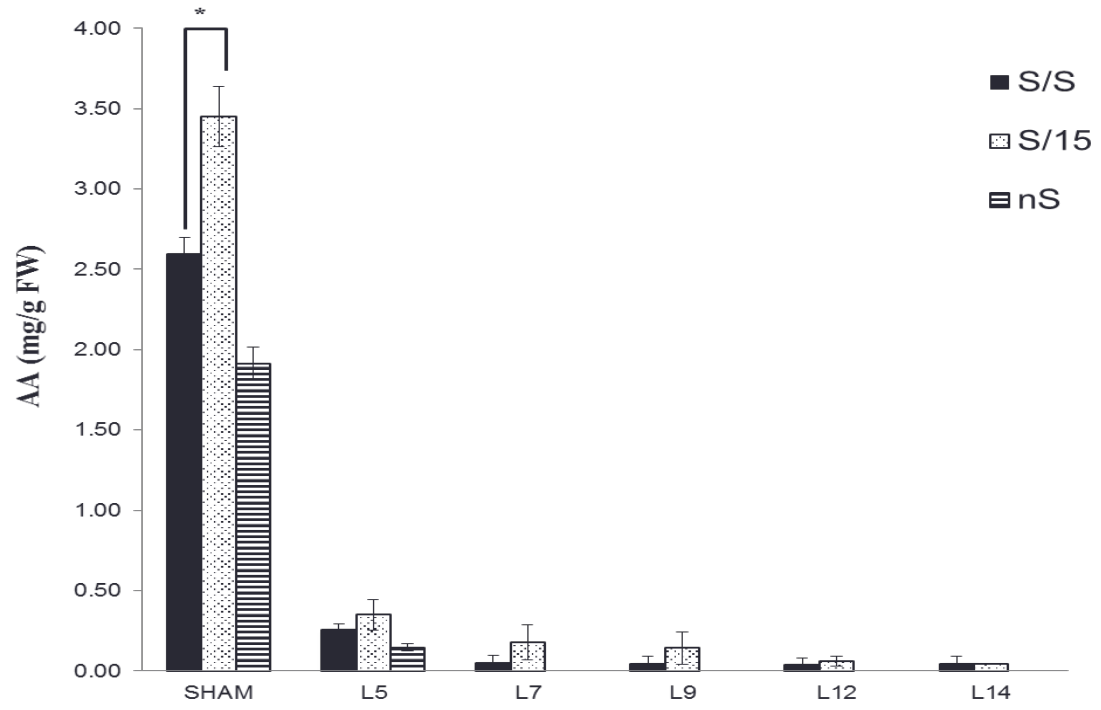
AA converts to AB via an apparently non-enzymatic photo-oxygenic reaction, and AB yield showed a trend similar to that of its precursor, AA. AB was highest in ShAMs and all leaves in S/15 grafts and significantly greater in ShAMs and young leaves (ShAM-L7) of S/S grafts (Fig.13E). Grafted plants also showed greater AB levels than ungrafted plants (Fig.13E).



C


DeoxyAN (mg/g FW)
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
SHAM
L5
L7
L9
L12
L14
S/S
S/15
15/S
15/15
nS
n15
G/S

D


AA (mg/g FW)
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
SHAM
L5
L7
L9
L12
L14
S/S
S/15
nS

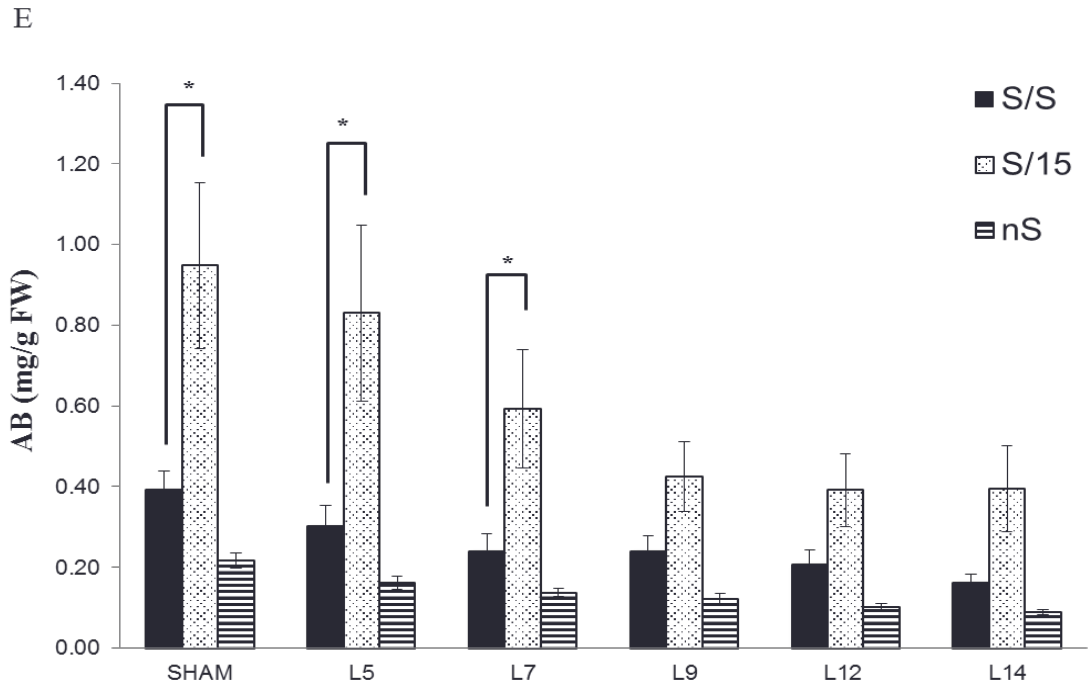


Figure 13 Artemisinic metabolite levels as leaves develop to leaf 14 for grafted and ungrafted A. annua cultivars. A, artemisinin(AN), B, deoxyartemisinin (deoxyAN), C, arteannuin B (AB), D, dihydroartemisinic acid (DHAA), E, artemisinic acid (AA). S, Sam; 15, #15; G, Glandless cultivar; nS, n15 and nG are ungrafted. Scion/rootstock of respective cultivars. *Error bars* are ±SE and n≥ 6 *$P \leq 0.05$, **$P \leq 0.01$.

4.4 Flavonoid levels among different grafted *A. annua* cultivars

Flavonoid content was highest in 15/S among all other ungrafted and grafted cultivars. The S rootstock is particular significantly elevated flavonoid levels in 15 scions, when comparing to 15 scions with a 15 rootstock. In addition, the flavonoid levels significantly decreased in S scions with a 15 rootstock (S/15) in comparison S scions with an S rootstock (S/S) (Fig.14). Self-grafts of both S and 15 produced significantly more than their corresponding ungrafted controls. Although the flavonoid levels of any G scions were very low, G self-grafts G/G were higher than nG and S/G in ShAM and young leaves (Fig.14). Both nS and n15 contained about the same amount of flavonoids with only slight difference in their ShAM. Flavonoid levels were hardly detectable among ungrafted G (nG) or any G grafted scions. Flavonoid data remained almost similar trends with GLT data. Relative differences between 15/S and 15/15 scions remained constant as leaves matured.

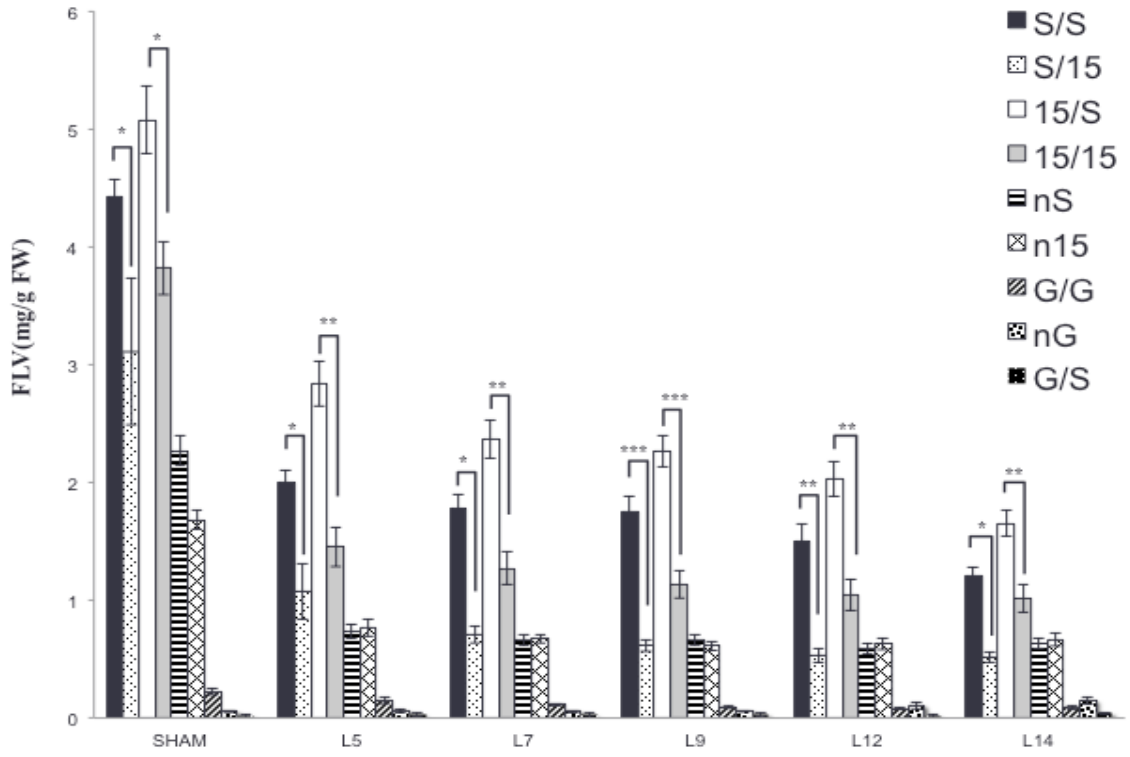
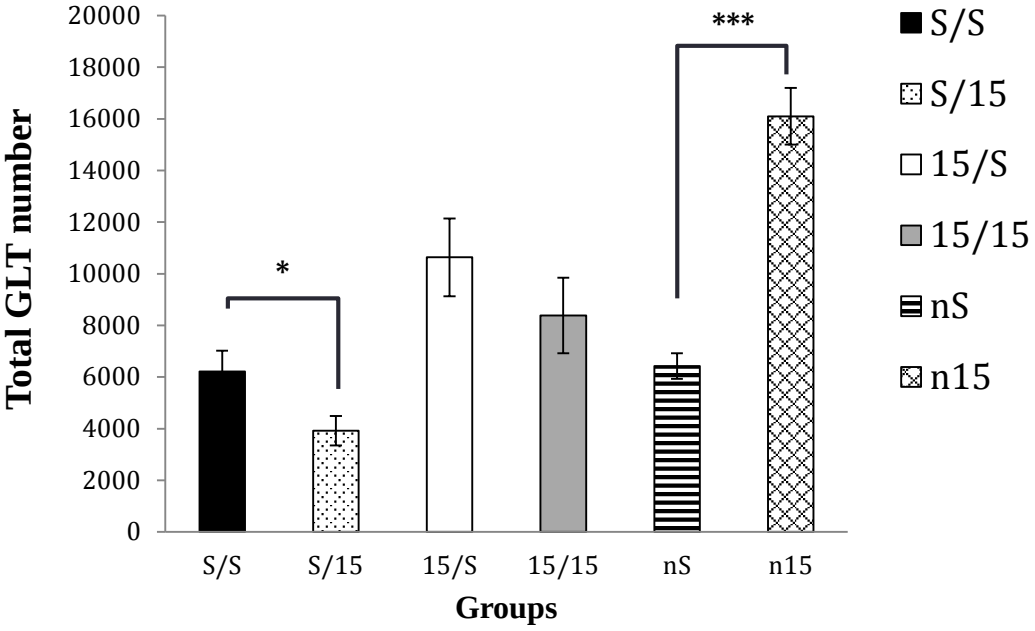


Figure 14 Flavonoid levels as leaves matured from ShAM to leaf 14 among different ungrafted and grafted A. annua cultivars. S, Sam; 15, #15; G, Glandless cultivar; nS, n15 and nG are ungrafted. Scion/rootstock of respective cultivars. *Error bars* are ±SE and n≥ 6 *$P \leq$ 0.05, **$P \leq$ 0.01, ***$P \leq$ 0.001.

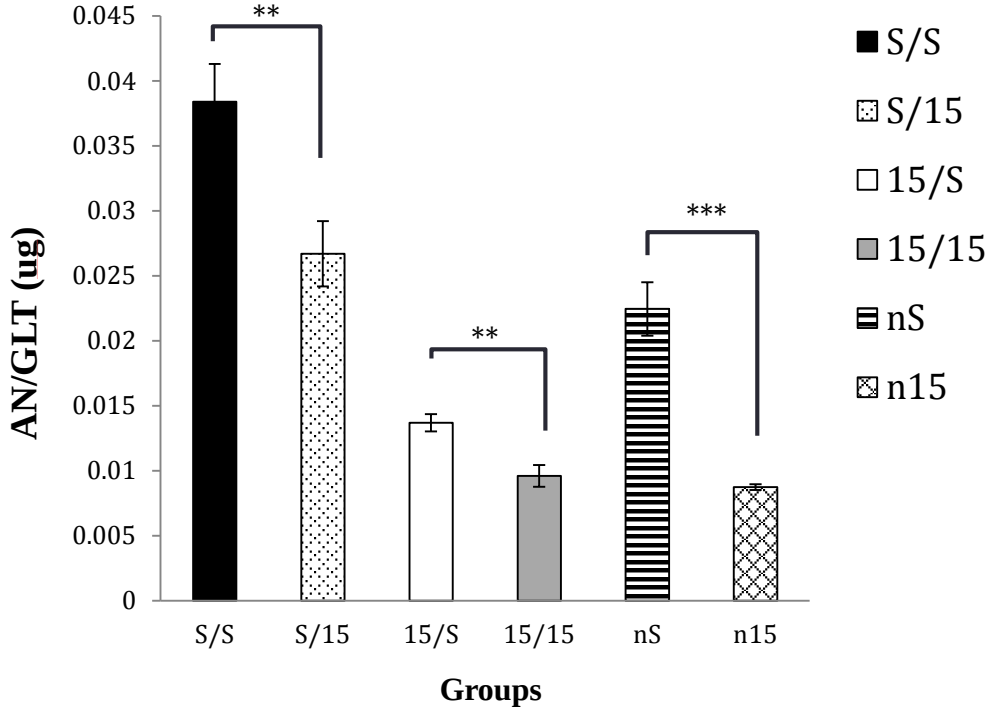
4.5 Artemisinin and flavonoids in glandular trichome

Total leaf areas were calculated along with total GLTs per leaf (Fig. 15A). When the AN content per GLT was calculated (Fig 15B), the S rootstock induced more AN production in the 15 scion compared to the 15 self-graft. When S was the scion and 15 was the rootstock, AN level per GLT decreased significantly compared to the S self-graft. Flavonoid content per GLT (Fig.15C) was also measured and similar to AN per GLT, the S rootstock stimulated AN production in 15 scions compared to the 15 self-grafts. When 15 was the rootstock, the flavonoids per GLT decreased in the S scion compared to S self-grafts.

A


Total GLT number
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
*
***
S/S
S/15
15/S
15/15
nS
n15
Groups

B


AN/GLT (ug)
0.045
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
**
**
***
S/S
S/15
15/S
15/15
nS
n15
Groups

C

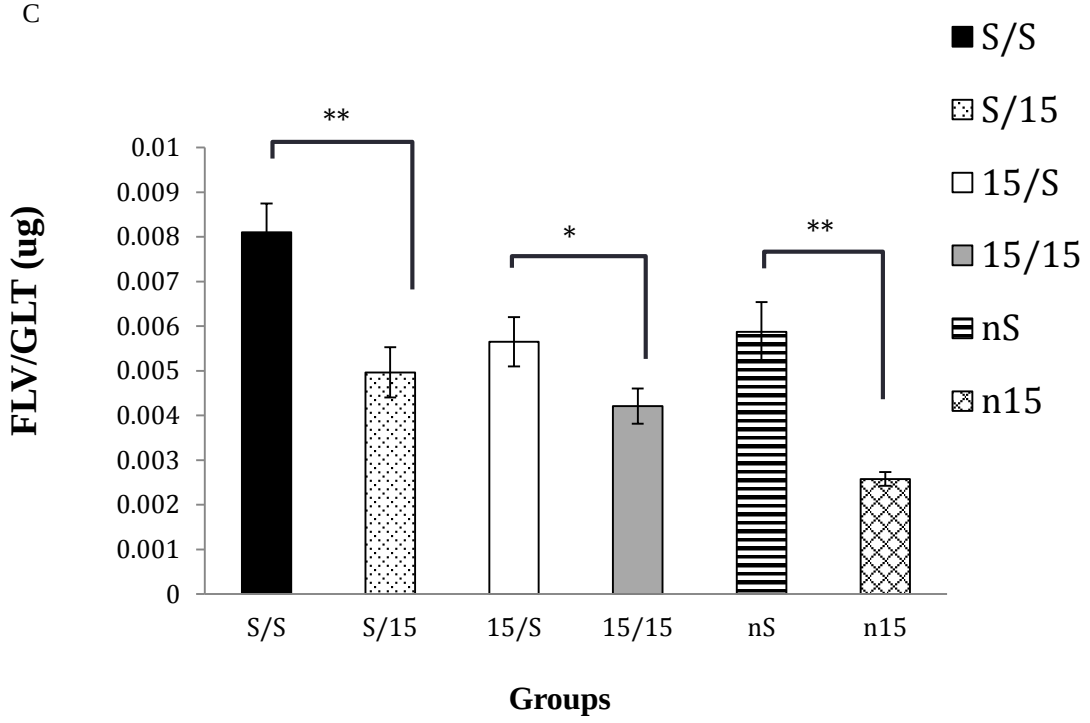


Figure 15 Artemisinin and flavonoid level per GLT in Leaf 10 among different ungrafted and grafted *A. annua* cultivars. A. Total glandular trichome number in L10. B, artemisinin level per GLT in L10. C. Flavonoid level per GLT in L10. S, Sam; 15, #15; nS, n15 and nG are ungrafted. Scion/rootstock of respective cultivars. *Error bars* are ± SE and n≥ 6 *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

5. Discussion

5.1 Rootstock effects on shoot biomass and morphology

Roots of high AN-producing plants significantly affected trichome development, and AN and FLV production in *A. annua*. Grafting stress, as confirmed via self-grafts, also markedly decreased the leaf biomass of all grafted cultivars. Although leaf biomass gradually declined in nS as leaves matured, neither n15 nor nG showed such a decline in this study, possibly because they were different cultivars. All self-grafts yielded significantly less fresh shoot mass than ungrafted controls and G/S and G/G apical regions of shoots exhibited floral-like leaf buds, suggesting grafting stress negatively affected leaf development. Similar results also occurred in peach and tomato grafts (Glenn and Scorza 1992; Kim et al. 2001). The scion of the null mutant, G was particularly sensitive to grafting stress, resulting in altered leaf morphology in both size and ShAM development. Furthermore, the lateral branches of nG also seemed to grow faster than either n15 or nS, and the G scion of G/G also seemed to grow more rapidly; ShAMs appeared more like those of floral meristems than shoot meristems. This lateral branch extension suggested some alteration in auxin signaling akin to loss of apical dominance. Auxins such as IAA are involved in regulating apical dominance and embryonic and post-embryonic development; they influence aspects of cell division, cell elongation and cell differentiation (Went 1935; Teale et al. 2006). Interestingly, the G rootstock also stimulated lateral branch growth on S scion in S/G grafts.

The Aux/IAA protein is a transcription inhibitor (Weijers et al. 2005). Mutations in the aux/iaa gene result in auxin response factor (ARF) disorder and plants exhibit a variety of auxin-related developmental phenotypes, including altered phototropism/gravitropism, root formation, apical dominance, stem/hypocotyl elongation, leaf expansion (Rouse et al. 1998; Reed 2001). Wang et al. (2005) showed down-regulation of *IAA9*, which belongs to the subfamily of *Aux/IAA* genes, led to an inverted pattern of axillary shoot development and reduced apical dominance, a phenotype that was consistent with this study. Together these studies may explain the interesting phenotypes observed in this study for the G cultivar

including clustered and unexpanded leaves of G/G and loss of apical dominance in G/G and S/G.

Additionally, the S rootstock significantly increased young and mature leaf biomass of G scions compared to G self-grafts, while the G rootstock significantly decreased leaf biomass of S scions compared to their self-grafts. However, this response was not observed for the other two cross grafts, S/15 and 15/S compared to G. S rootstock seemed to induce more leaf biomass suggesting that rootstocks of these larger leaf cultivars stimulated leaf biomass in the smaller leaf cultivars. These phenotypes were possibly caused by long distance signals. Rahayu et al. (2005) showed root-derived cytokinins functioned as long-distance signals to mediate NO_3^- induced leaf growth. In addition Maes et al. (2011) also showed BAP increased both glandular and filamentous trichome development in high and low AN-producing cultivars. In this study scions of 15/S had significantly more GLTs and FLTs than S/S, leaf size, however, did not change. G scions of G/S also showed increased FLTs and larger leaf size than nG.

Gibberellin A3 and gibberellin-like substances could translocate in grafts in grafts between normal, dwarf1, and dwarf5 seedlings of *Zea mays* L. and resulted in leaf-sheath elongation (Katsumi et al. 1983). In *A. annua* Banyai et al. (2011) showed that GA_3 treatment improved leaf biomass and AN level. After GA_3 treatment FLT populations and GLTs size also increased, but only in low AN-producing cultivar (Maes et al. 2011). My study was consistent with these prior studies. The high AN-producing S rootstock significantly increased AN level in low-AN producing (15) scions vs. 15 self-grafts. Furthermore, leaf biomass of the null mutant (G) scion was also greater than G self-grafts, and the FLT population of the G scion was also greater than either the low-AN producing cultivar or the null mutant (nG). Taken together these data, suggested that root-derived GA could also be involved in the regulation of leaf size and affect GLT development on leaves.

ABA-deficient maize mutants produced less leaf biomass than wild type suggesting an important function of ABA as an inhibitor of leaf expansion (Sharp and LeNoble 2002). Sobeih et al. (2004) also showed ABA travelled long-distance from root to shoot to regulate leaf growth in tomato. Thus, ABA cannot be ignored as a root-derived signal. Depending on cultivar,

rootstocks of tomato also showed significantly different effects on leaf biomass (Rick 1952). Combined these studies suggested that phenotypic changes may be caused by a complex of phytohormones, which would not be surprising.

5.2 Rootstock effects on trichome size and populations.

There are two kinds of trichomes in *A. annua*: glandular (GLTs) and filamentous (FLTs) (Duke 1994). Although measurements in this study mainly focused on the GLTs, which produce and store AN (Duke et al. 1994), grafts including G as the scion showed that both trichome types seemed to have been affected. Whenever S was the rootstock, GLT populations in grafted scions significantly increased, but especially when 15 was the scion. On the other hand, the 15 rootstock seemed to have little to no effect on 15 scions when self-grafted or when compared to either n15 or S/15 grafts. In particular the GLT population, while similar to the self-grafts S/S and 15/15, increased fourfold when S was the rootstock for 15 vs. when 15 was the rootstock for S. While nS and S/15 were similar in GLT populations, both the S/15 and S/G grafts had significantly fewer GLTs than S/S indicating a suppression of the grafting stress response in that cultivar. Taken together these results suggested that the S rootstock stimulated GLT production in the 15 scion, but the 15 and G rootstocks both decreased GLT production in the S scion.

These differences in GLT populations could be an effect of altered levels of and/or sensitivity to jasmonic acid (JA). Wang et al. (2010a) demonstrated MeJA treatment also increased the production of AN in intact plants. MeJA similarly increased glandular trichome density on *Lycopersicon esculentum* leaves (Boughton et al. 2005).

In contrast, artificial wounding, JA treatment and SA treatment all had the same effect, increasing trichome density in *Arabidopsis* (Traw and Bergelson 2003). JA, SA, ABA (Dong 1998; Kunkel and Brooks 2002) and trichomes are all are involved in plant defense (Levin 1973). S and 15 are high and low-AN producing cultivars, respectively, so results of this study are consistent with JA affects on *A. annua* observed by Maes et al. (2010/2011). They showed that in low AN-producing cultivars, JA increased the number of GLTs and FLTs respectively,

glandular trichome size, and AN levels. On the other hand, when high AN-producing cultivars were treated with JA, GLT size did not change, and neither did AN levels; GLT numbers increased.

Although the S rootstock significantly increased the GLT populations in S and 15 scions, there was no effect on G scions; no GLTs were produced. Thus, whatever the stimulus was from S rootstocks on scions already capable of producing GLTs, it was unable to initiate GLTs in a cultivar where previously there had been none.

5.3 Rootstock effects on flavonoids

Flavonoids are also important metabolites produced in *A. annua*. Of the more than 40 flavonoids found in the plant (Ferreyra et al. 2012), >10 have some weak antimalarial activity (Lehane and Saliba 2008; Ganesh et al. 2012; Suberu et al. 2013), with some also reported to work synergistically with AN against falciparum malaria (Liu et al., 1992).

There seemed to be a close correlation between flavonoid levels and GLT populations; high flavonoid levels were found in scions that also had high GLT populations. When S was the rootstock, flavonoid levels increased in both S and 15 scions, while 15 rootstocks decreased the levels in both 15 and S scions. Flavonoids were barely detectable in G scions of ungrafted nG, or in the grafts G/G or G/S, which was not surprising since G has no GLTs (Duke et al., 1994). This observation was substantiated by a transcriptome analysis of *A. annua* GLTs, which yielded transcripts of many of the genes in flavonoid biosynthesis including phenylalanine and chalcone synthases (Wang et al. 2010b; Maes et al. 2011). Furthermore, Soetaert et al. (2013) recently showed that two dioxygenases annotated to flavonoid pathways were located in GLTs, but not FLTs. Taken together these results demonstrated that the S rootstock stimulated production of flavonoids in both S and 15 scions, suggesting there was some mobile signal moving from the root of S to the shoots. Similarly, the 15 rootstock may have a signal that moved to inhibit production of flavonoids in both S and 15 scions. While the total FLV level showed no difference between S and 15 cultivars per DW, the FLV data trended with the GLT data.

5.4 Rootstock effects on artemisinic metabolites

AN levels were measured in the ShAMs, and young and mature leaves of all ungrafted and grafted scions. Rootstocks of the high-AN producing S cultivar significantly increased the AN level in the low-AN producing 15 scion. The response was greater than in the scions of the 15 self-graft and either nS or n15. However, the highest overall AN production was in the scions of S self-grafts, demonstrating a strong grafting stress response. Overall levels were: S/S > 15/S > S/15 > 15/15 > nS $\approx$ n15 with all greater than any G scions where AN was not detectable. The precursor to AN is DHAA and its production in all scions was similar to AN production levels. DeoxyAN is an alternate product stemming from DHAA. In contrast to AN, deoxyAN varied slightly in its level in grafted and ungrafted scions; highest levels were in the 15/S grafts vs. in S/S grafts as observed for AN and DHAA. Although the DHAA level was at about the same level in scions of 15/S and S/15, 15/S converted more to AN and deoxyAN than did S/15. Together these results demonstrated that the S rootstock stimulated production of DHAA, AN and deoxyAN in both S and 15 scions, suggesting there was some mobile signal moving from the root of S to the shoots resulting in stimulation of the AN/deoxyAN branch of the AN biosynthetic pathway.

There is a close relationship between GLT populations, leaf maturity and AN level (Ferreira and Janick 1996; Kapoor et al. 2007; Arsenault et al. 2010), but AN levels in this study did not correlate well with GLT populations. Nguyen et al. (2013) showed that GLTs increased in size and fullness as leaves matured and Graham et al. (2010) also observed AN level reached relatively maximum and stable at about leaf 10th and older, suggested completely maturity of GLT. Maes et al. (2011) showed that when low AN-producing and high AN-producing *A. annua* were treated with BAP, both of them significantly increased their populations of GLTs with the low AN-producers showing a greater change than high AN-producers including decreased GLT size. Artemisinic metabolites, on the other hand, showed no response to BAP and did not increase, suggesting the population of GLTs was not the only factor affecting artemisinic metabolite level. In this study, 15 and S are also low- and high-AN producing

cultivars and the S rootstock similarly increased the GLT population in both 15 and S scions, but the greater changes were in the low-AN producing 15 scion.

Compared to AN, the other artemisinic metabolite levels did not trend with GLT data. There were also considerable differences in fluorescence brightness and distribution among scions. Brightly fluorescing GLTs probably contain more secondary metabolites than GLTs with less fluorescence. S/S had large highly fluorescent GLTs suggesting they were replete with secondary metabolites, while 15/15 was brilliant overall, suggesting lower content level. Interestingly, the GLTs from 15/S scions had barely detectable fluorescence on the end perimeter of each column of trichome cells, but a more brilliant center. The alternate S/15 scion had a barely detectable fluorescent center and instead had greater fluorescence over each of the two cell columns of the GLT. Taken together with these, it seemed the varied fluorescence in GLT might correlate with varied secondary metabolite levels.

ROS plays an important role in the AN biosynthetic pathway, especially the last non-enzymatic photo-oxidative steps from DHAA→AN and DeoxyAN, AA→AB (Covello 2008; Mannan et al. 2010). Potters et al. (2009) showed auxin, ethylene and ROS were involving in mediating stress-induced morphogenic responses, suggesting there was a close relationship among root ROS, auxin, and shoot and GLT morphogenic responses. Nguyen et al. (2011) documented the close relationship among reactive oxygen species (ROS), roots, trichome development and artemisinic metabolites. ROS interacts with JA and SA in the plant defense signaling system (Klessig et al. 2000). NAA and IAA are also reported to induce ROS in roots (Joo et al. 2001). However, there seems to be little known about specific effects of auxins on AN biosynthesis. Woerdenbag et al. (1993) showed that *A. annua* shoots grown in MS-medium containing 0.2 mg L^{-1} NAA yielded the maximum AN level, suggesting exogenous auxin affected AN biosynthesis. Mannan et al. (2010) showed ROS was induced when in vitro-cultured *A. annua* rooted shoots were treated with DMSO resulting in increased AN in shoots. Nguyen et al. (2013) indicated that NAA might mediate the conversion of AA→AB.

Production of AB via AA constitutes an important branch in the AN biosynthetic pathway. Neither AA nor AB was detected in any 15 scion including ungrafted 15 (n15). This result

suggested that either the AA→AB branch was inhibited in the 15 scions or that it was nonexistent in the 15 cultivar. Of particular interest is the stimulation of both AA and AB levels in the S scion when 15 was the rootstock vs. when S was the rootstock. The 15 rootstock also inhibited the production of AN, DHAA and deoxyAN in the S scions. Considering these results, it seemed that in cultivar 15 there was a shift in metabolite flux between these two pathway branches possibly where they split in a step catalyzed by DBR2. Jiang et al. (2014) showed the *DBR2* promoter is trichome-specific with the same *cis*-element also present in *ADS* and *CYP71AV1* promoters. TFs such as AaWRKY1 and AaORA may also bind to the promoter of *DBR2*, which has a key role to play in shifting carbon between the AB and AN routes in the pathway (Yang et al. 2015). It would be interesting to measure the transcripts of this gene in the various grafted and ungrafted scions of S and 15.

RNAs (mRNA, siRNA, miRNA) can function as long-distance signals from source to sink via the phloem, which connects even the most distant parts of a plant; phloem provides fast and directed systemic information transfer (Kehr and Buhtz 2008; Goldschmidt 2014). Tzarfati et al. (2013) showed grafting indeed induced changes in miRNA expression pattern in leaf petioles of *Citrus*, especially miRNA 156 and 157; these same miRNAs were also recently found in *A. annua*.

In *A. annua*, FPP serves as a common precursor of amorpha-4,11-diene and of other sesquiterpenes diverted by at least three other sesquiterpene synthases (Weathers et al. 2006) through competitive pathways. In another example, squalene synthase (SQS) is the key enzyme catalyzing the first step of the sterol biosynthetic pathway, which competes with AN biosynthesis pathway (Liu et al. 2006), so a miRNA signal may move from rootstock to GLTs on the scion to regulate or balance GLT-localized secondary metabolisms. In this study, one could consider that a rootstock-derived miRNA may translocate to the shoot and repress other pathways thereby enhancing the AN biosynthetic pathway.

5.5 Phenotype duration

Since the various scion phenotypes were analyzed several months after grafting, this suggests a persistent change including in shoot and trichome morphology as well as artemisinic and flavonoid metabolites. The duration of the long-term phenotypic changes therefore suggested there was an epigenetic alteration of the scion. This was consistent with observations by Brosnan et al. (2007) and Molnar et al. (2010) who demonstrated that an sRNA was transmitted from root to shoot and subsequently directed epigenetic modification of the genome of the recipient cells in *Arabidopsis*.

Eukaryotic genomes are modified with a diverse set of chromatin markers, which are involved in both DNA, e.g. DNA methylation, and associated histones, e.g. histone modifications. Although these changes do not alter the primary DNA sequence, they are frequently heritable through cell division, sometimes for multiple generations, and remain as altered phenotypes for the long-term (Henderson and Jacobsen 2007). Recently Fuentes et al. (2014) demonstrated that an entire nuclear genome could be transferred between rootstock and scion yielding new allopolyploids that changed chromosome sets and primary DNA sequence, suggesting even another explanation for persistent and varied phenotypes in this study.

6. Conclusions and Future Work

6.1 Conclusions

Roots are involved in regulation of artemisinin and flavonoid metabolism in *A. annua* shoots and also in trichome development. In the scion of the low-AN producing cultivar, 15, high-AN producing S rootstocks markedly enhanced GLT size and populations, as well as production of AN, deoxyAN and their precursor, DHAA. Conversely, the rootstock of the low-AN producing 15 cultivar inhibited leaf biomass, GLT size and populations, and AN, deoxyAN and DHAA levels in the scions of S, the high-AN producing cultivar. There was also a shift in the metabolite flux between the two artemisinic biosynthetic pathways from DHAA → AN/DeoxyAN to AA→AB with the latter branch favored when 15 was the rootstock and the former branch favored with the S rootstock. Taken together these results are consistent with one or more mobile signals that are transmitted from roots to shoots that not only regulate two branches of the AN pathway, but also the fullness and number of GLTs on the plant.

6.2 Future work

Future studies are warranted. First, the roots indeed play a pivotal role in regulating AN biosynthesis and trichome development probably by sending a mobile signal to the shoots. Identification of the chemical nature of the signal, its pathway to the shoots, its directionality (only upwards?), and eventual target all still must be studied.

Last but not least, although the root significantly affects AN and FLV biosynthesis and trichome development by possibly translocating some mobile signal factor to the shoot, it is not clear if the signal(s) either continuously or intermittently modulates the responsible genes in the pathway. Do these affects result from a permanent genetic change or are they concentration dependent to give a stimulating or inhibiting response?

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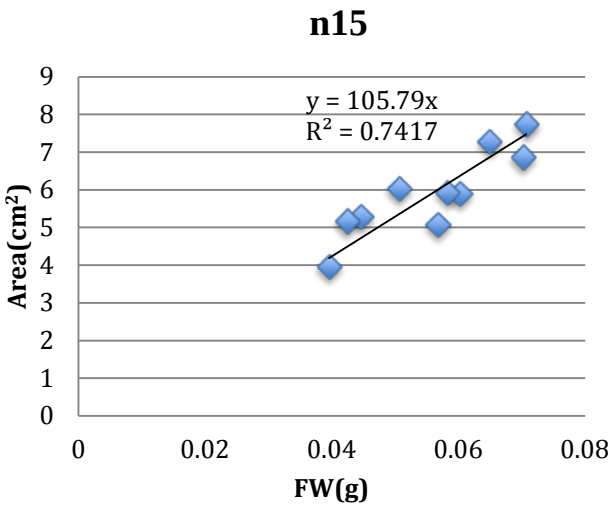
8. Appendix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Area(cm²)
FW(g)
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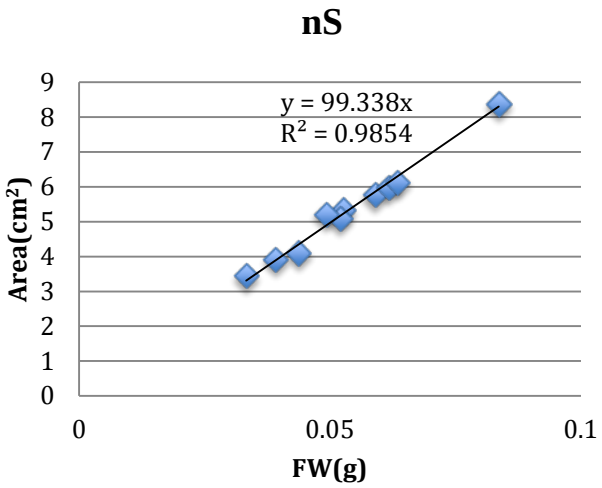
2
3
4
5
6
7
8
9
0
0.02
0.04
0.06
0.08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R² = 0.9854
Area(cm²)
FW(g)
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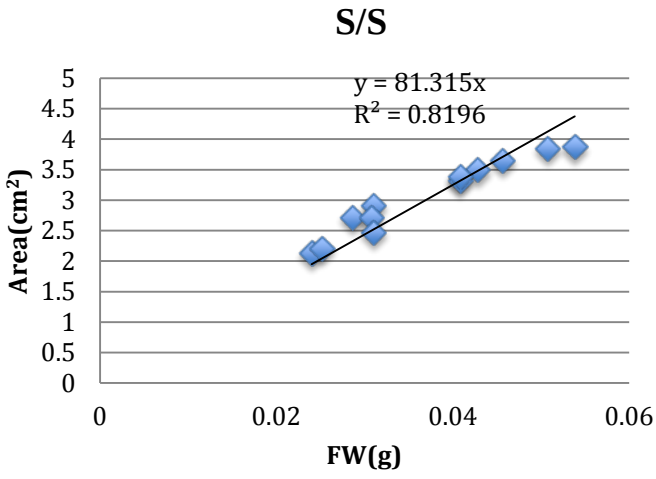
2
3
4
5
6
7
8
9
0
0.05
0.1


S/S
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R² = 0.8196
Area(cm²)
FW(g)
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1.5
2
2.5
3
3.5
4
4.5
5
0
0.02
0.04
0.06

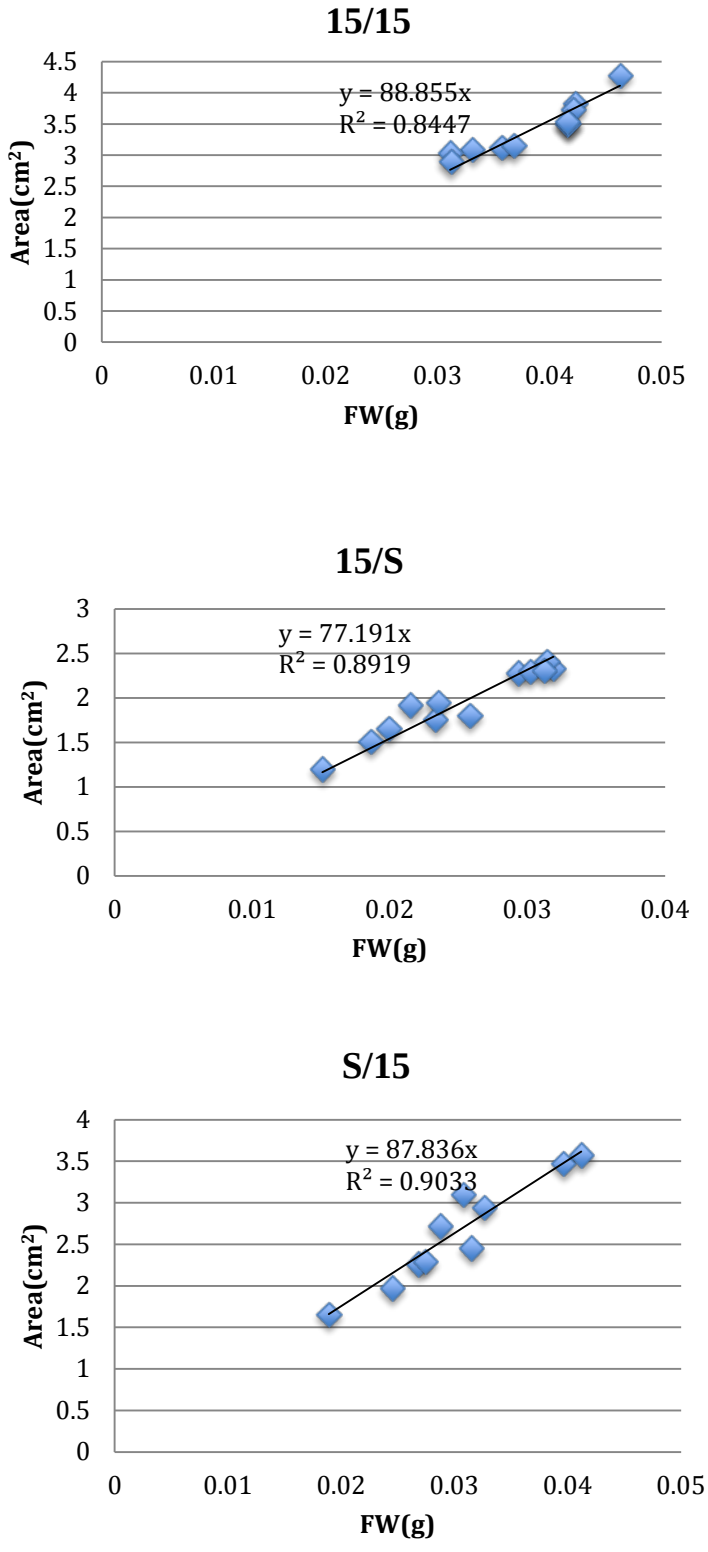

Figure 16 Correlation between leaf biomass and leaf area among different grafted and ungrafted cultivar. ≥ 10 leaves coming from 3 individuals of each type of grafted and ungrafted cultivar, which were used for calculating. S, Sam; 15, #15; G, Glandless cultivar; nS, n15 and nG are ungrafted. Scion/rootstock of respective cultivars.

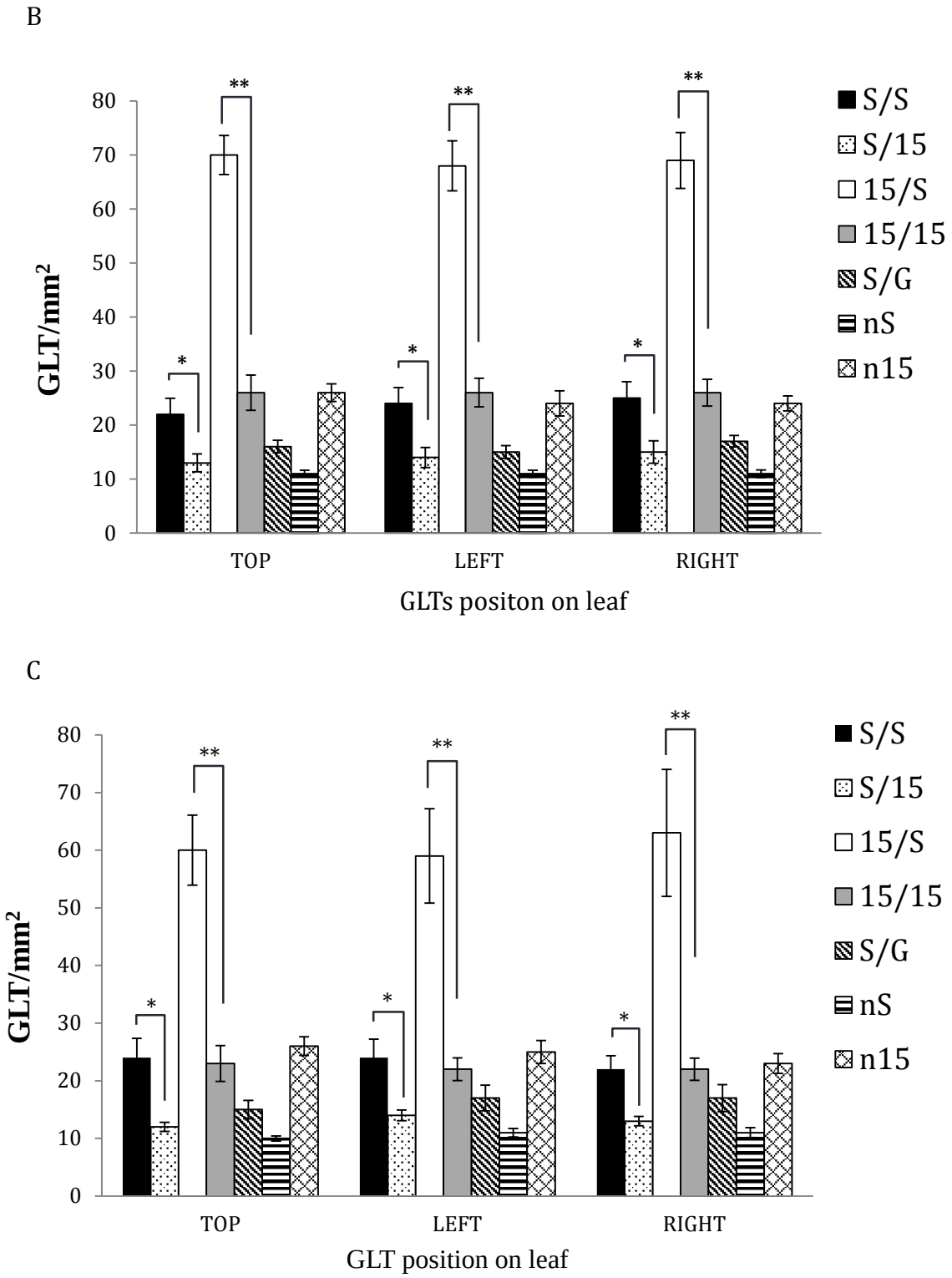

Figure 17 GLT population density. A, GLT population density of leaf 11 for all grafted and ungrafted plants in the three leaf regions. B, GLT population density of leaf 13 for all grafted and ungrafted plants in the three leaf regions. S, Sam; 15, #15; G, Glandless cultivar; nS, n15 and nG are ungrafted. Scion/rootstock of respective cultivars. *Error bars* are ±SE, n≥ 6, where * is $p \leq 0.05$ and ** is $p \leq 0.01$.