CHARACTERIZATION OF PROANTHOCYANIDINS AS A BIOMARKER FOR WOOD QUALITY IN THE NATIVE TIMBER-WOOD TREE *ACACIA KOA*

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Abstract

Acacia koa (koa) is an economically valuable timber wood tree playing an important role for Hawaiian culture and ecology. Wood color of koa can range from blond to dark red while its grain figure ranges from plain to curly. Given that koa populations are highly heterozygous and heterogeneous due to cross-pollinating nature, there is no current screening method to select seedlings for superior wood quality. Proanthocyanidins (PA), a subclass of tannins, are shown to be involved in wood quality. The goal of this study was to quantify PAs and identify PA biosynthesis genes as a biomarker for wood color. PA biosynthesis genes in koa were identified and isolated including dihydroflavonol reductase, anthocyanidin synthase, anthocyanidin reductase, and leucoanthocyanidin reductase. To correlate wood color, PAs, and expression of PA biosynthesis genes, koa seedlings expressing green to red hues were compared from Hawai‘i, O‘ahu, Kaua‘i, and Maui. Measurement of total tannin content was analyzed by the Folin-Ciocalteau method and PA content was measured by acid butanol assay in dry matter. Red-hued koa from Maui contained the highest total tannin and PA content. Distribution of the total tannin and PA content was assessed within koa seedlings expressing either green or red hues (O‘ahu and Maui). Total tannin content varied significantly among tissue type (leaf, stem, root). Aside from PA content also varying among tissue type, an opposing trend of lowest to highest PA content dependent on the hue expression of the family was observed. This suggests PA content is correlated to the overall hue of each family. qRT-PCR analysis of PA biosynthesis gene expression showed an upregulation in red-hued koa (Maui) compared to green-hued koa (Hawai‘i and O‘ahu). This suggests PA biosynthesis gene expression is also correlated to the overall hue of each family. Liquid chromatography-mass spectrometry (LC-MS) confirmed procyanidin B2 as the type of proanthocyanidin in koa. Characterizing proanthocyanidins as a biomarker for wood quality will be useful as a rapid screening method among progenies of different families in koa improvement programs.

Table of Contents

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

PA: proanthocyanidin

WGTIP: Western Gulf Tree Improvement Program

GSPE: grape seed proanthocyanidin extract

ROS: reactive oxygen species

PSE: peanut skin extract

UV: ultra-violet

CHS: chalcone synthase

CHI: chalcone isomerase

F3'H: Flavanone 3'-hydroxylase

DFR: dihydroflavonol reductase

ANS: anthocyanidin synthase

ANR: anthocyanidin reductase

LAR: leucoanthocyanidin reductase

HPLC: high performance liquid chromatography

LC-ESI-MS: liquid chromatography electrospray ionization mass spectrometry

HARC: Hawai'i Agriculture Research Center

RNA: ribonucleic acid

cDNA: complementary deoxyribonucleic acid

PCR: polymerase chain reaction

qRT-PCR: quantitative real-time polymerase chain reaction

NCBI TSA: National Center for Biotechnology Information Transcriptome Shotgun Assembly

TAE: tannic acid equivalent

LCE: leucocyanidin equivalent

DW: dry weight

MeOH: methanol

NADPH: nicotinamide adenine dinucleotide phosphate

ANOVA: analysis of variance

CHAPTER 1

INTRODUCTION

Evolution and importance of *Acacia koa* as a timber-wood tree on the Hawai‘ian Islands

Acacia koa (koa) is a leguminous native timber-wood tree endemic to the Hawai‘ian Islands. Koa is a member of the family Leguminosae, subfamily Mimosoideae, genus *Acacia*, subgenus *Phyllodineae*. Generally, koa have been found thriving in a broad range of habitats: near sea level to 2000 m in wet or mesic forests with annual rainfall from 1850 to 5000 mm (Harrington et al., 1995; Anderson et al., 2002; Wilkinson and Elevitch, 2003; Baker et al., 2009). These koa habitats are distributed among the five major Hawai‘ian islands including Hawai‘i, Moloka‘i, Maui, Lāna‘i, O‘ahu, and Kaua‘i (Wagner et al., 1999).

Of the 950 *Acacia* species known, 18 species including *Acacia koa* reside outside of Australia (Ishihara et al., 2017; Robinson and Harris, 2000; Orchard and Maslin, 2003 Brown et al., 2012). The evolution of koa date back to more than 5.1 million years from its descendant *Acacia melanoxylon*, which is another important woody tree originally from Australia (the age of Kaua‘i, the oldest Hawai‘ian island; Le Roux et al., 2014). The dispersal of koa is still being investigated; birds and free-floating seeds are the most probable dispersal strategies (Ishihara et al., 2017). The closest relative to koa is *Acacia heterophylla*, an endemic species to Réunion Island located east of Madagascar in the Indian Ocean (St. John, 1979; Le Roux et al., 2014).

The koa tree is ecologically, culturally, and economically important to Hawai’i. Ecologically, koa is a nitrogen-fixing legume that enhances soil fertilities of forests. In addition, Hawai‘ian fauna and flora such as the endangered Hawai‘ian honeycreepers, ‘akiapōlā‘au (*Hemignathus munroi*), and ‘ākepa (*Loxops coccinea*) as well as epiphytes *Korthalsella platyphylla* and lichens, prefer habitat on koa trees (Sakai, 1988; Whitesell, 1990; Elevitch et al., 2006; Baker et al., 2009). Culturally, Hawai‘ian ancestors used koa to produce spears for fishing as well as building tools, canoes, and houses. Koa is also

traditionally associated with Hawai‘ian royalty. Economically, koa timber and wood products provide a gross value of $20-30 million annually (Yanagida et al., 2004; Baker et al., 2009). Koa timber is highly priced, up to $125 per board foot at market value (Baker et al., 2009). Items made from koa wood include elegant furniture, musical instruments, ornamental pieces, and jewelry (Abbott, 1992; Krauss, 1993; Elevitch et al., 2006; Baker et al., 2009).

Environmental differences within and among the Hawai‘ian Islands such as altitude, rainfall, winds, etc. can affect the morphological characteristics of koa. On the leeward side of each island at elevations below 1000 m, the common relative of koa known as *Acacia koaia* Hillebr. (koai‘a) can be found flourishing. Koai‘a is a small, bushy, and hardy leguminous tree that normally resides in dry, windy, and open conditions. Unlike koa, the koai‘a wood is harder and more gnarled (Ishihara et al., 2017; Baker et al., 2009). These traits are characteristics of other species that live in dry habitats showing small leaves and dense woods whereas large leaves and soft woods are consistent with those species in wet habitats.

Koa is predominantly an outcrossing tree whose populations show a wide genetic diversity (Ishihara et al., 2017). Koa is a tetraploid species ($2n = 4x = 52$) that is highly heterozygous and heterogeneous due to its cross-pollinating nature (Atchison, 1948; Carr, 1978; Conkle, 1996; Shi 2003; Hipkins, 2004). Therefore, assessing the genetics of koa populations prove to be difficult. Phenotypic variation among traits such as tree form, canopy structure, height, phyllode development, pods, and seed arrangement appear to be highly heritable (Sun, 1996; Brewbaker, 1997; Sun et al., 1997; Daehler et al., 1999). In koa improvement programs, half-sib selection is often used as a method of breeding (Daehler et al., 1999). Half-sib progeny of koa, obtained by growing seeds from one mother tree, constitute a family. In the present study, seedlings of koa families were shown to vary in foliage color such as green to red hues of leaflet and stem.

The timber-wood quality of koa has no current screening method for superior wood quality. The quality includes traits such as texture, hardiness, and color. For example,

the color can range from light blond to dark red (Fig. 1). Grain figure patterns are identified as straight-grained wood, and wavy or curly grain (Dudley and Yamasaki, 2000). Koa wood quality research is needed to produce “elite trees” for agroforestry and koa wood industry. Koa seedling selection is a key to furthering the wood quality that requires a set of breeding objectives. For some eucalyptus and pine species of woody plants, there is a development of economic breeding objectives for forestry enterprises (Borralho et al., 1993; Ivkovic et al., 2006). In *Pinus taeda* (loblolly pine tree), the wood quality is assessed by stem straightness, wood specific gravity, and microfibril angle (Bridgwater et al., 2005). Currently, industrial and private landowners are compensated for their timber primarily based on the weight or volume of the green wood. Selection is economically focused on adaptability and volume growth that result in decreased wood quality over time. Loblolly pine is the focus for The Western Gulf Forest Tree Improvement Program (WGFTIP), which is set in place to identify breeding objectives for improved wood quality.

Elite wood quality populations are being developed based on the goal of improvement of wood quality for some pine and eucalyptus woody trees (McKeand & Bridgwater, 1998; Bridgwater et al., 2005; Resende & de Assis, 2008; Resende et al., 2012). As a technological tool for developing elite wood quality populations, molecular marker selection and breeding have been associated for temperate hardwood tree improvements (see review by Pijut et al., 2007). The use of molecular markers aids in developing knowledge of the genetic quality and population structure for natural forests and plantations, and the quantitative genes of superior trees. To produce elite or genetically improved genotypes, *in vitro* and clonal vegetative propagation methods are important for temperate hardwood species providing improved planting stock for use in progeny testing and for production forestry (Pijut et al., 2007). Genomic selection for hardwood forest tree improvement is a recent topic thoroughly studied in eucalyptus. It has shown to be a more powerful approach to applied breeding selection than the conventional phenotypic selection for growth and wood quality traits (Wong & Bernardo, 2008; Jannink et al., 2010; Grattapaglia & Resende, 2011; Resende et al., 2012). The use of biochemical markers or biomarkers to predict wood quality and color in koa has

not been done. Secondary metabolites in woody trees can serve as a biochemical marker for various functions.

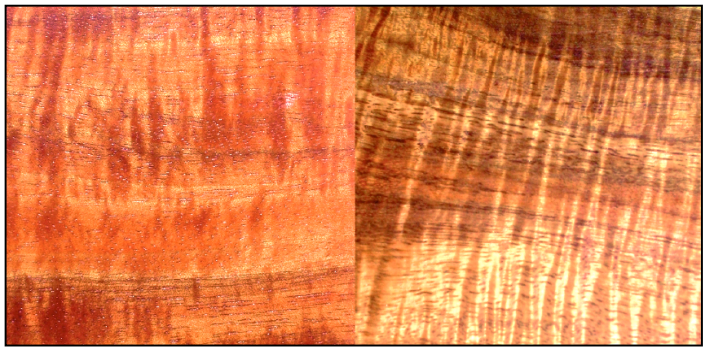


Fig. 1 Koa timber and wood products range in wood color. The wood color ranges from blond to dark red while the grain figure ranges from plain to curly.

Proanthocyanidins are a subcategory of plant tannins that are a secondary metabolite from the phenylpropanoid pathway found to be involved in many biological functions including wood quality (Hagerman and Butler, 1981; Xie and Dixon, 2005; Zhao et al., 2010; Barnett and Jeronimidis, 2009; Saito et al., 2013). Sufficient research of tannins in wood quality especially proanthocyanidins has yet to be completed to expose pigmentation of heartwood in woody trees. In doing so, this is economically important for manufacturing more desirable, elegant furniture or ornamental decoration. Wood Chemistry by Sjostrom (2013) describes the phenolic extracts found in woody trees such as chestnut wood or Eucalyptus as the natural color of the wood. Hydrolysable tannins are not usually found in wood, therefore proanthocyanidins of koa are our primary focus (Sjostrom, 2013). In a study of 16 woody species of softwood and heartwood, woody trees with higher tannin content generally have a deeper degree of staining when treated with ferric chloride or iron-ion solution (Hon and Shiraishi, 2000). This suggests that tannins, more specifically proanthocyanidins that are known to react with iron ion molecules, are responsible for coloration change in wood.

The long-term goals of improving koa are: (1) wood quality, (2) disease resistance, and (3) desirable tree structure. The first two long-term goals are regulated by phytochemicals governed by the genetics of koa. It is found that phenolic compounds such as anthocyanins, flavonols, and condensed tannins (proanthocyanidins) are

involved in both plant disease resistance and wood quality (Treutter 2006; He et al., 2008; Barnett and Jeronimidis, 2009; Saito et al., 2013).

Overall aims of research:

The aims of this study were to identify the role of proanthocyanidins in *Acacia koa* as a potential biomarker for wood quality and color. Currently, there is no screening method for superior wood quality in koa improvement programs.

Hypothesis:

It is hypothesized that the color difference of seedlings among koa families may be due to differential production of PAs.

Specific objectives:

1) Identifying and isolating PA biosynthesis genes
2) Detect and quantify tannins and PAs in koa
3) Determine PA biosynthesis gene expression
4) Confirming the presence and type of proanthocyanidin in koa

CHAPTER 2

LITERATURE REVIEW

Proanthocyanidin biosynthesis and its role in human, animal, and plant health

Condensed tannins or proanthocyanidins (PAs) are a class of polyphenols or oligomeric flavonoids found in plants. PAs are end-oligomers of the flavonoid and phenylpropanoid pathways (Haslam 1966; Xie and Dixon, 2005; Saito et al., 2013). These oligomers are a subclass of tannins, which are known as tanning agents for animal skins to produce leather as well as the source of astringent taste in wine, beer, and juices (Rosenheim 1920; Foo and Porter, 1981; Deshpande et al., 1986; Covington 2009; Garcia-Ruiz et al., 2009; Romer et al., 2011). Pioneer studies of plant tannin chemistry characterized various tannin structures, including leucoanthocyanidins and PAs in fruits such as varieties of apples and berries as well as in shrubby trees (Haslam 1977; Thompson 1972; Foo and Porter, 1981). This review concentrates on the contributions of PAs to biological functions in human health, animal nutrition, and most importantly, in plants. This literature review will also highlight the biosynthesis of PAs in woody plants. In the future, these studies may aid in the improvement of *Acacia koa* wood quality and disease resistance.

2.1 PA biochemistry

The classification of tannins includes four chemically distinct groups including gallotannins, ellagitannins, complex tannins, and **condensed tannins** (or **PAs**) (Fig. 2) (Freudenberg 1919; Chung et al., 1998; Khanbabaee and van Ree, 2001). The four types of tannins can also be classified as hydrolysable and non-hydrolysable (Khanbabaee and van Ree, 2001). Hydrolysable tannins include ellagitannins and gallotannins; they can be fractionated hydrolytically by hot water or tannases into their precursor(s). Non-hydrolysable tannins include condensed tannins (or PAs) and complex tannins. There has been much research and review of literature regarding the building blocks of PAs (Bate-Smith and Metcalfe 1957; Haslam 1977). PAs are subdivided by the types of flavan-3-ol units that polymerize/oligomerize. B-type PAs have a single interflavan linkage between C4 and C8, whereas A-type PAs have an

additional interflavan linkage between C2 and O7 (Fig. 3; Foo and Porter, 1981; Gu et al., 2003). PA monomers, epicatechins and catechins with two (3'4') B-ring hydroxyl groups, are homo-oligomeric and called procyanidins. Mixed PA oligomers, including epiafzelechin with one unit containing a 4'-OH and epigallocatechin with the 3'4'5'-tri-hydroxy pattern, are called propelargonidins and prodelphinidins, respectively (Fig. 3; Xie and Dixon, 2005).

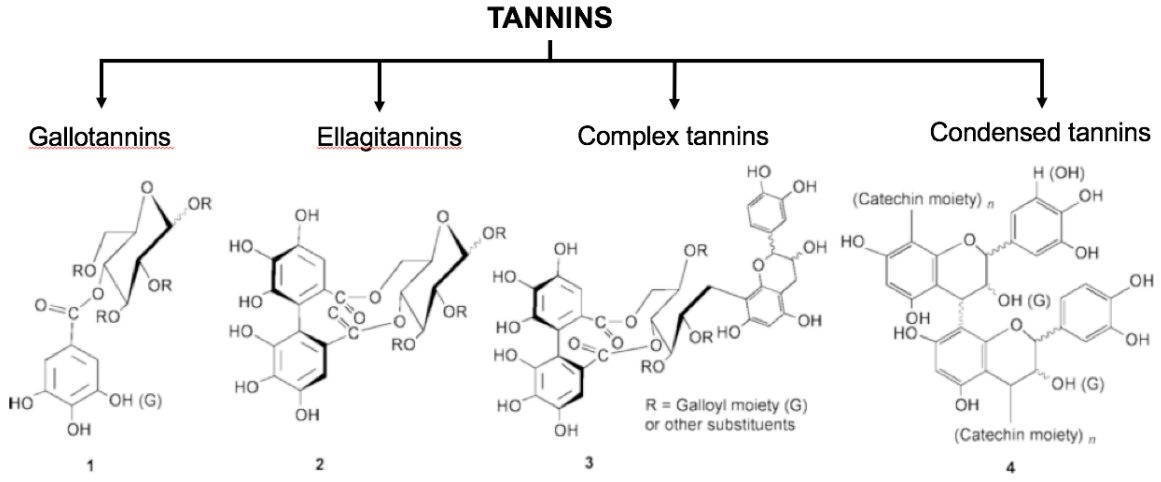


Fig. 2 Subcategories of tannins are based on structural diversity.

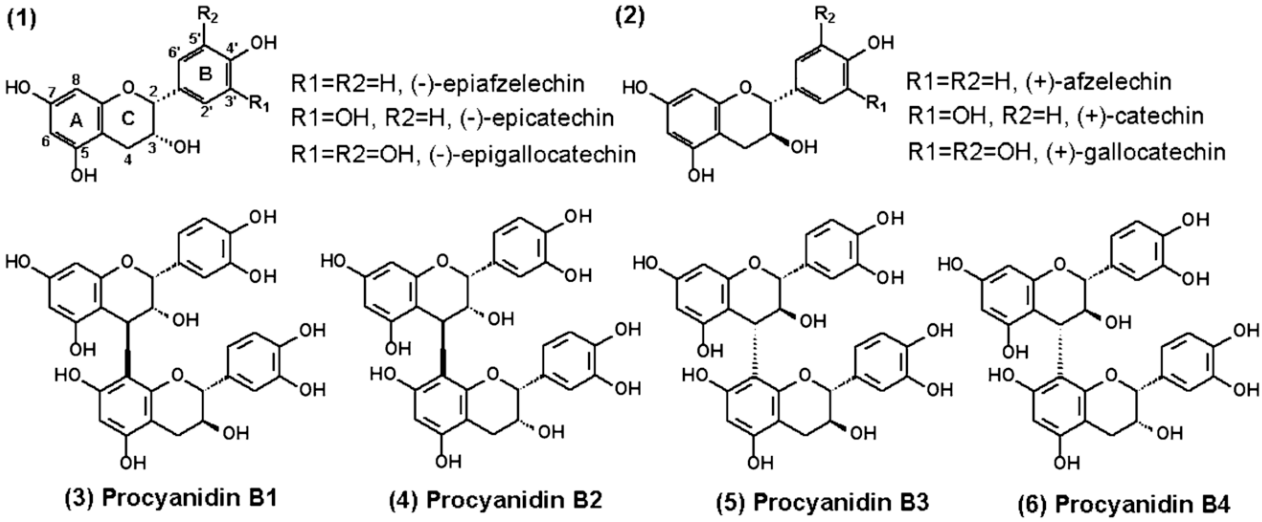


Fig. 3 PA oligomers are classified according to their combination of extension units. Above are different types of PAs based on the extension units polymerized. Examples include polymerized epicatechins called procyanidins or combinations of epiafzelechin and/or epigallocatechin to form propelargonidins and prodelphinidins, respectively.

Stereochemistry and structure of PAs can vary widely among different plant groups such as dicots and monocots, and sometimes even among closely related plant species (Ayres et al., 1997; Xie and Dixon, 2005). It was determined that the types of procyanidins are characteristic of specific plant species (Haslam, 1977; Xie and Dixon, 2005). Haslam (1977) outlined foods with specific procyanidins; for example, procyanidin B1 found in grape, B2 in apple, B3 in strawberry, and B4 in blackberry.

2.2 PAs role in human health

Green tea, grapes, and cranberries containing various tannins are being reviewed for already claimed beneficial dietary effects (Xie and Dixon, 2005). A popular study on (-)-epicatechins in chocolate by Serafini et al. (2003) suggested that consumption of dark chocolate (but not milk chocolate) was potentially cardioprotective because of increases in blood plasma antioxidant potential associated with raised plasma epicatechin levels. In contrast, ingestion of milk with the dark chocolate prompted the milk proteins to complex with the polyphenols and reduced their bioavailability (Serafini et al., 2003). Another plant source for PAs is grape seed proanthocyanidin extract (GSPE), which is tested in many animal/cell model systems for effects on human health (Table 2 in Xie and Dixon, 2005; Bagchi et al., 2000; Caimari et al., 2013). Rats fed with GSPE showed improved cardiac recovery during reperfusion after experiencing a heart attack and was associated with the significant reduction in free radical levels (Pataki et al., 2002). Clinical trials with human subjects suggested that consumption of GSPE could significantly reduce oxidized low-density lipoprotein, which is a marker for cardiovascular disease and reduction of plasma lipid hydroperoxide levels during the postprandial phase. This is one possible reason for the benefits of drinking red wine during meals (Xie and Dixon, 2005).

Polyphenols, specifically PAs, are indicated to play a role as anti-carcinogens. Catechin derivatives, which are PA monomers found in green tea, have been found to protect mammalian cells against free radical-mediated oxidative stress and to cause apoptosis of cancer cells (Zhu et al., 1997; Muhktar and Ahmad et al., 2000; Spencer et al., 2001). In addition, PAs have potential as a chemoprevention method against

cancers (Ouédraogo et al., 2011). The definition of cancer chemoprevention is the administration of agents to prevent cancer induction, to inhibit or delay its progression, or to inhibit or reverse carcinogenesis at a premalignant stage (Kelloff, 1999; Patel et al., 2007). Cancers can occur due to excessive production of reactive oxygen species (ROS) or depletion in antioxidants of nutritional origin, which is also known as oxidative stress. This problem is found in pathologies related to anemia, asthma, arthritis, inflammation, neurodegeneration, Parkinsons, and Alzheimer's disease, ageing process, atherosclerosis, and cancer (Bermudez-Soto et al., 2007a,b; Iglesia et al., 2010). Therefore, antioxidants that scavenge a wide range of ROS and/or inhibit their formation are considered important for cancer prevention. PA-rich plant extracts were found to have phenomenal antioxidant activities in scavenging assays toward 1,1-diphenyl-2-picrylhydrazyl (DPPH), ABTS [2,2'-azino-bis (3- ethylbenzthiazoline-6-sulphonic acid)], hydroxyl and superoxide anion radicals. PA-rich plant extracts also showed ferrous ions chelating ability and of their capability to inhibit Fe (II)-induced lipid peroxidation. Further studies of PAs in cancer chemoprevention are necessary to validate current findings but these findings indicate a promising future for PAs as a therapeutic cancer agent.

PAs are not only are known for their anti-carcinogenic effects, but also for their role as an anti-inflammatory agent. A new study of PA characterization from Cat's Claw (*Uncaria tomentosa*) determined that the anti-inflammatory and anti-carcinogen properties may not only be due to its alkaloid content, but also the phenolic content with an emphasis on PAs (Navarro-Hoyos et al., 2017; Sandoval et al., 2002). Berries (*Vaccinium floribundum* and *Aristotelia chilensis*) in South Africa were evaluated for the ability of their phenolic extracts containing anthocyanins and PAs to reduce adipogenesis and lipid accumulation in 3T3-L1 adipocytes, *in vivo* (Schreckinger et al., 2010). The anti-inflammatory property of the phenolic extracts inhibited lipid accumulation by 4-10.8% at maturity. Tatsuno et al. (2012) studied the beneficial effects of PAs on skin by extracting PA oligomers from peanut skin (*Arachis hypogaea L.*, Fabacaeae). The goal was to determine whether the PA oligomers showed inhibitory activity on inflammatory cytokine production and melanin synthesis in cultured cell lines.

The suppressive activity of peanut skin extract (PSE) decreased melanogenesis (PSE, 200 µg/ml) and decreased inflammatory cytokine production (PSE, 100 µg/ml). The type of PA showed to have a differential effect; the dimeric or trimeric form had a much stronger effect than the PA monomers and tetramers. PA oligomers from peanut skin showed the potential to reduce dermatological conditions involving inflammation and melanogenesis (Tatsuno et al., 2012). A similar observation was shown of the anti-inflammatory effects of cocoa procyanidins by Bitzer et al. (2015). They studied effects of monomeric, oligomeric, and polymeric cocoa procyanidins on human colon cells for anti-inflammatory activity. Results showed the high-molecular-weight polymeric procyanidins to be most effective for preventing loss of gut barrier function and epithelial inflammation (Bitzer et al., 2015). These studies explain the role of PAs as an anti-inflammatory agent that are extracted from different plant sources and show an emphasis of antioxidants in human health.

A review by Salvado et al. (2015) summarized data of PA intake in human diet across U.S. and 14 European countries. Spain had the highest daily PA intake and lowest in the Netherlands. It is known that PAs are the most common form of polyphenols in the Western diet. It is also the second most abundant polyphenol following lignin (He et al., 2008). A closer look showed the PAs ingested by U.S. adults per day to be 95 mg that includes polymers (30%), monomers (22%), dimers (16%), trimers (4%), 4-6 mers (15%), and 7-10 mers (11%). These PAs are commonly found in foods such as legumes, fruits, vegetables, and beverages, such as wine and tea (Quesada et al., 2009; Terra et al., 2009). Another highlight of PAs role in human health, studied *in vitro*, is its effects on fat and body weight. The effects of GSPE when ingested with a standard diet or high-fat diet have been studied in rodent models. Results showed a significant decrease in body weight gain and also in white adipose tissue accumulation. Similarly, studies of rodent models eating a high-fat diet supplemented with cocoa flavonol extract or a flavonol fraction enriched with monomeric, oligomeric, or polymeric procyanidins prevented weight gain, increases in fat mass, impairment of glucose tolerance, and insulin resistance (Dorenkott et al.,

2014). Therefore, these studies indicate PAs are influential on reducing fat and body weight.

2.3 PAs role in animal nutrition and health

Herbivore-tannin interaction studies are vast to date and reviewed extensively. Research on this topic seems to argue different theories of the herbivore-tannin interactions. One past argument for the widespread occurrence and high concentrations of PAs in plant species is due to selective pressures by herbivores (Coley 1983, 1986; Herms and Mattson, 1992; Skogsmyr and Fagerstrom, 1992). High levels of PAs from many plants have antiherbivore activity due to its ability to complex with dietary proteins and digestive enzymes in the gut of ruminants causing reduced digestibility. In contrast, there has been research showing a nutritive value or beneficial effect of tannins from specific plants on herbivore gut. Mueller-Harvey (2006) list the beneficial effects of some tannins from fodder legumes, browse leaves, and fruits, including species of *Acacia*, *Dichrostachys*, *Dorycnium*, *Hedysarum*, *Leucaena*, *Lotus*, *Onobrychis*, *Populus*, *Rumex*, and *Salix*. These species provided tannins involved in better utilization of dietary protein, faster growth rate of liveweight or wool, higher yields of milk, increased fertility, and improved animal welfare and health through prevention of bloat and lower worm burdens.

Bloat in pasture ruminants are sometimes thought to occur by tannins found in animal feeds, but most research shows that it is rather beneficial, allowing prevention of bloat. The preventative mechanism of tannins against pasture bloat remains unclear, but PA concentrations as low as 1-5 g kg^{-1} from *Lotus* spp. and *Onobrychis* spp. “sainfoin” varieties are sufficient enough to prevent it (Li et al., 1996). It is also known that ruminants are producers of greenhouse gases such as ammonia and methane (Mueller-Harvey, 2006). It is well known that low-quality feeds account for 90% of the world’s methane production by ruminants. Recent research indicates that tannin-containing diets can reduce methane emissions from ruminants such as freshly fed *Lespedeza cuneata* (180 g PAs kg^{-1}) and *L. pedunculatus* that reduced emissions by

16-20% per unit intake. Whether lower methane losses also result in lower energy losses from ruminants is an important question still unanswered.

A suggested concentration of PAs for animal feed intake was < 50 g PAs kg^{-1} as beneficial (Barry and McNabb, 1999; Mueller-Harvey, 2006). These studies were mainly based on *Lotus* spp. that may not be applicable to other sources of feeds. In addition, it was shown that in tropical forage *L. cuneata* (Dums. Cours.) with 5-12 g PAs kg^{-1} had higher nutritive values than similar forages without PAs. Structural diversity of tannins is vast, which may account for the broad results seen in animal nutrition and health. Most plant species have unique tannin characteristics, specifically the types of tannins and degree of polymerization. Therefore, the case of whether tannins especially PAs are detrimental or beneficial to animal health is a difficult question to directly answer.

2.4 PAs role in plants

One of the first to review PA biochemistry, Haslam (1977) described anthocyanidin as a pigmentation in fruits and flowers for attraction to birds and insects. PAs in plants play a role in UV protection, microbial and insect pest defense, and disease resistance. Treutter (2006) lists evidence for flavonoids produced in response to UV radiation. Reuber et al. (1996) found epidermal flavonoids that absorb UV-radiation protect the internal tissues of leaves and stems. Enhancement of flavonols in Norway spruce (*Picea abies*) was shown in response to near-ambient UV-B irradiation when compared to close-to-zero treatments (Fishbach et al., 1999). In silver birch and grape leaves, UV-B irradiation also induced flavonol production (Kolb et al., 2001; Tegelberg et al., 2004). In a more recent study, two reindeer forage plants (*Epilobium angustifolium L*. and *Eriophorum russeolum*) was tested in response to UV-B radiation treatment increased production of hydrolyzable tannins (69%) and PAs (66%) in leaves (Martz et al., 2011). No UV effect was detected in content/composition of soluble phenolics in *Epilobium* leaf but significant UV effects were detected in *Eriophorum* leaves in a developmental-specific manner. Their results indicated PAs are species-specific and detectable only at certain developmental stages produced in response to UV radiation. Studies

highlighting not only flavonoids but also PAs against UV radiation need to be conducted to further validate the role of PAs in UV protection.

PAs are also shown to function in microbial- and insect-herbivore defense in plants. A pioneer study by Feeny (1970) on plant-herbivore interaction showed tannins in oak leaf reduced feeding by winter moth caterpillars. The oak leaf tannins, which increase during the summer, were shown to inhibit the growth of winter moth larvae, making leaves less suitable for insect growth due to reduction of nitrogen availability and perhaps influencing leaf palatability. Scalbert (1991) reviewed the literature regarding antimicrobial properties of tannins that lists a table of microorganisms susceptible to the toxicity of specific plant tannins. Some filamentous fungi were shown to be susceptible to PA toxicity such as *Botrytis cinerea*, *Colletotrichum graminicola*, and *Trichoderma viride*. The toxicity of these PAs was measured by reduction of *in vitro* growth of mycelium. Bacteria that are susceptible to PAs included *Pseudomonas maltophilla*, *Nitrosomonas*, and *Streptococcus mutans*. These bacteria were tested for susceptibility to PA toxicity by plate count methods, disk diffusion methods, and nephelometry.

Ayers et al. (1997) analyzed insect herbivore-tannin interactions from 16 different woody plant species. They showed high doses of PAs did not have strong anti-herbivore activity, even on insects that had never evolutionarily come into contact with said PAs. Their research suggested that selective pressures by herbivores specifically folivorous insects do not contribute to the synthesis of PAs in so many plant species. A review of phenolic metabolites in northern forest trees to pathogens by Witzell and Martin (2008) gave extensive insight into woody plant-herbivore interaction. Northern forest trees examined include pine, spruce, birch, poplar, and willow species (*Pinus*, *Picea*, *Betula*, *Populus*, and *Salix spp.*). Flavonoid and PA monomer, (+)-catechin were found in two spruce tree species at high concentration relative to enhanced resistance of both tree species (Brignolas et al., 1998; Bahnweg et al., 2000). Interestingly, clones of Norway spruce with different susceptibility to *Ceratocystis polonica*, showed varied (+)-catechin content dependent on provenance origin and altitude.

Disease resistance by PAs and other phytochemicals can be categorized into two groups, innate and induced. Innate or “preformed” compounds are synthesized during the normal development of the plant tissue, and “induced” compounds are formed in response to plant injury or infection. Barley mutants, deficient in “preformed” PAs, were susceptible to *Fusarium* (Skadhauge et al., 1997). Also, proanthocyanidins were “induced” to accompany wound periderm formation in *Eucalyptus globulus* in response to infection by *Cytonaema sp*. (Eyles et al., 2003). Plants exposed to long periods of abiotic stress, such as drought and elevated light, are shown to enhance secondary metabolite production to protect from oxidative damage (Sies 1993; Varela et al., 2016). Varela et al. (2016) showed elevated production and accumulation of flavonoids in two Patagonian native shrubs in response to drought stress, which may be used as an indicator of drought tolerance. Stressors such as mechanical wounding, elevated light, and pathogens were tested for possible effects on PA accumulation through regulation of transcription factor gene, *MYB134*, involved in PA biosynthesis in poplar trees (*Populus spp.*) (Mellway et al., 2009). Their results showed that the MYB factors worked to control PA synthesis in response to biotic and abiotic stresses.

2.5 PA biosynthesis in plants

The mechanism of PA biosynthesis in various plant species such as *Arabidopsis*, maize, and barley are well studied (Xie and Dixon, 2005; He et al., 2008). Mutants are available of these plants to identify key genes in the flavonoid pathway that lead to downstream synthesis of PAs. In *Arabidopsis*, maize, and barley there are mutants on genes such as chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3’-hydroxylase (F3’H), dihydroflavonol reductase (DFR), anthocyanidin synthase (ANS), and anthocyanidin reductase (ANR). A pathway for PA biosynthesis was proposed by Xie and Dixon (2005) based on studies in *Arabidopsis*, maize, and barley. Mellway et al. (2009) suggested a similar pathway of PA biosynthesis for poplar also (Fig. 4).

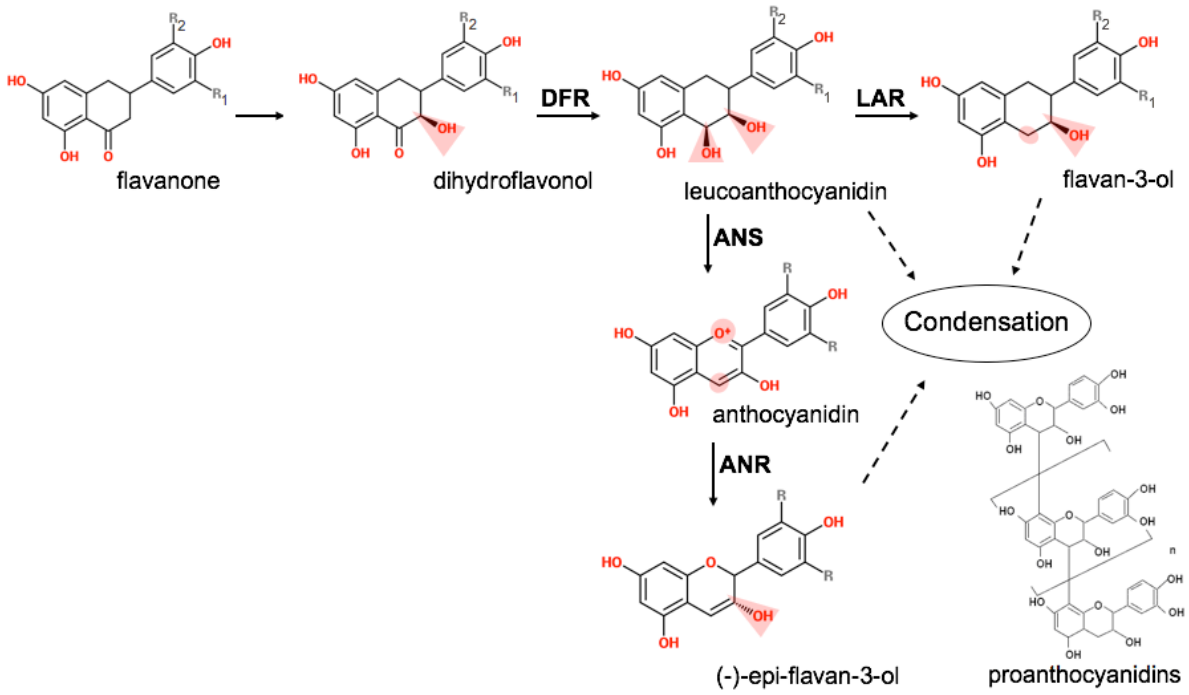


Fig. 4 A possible pathway for PA biosynthesis in koa. Based on other homologous species, four genes may be involved for biosynthesis of PAs in koa. These are dihydroflavonol reductase (DFR), anthocyanidin synthase (ANS), anthocyanidin reductase (ANR), and leucoanthocyanidin reductase (LAR). DFR converts dihydroflavonol to leucoanthocyanidin that will then be converted to PA monomer flavan-3-ol by LAR. In addition, leucoanthocyanidin may also be converted by ANS to anthocyanidin and further converted to another PA monomer (-)-epi-flavan-3-ol by ANR. Condensation of three precursors: flavan-3-ol, (-)-epi-flavan-3-ol, and leucoanthocyanidin will oligomerize to form PAs.

Research has determined the function of each enzyme leading to PA biosynthesis. DFR is known to catalyze the conversion of dihydrokaempferol into leucoanthocyanidins (Martens et al., 2002). Leucoanthocyanidins, an extension unit of PAs, were first investigated in plants circa 1920's by Robinson and Robinson (reviewed by Haslam 1977). Bate-Smith (1957) later found the presence of leucoanthocyanidins to be confined to plants with a woody habit of growth. DFR cloning and expression were highlighted in a study of molecular analysis of herbivore-induced PA synthesis in trembling aspen (*Populus tremuloides*) (Peters and Constabel, 2002). Their results indicated the significance of DFR in PA synthesis by mechanically wounding the plants as well as studying the feeding of forest tent caterpillar and satin moth. DFR was dramatically induced and increased in expression in response to the herbivores feeding on leaves. This increased expression translated to increased DFR enzyme activity that

upregulated concentrations of PA synthesis in the leaves. Conversion of these leucoanthocyanidins to catechins or flavan-3-ols (2R, 3S-flavan-3-ol) is catalyzed by LAR enzyme. Tanner et al. (2003) identified the LAR gene as a unique sequence to be involved in the conversion of leucoanthocyanidins to (+)-catechins. By cloning and monitoring the enzymatic LAR activity in *Desmodium uncinatum*, the study showed LAR enzyme was not a part of DFR activity. ANS, previously known as leucocyanidin dioxygenase, was shown to not only play a major role in first pivotal step of anthocyanin formation (red color seen in many flowering plants), but also in PA biosynthesis (Pelletier et al., 1997). The ANS enzyme converts leucoanthocyanidin into anthocyanidin by an oxidation-reduction reaction. Further downstream is the reaction of anthocyanidin converting to (-)-epi-flavan-3-ols or epicatechins (2R, 3R-flavan-3-ol) by the ANR enzyme. ANR was shown to be encoded by the gene *BANYULS* (BAN) in legume *Medicago truncatula* (Xie et al., 2003). Overexpression of *Medicago* BAN in tobacco led to accumulation of PAs throughout the pigmented portions of the petals, with concomitant reduction in anthocyanin levels. This suggested a role of ANR specific to PA biosynthesis that possibly reduces anthocyanin synthesis.

Both (+)-catechin and (-)-epicatechin are PA monomers that are well studied, but the mechanism of polymerization of these monomers has not been determined. He et al. (2008) explained the possible routes of PA precursors to convert into the stereospecific conformation that polymerize (Fig. 1.4 in He et al., 2008). PA biosynthesis is proposed to involve conversion from leucoanthocyanidins to quinone methides, which is a loss of the 4'-OH group, to form carbocations (nucleophilic site) at the C-4 position to polymerize with the C-8 of either (-)-epicatechin or (+)-catechin. Besides the characterization of PA polymerization, how the PAs are synthesized within a given plant cell has not been determined. There are some major studies to outline PA biosynthesis, but still the question of polymerization mechanism and where it occurs remains. A review by Koes et al. (2005) shows a colorful model for the regulation and evolution of flavonoid pathway leading into PA biosynthesis. From phenylalanine precursor in the cytosol of the plant cell, 4-coumaroyl-CoA is synthesized, chalcone and naringenin thereafter. Steps leading to formation of (+)-catechin and (-)-epicatechin by LAR and

ANR, respectively, occur in the cytosol. A transporter protein is available in the vacuolar membrane to allow for transport into the vacuole that will be stored as monomers or polymerized into PAs. Zhao et al. (2010) showed a more detailed orientation of PA biosynthesis in the plant cell, which suggests the PA biosynthesis proteins are attached to the cytosolic side of the endoplasmic reticulum. PA monomers are synthesized in the cytosolic area, which may have a few possible ways of being transported to the vacuole for further processing. The catechins/epicatechins formed are glycosylated and transported in membrane-bound vesicles that bud to fuse with the vacuole. Here, the precursors are stored as mentioned previously, or polymerized into oligomeric PAs that will be further shuttled to the plant cell wall. This mechanism may be stimulated as a plant's response to infection or environmental stress.

2.6 Methods of analyzing PAs

Paper chromatography was first applied to separating flavonoid pigments by Bate-Smith (1948) and then for the characterization of leucoanthocyanidins (Bate-Smith 1953). Identification of phenolic compounds commonly use UV-visible spectrophotometry, mass spectrometry (MS), and nuclear magnetic resonance (NMR). When high performance liquid chromatography was introduced in the 1980s it offered better resolution and quantitative analysis of numerous phenolics much faster that quickly replaced classical techniques. By these three applications, analysis of the structural diversity of PAs can be determined and more in-depth by coupling techniques such as liquid chromatography UV diode array detector (LC-UV-DAD) and LC-electrospray ionization-MS (LC-ESI-MS).

Acid butanol assay or acid hydrolysis is a well-referenced method proposed by Porter et al. (1986) as a depolymerization method of PAs to quantify PA amounts based on the anthocyanidin released. Spectrophotometry is used to visualize the colorimetric assay that calibrates the PAs present using cyanidin as a standard. It has been shown that this method commonly overestimates the PA content in a given extract specific to the source of which the cyanidin was purified from. This may be due to the fact that this method is not quantitative and dependent on inter-flavanoid linkages (4 → 8 *vs* 6 → 8

bonds; A- *vs* B-type tannins) and the presence or absence of 5'-OH groups (Ferreira and Bekker, 1996). So, this explains why *Quebracho* tannins which do not possess 5'-OH yield less color than *Lotus* tannins that possess 5'-OH groups when used as standards to calibrate the acid butanol assay and cause for miscalculation of PA amounts (Mueller-Harvey 2006).

CHAPTER 3

METHODS

3.1 Identification and isolation of PA biosynthesis genes and measuring gene expression

3.1.1 Koa families in present study

Acacia koa seeds were obtained from Hawai‘i Agriculture Research Center (HARC). Previously, it was observed that koa families from different islands expressed varied hues in overall appearance at the seedling stage. To test the varied hues, we used several families from Kaua‘i, O‘ahu, Maui, and Hawai‘i (Fig. 5). PA biosynthesis gene expression and quantification of total tannin and PA content were assessed among the koa families. Green-hued koa (Hawai‘i, Koala-27), slight red-hued koa (Kaua‘i, Lapa Exclosure), and red-hued koa (Maui, S-1) were compared at one month. To test a later stage of seedling development, green-hued koa (O‘ahu, MW-99 #37) and red-hued koa (Maui, MLPF-26 #20) were compared at three months. For HPLC and LC-ESI-MS analysis, green-hued seedlings from Hawai’i (Pii-138) at three months were sampled. Total tannin contents for the 3-yr-old koa tree were sampled at HARC.

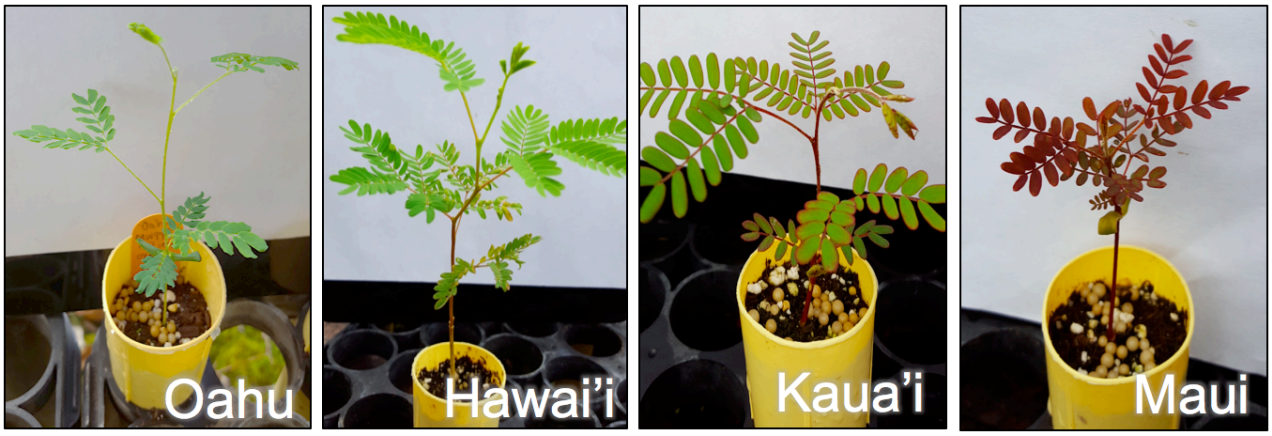


Fig. 5 Seedling hue varied from koa families on different islands. Using PA as a biomarker for wood color may be able to predict the color of the mature wood in advance as a screening method for superior wood quality. Koa families from Kaua’i, Maui, O‘ahu, and Hawai’i expressed variations of green to red hues at seedling stage. The red hue of koa seedling may indicate more PA whereas green hue may suggest less PA, therefore less color. Plastic container size is 3.8 cm x 18.4 cm (diameter x depth).

3.1.2 Growing koa seedlings

Growth of seedlings was completed at the Maunawili station of HARC. For germination, a widely used protocol was adapted from HARC. Seeds were soaked in a 10% bleach solution for 5-10 min. Scarification of seeds was done by clipping a small fragment of the seed coat at a position away from the embryo. Clipped seeds were soaked in water for 12 hours then transferred to a plastic tray containing 1:1 mixture of perlite (RedcoII, North Hollywood, CA) and vermiculite (Sta-Green Horticultural Vermiculite, St. Louis, MO). Prior to sowing seeds, the perlite and vermiculite mixture was moistened with a solution of 30 ml of ZeroTol™ (BioSafe Systems) in one gallon of water. Seeds were incubated for 2-3 days in the plastic tray with a cover to create a humidifying chamber for germination. A heating mat was supplied under the plastic tray at 85-90 ˚F during germination. Once the radicle formed from each seed (about size of cotyledon), these were transplanted into a commercial peat moss/perlite media (Sunshine® Mix 4, Aggregate Plus, Sungro Horticulture, Belleview,WA) in 115 ml plastic containers (RLC7, Stuewe and Sons, Tangent, OR).

3.1.3 RNA extraction and cDNA synthesis

At the point of collection, koa seedling tissue was immediately frozen using liquid nitrogen to prevent any mRNA degradation after removal from the soil-less mixture. A mortar and pestle, previously frozen to ≥ -80 ˚C, were used to grind frozen koa plant tissue. Ground, frozen tissue (above method) was used to extract total RNA following the Qiagen RNeasy Plant kit (Qiagen, Valencia, CA) with modification to the extraction method. Fifty mg aliquots of tissue powder were taken in 1.5 mL Eppendorf tubes. A lysis buffer was made using 100 µL of Fruit Mate™ (cat. no. 9192, Takara, Japan), 400 µL of Buffer RLT from RNeasy Plant (cat. no. 74904, Qiagen), and 5 µL of ß-mercaptoethanol (Ishihara et al., 2016). The 505 µL lysis buffer suspension was added to a 50 mg plant tissue powder which formed a cloudy white precipitate. The tube was vortexed vigorously to homogenize the extraction mixture. Further steps were followed according to the protocol of Qiagen RNeasy Plant kit except for the additional wash using 75% ethanol after the second wash with Buffer RPE. Purified total RNA was eluted with 30 µL of DNase/RNase-free water. Using the NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies, DE, USA) quality and quantity of total RNA were

determined at wavelengths of 230, 260, and 280 nm. The acceptable range for pure RNA was 1.8 – 2.0. This value is a ratio of the absorbance value at 260 and 280 nm for nucleic acid to protein and a ratio of A260/A230 for nucleic acid to residual chaotropic agent/phenol. Purified total RNA was treated for genomic DNA contamination using the Turbo DNA-free kit (Ambion, CA, USA). Synthesis of cDNA was completed using M-MLV Reverse Transcriptase (Promega, WI, USA) with an oligo(dT) primer and 500 ng of purified DNAse-free total RNA.

3.1.4 PCR and sequencing

cDNA synthesized above served as the template for PCR amplification of dihydryoflavonol reductase (DFR), anthocyanidin synthase (ANS), anthocyanidin reductase (ANR), and leucoanthocyanidin reductase (LAR). Two legumes *M. truncatula* and *Glycine max*, as well as a woody tree *Populus trichocarpa* were compared to the NCBI Transcriptome Shotgun Assembly (TSA) database to identify partial orthologs of koa cDNA for DFR, ANS, ANR, and LAR (Ishihara et al., 2015). Primers were designed using the conserved regions to optimize the complete coding sequence of DFR, ANS, ANR, and LAR in PCR. Each reaction sample was made up in a 20 µL volume that consisted of 0.5 µL (10 µM) of forward primer, 0.5 µL (10 µM) of reverse primer, 10 µL of Phire Plant Direct PCR Mastermix (Thermo Scientific cat. no. F-160, USA), 8 µL of DNase/RNase free water, and 1 µL of synthesized cDNA. The PCR reaction conditions were: initial denaturation step at 98 ˚C for 5 min, then 35 cycles of 98 ˚C for 5 sec, 54 ˚C for 5 sec, 72 ˚C for 1 min, and a final extension step of 72 ˚C for 5 min. PCR reaction was held at 4 ˚C before gel electrophoresis was done to visualize PCR product(s). DNA fragments were excised from the gel and purified using the QIAquick® Gel Extraction kit (cat. no. 28704, Qiagen) before being sent for sequencing. Sequencing was done at the Advanced Studies in Genomics, Proteomics, and Bioinformatics (ASGPB), University of Hawai‘i at Manoa, Honolulu, HI.

To analyze the resulting sequences to confirm transcriptome sequences for the genes of interest, contiguous coding sequences were generated by CAP3 Sequence Assembly Program (PRABI-Doua). Translation to protein was done using ExPasy

Translate bioinformatics web tool. Protein sequence alignment was compared on BlastP analysis of the homologous species to koa.

3.1.5 Gene Expression

Gene expression data was collected by quantitative real-time PCR (qRT-PCR) to assess the gene expression difference of PA biosynthesis genes between koa families from different islands of Hawai‘i. Reference genes: actin, tubulin, ubiquitin, 18SrRNA, 5.8SrRNA, and ef1- α (elongation factor 1-α) were used to normalize and calculate the fold change of each PA biosynthesis gene expression (Negi et al., 2011). *Ct* values for the six candidate reference genes were linearized for normalization. The Normfinder applet for Microsoft Excel was used to analyze the linearized reference gene expression data determining the lowest inter- and intra-variance value and stability value for each gene (Andersen et al., 2004).

Primers optimized for qRT-PCR were designed from the koa transcriptome sequences using NCBI Primer-Blast software. Parameters for primer design included maximum self-complementarity set to 4.00, maximum 3’ compatibility set to 2.00 and product size of 100 to 300 bp. Confirmation of primer specificity and amplification of desired genes was carried out by melting curve analysis and sequencing of the PCR products. Statistical significance was determined using GraphPad Prism® 7 software. A Student’s two-tailed t-test was performed with significant differences for $p<0.05$.

3.2 Quantification of total tannin and PA content

3.2.1 Sample Preparation

Chemical analysis of total tannin and PA content in tissues of koa was quantified by the Folin-Ciocalteau method and acid butanol assay. Immediately after collecting from HARC, tissues were dried in a drying oven (50-52 ˚C) for 24 hours. Dry matter was calculated by re-drying the dried material for 1 hour. Using 200 mg of dried tissue, phenolic extracts were diluted in 10 ml of 70% acetone. Various koa tissues including leaflet, phyllode, stem, root, and bark were analyzed.

3.2.2 Total tannin quantification

Total phenol in extracts were measured by the Folin-Ciocalteau reagent (Makkar 2000) (cat. no. F9252, Sigma Aldrich, USA). Thereafter, a simple phenol measurement was conducted by binding oligomeric tannin-phenolics in a given koa plant extract to the insoluble matrix polyvinylpolypyrrolidone (PVPP) (cat. no. P-6755, Sigma-Aldrich). Phenol content in koa extract was seen as colored hues of light to dark violet by the Folin-Ciocalteau reagent and best absorbed at 725 nm in spectrophotometry. A standard curve was generated using tannic acid (cat. no. 403040, Sigma-Aldrich) for reference. Total tannins were quantified on a tannic acid equivalent (TAE) basis in the sampled extract. Calculating the difference of total phenol to simple phenol represented as a TAE (mg) in 100 g of dry weight (DW).

Total tannin = (Total phenols – simple phenols)
(mg TAE in 100 g DW)

3.2.3 PA quantification

PA content in koa tissue extracts were quantified by the acid butanol or butanol-HCl assay (Makkar 2000). Koa tissue extracts produced above (0.5 ml), were prepared in a glass vial with 0.1 ml of ferric reagent (2% ferric-ammonia sulfate in 2N HCl) and 3 ml of butanol-HCl reagent (butanol-HCl 95:5 v/v). All acidic solutions were subjected to one hour of boiling in a water bath ≤ 97 ˚C. After cooling to room temperature, PAs in acidic solution were then analyzed in spectrophotometry at 550 nm. Calculation of PAs (% in dry matter) as a leucocyanidin equivalent (LCE) used the formula:

LCE = (A 550 nm x 78.26 x dilution factor) / (% in dry matter)

3.2.4 Aged plant material sampling

Total tannin assessment of aged-plant material was collected from a three-year-old tree at the HARC Maunawili station. Two types of aged material along a tree branch of the koa tree was sampled to compare (Fig. 6). Young newly formed plant material, leaflets and stems were sampled from the tip of the tree branch. To signify old established plant material, the same tissues were sampled from a point closest to the tree trunk. Aged root tissue was also examined for any difference of total tannin content by sampling surface lateral root as a young, newly-formed root compared to root sample collected from the taproot.

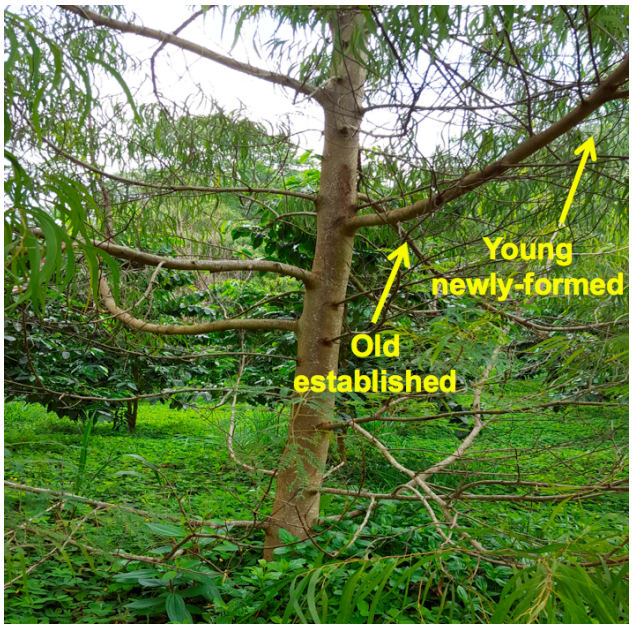


Fig. 6 Sampling from a koa tree at HARC. To begin characterizing total tannins and PAs in koa, we sampled from a tree at HARC which was about three years old. We compared leaflets and stems from the tip of a branch to signify young and newly-formed tissue. Thereafter, we also sampled dark green leaflets and stems from a position along the same branch closest to the tree trunk to signify old and established tissues.

3.3 Determination of the type of PA

3.3.2 Hydrolysis

HPLC standards (-)-epicatechin (cat. no. E4018) and procyanidin B2 (cat. no. 42157) were purchased from Sigma-Aldrich, USA. Sample preparation prior to HPLC analysis included acetone extraction, followed by removal of sugar residues for flavonol identification using acid hydrolysis. Koa seedling stem tissue was collected and immediately dried in a drying oven (50-52 ˚C) for 24 hours (Fig. 8). Koa stem tissue was extracted using 100 mg of dried plant powder in 1 ml of 70% acetone (aq.). The plant extract was vortexed, spun down, and sonicated at room temperature for 20 min. After centrifuging the plant extract at 4 ˚C for 10 min at ≤ 15,000 rpm, the supernatant was transferred to a new tube. The plant extracts were dried by rotary evaporation at 45 ˚C prior to hydrolysis.

To conduct acid hydrolysis of the plant extracts made above, the following methods were adapted from Hertog et al. (1992; Fig. 7). The plant extracts were hydrolyzed at 95 ˚C in 1.2 M HCl, 50% v/v aq. MeOH (Figs. 3.3 and 3.4). Standards including the monomer (-)-epicatechin and the oligomer isoform procyanidin B2 were each hydrolyzed to compare under similar conditions. Hydrolysis times were optimized to obtain the highest quantification and minimal degradation. Increments of 0 min, 5 min,

10 min, 15 min, and 20 min were tested. By comparing HPLC retention times of plant extract to standards, a hydrolysis time was chosen.

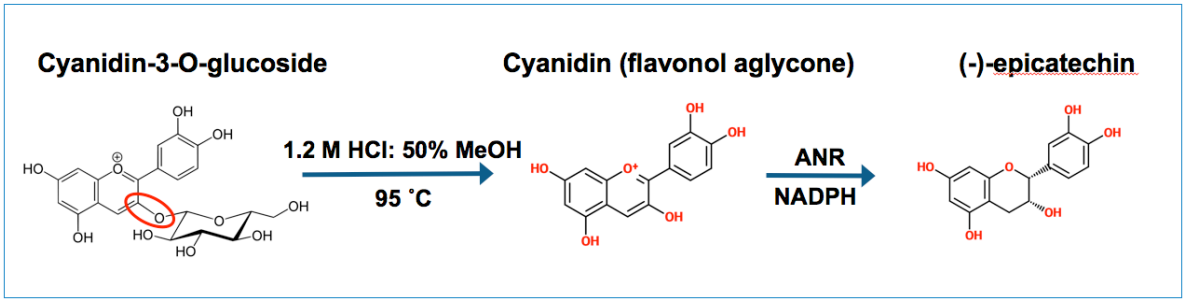


Fig. 7 De-glycosylation of sugars from flavonoid backbone. A schematic diagram above shows the PA precursor, cyanidin-3-O-glucoside, glycosylated at 3' position of the B-ring. Once the molecule undergoes acid hydrolysis using 1.2 M HCl with 50% MeOH (aq.) and heated for 95 ˚C, the cyanidin (flavonol aglycone) is exposed. In the plant, cyanidin is converted to (-)-epicatechin by ANR enzyme and NADPH as the donor group to form dimeric/oligomeric PAs.

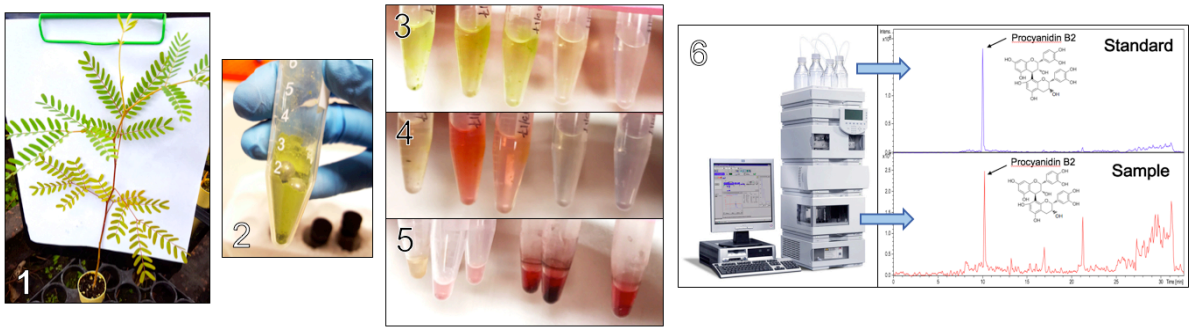


Fig. 8 Extraction and hydrolysis of koa stem extract in a flowchart. Steps include - 1. collection of koa seedling, 2. koa tissues freeze-dried, 3. extraction of 100 mg freeze-dried plant with 1 ml of 70% acetone (aq.), 4. rotary evaporation and addition of 1 ml of 1.2 M HCl, 50% v/v aq. MeOH, 5. incubation of plant extract at 95 ˚C, and 6. analysis of hydrolyzed plant extract and standard in HPLC.

3.3.3 HPLC analysis

High performance liquid chromatography (HPLC) was performed using a HPLC Waters 2695 (Waters Corp., Milford, MA, USA) with a PDA (photodiode array detector) Waters 996 (Waters Corp.) and autosampler (Waters Corp.). Instrumentation for HPLC required a Kinetex® 2.6 µm C-18 100Å column (100 x 4.6 mm). The mobile phase consisted of 2% acetic acid in water (A) and acetonitrile (B). The injection volume was 100 µL and flow rate was 0.7ml/min until 29 min at which the flow increased to 1.0 ml/min from 29-49 min. The following multi-step linear gradient was applied: 0 min, 5%

of B; 0-5 min, 5% of B; 5-27 min, 31.4% of B; 27-28 min, 100% of B; 28-29 min, 100% of B (flow change to 1.0 ml/min); and 29-49 min, 100% of B.

3.3.4 LC-ESI-MS analysis

To conduct liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS), acid hydrolyzed plant extract fractions were collected from HPLC to analyze in LC-MS ion trap (MS/MS). Instrumentation for LC-MS was Amazon Speed Nano-LC ion trap mass spectrometer (Bruker Daltonics Inc., Fremont, CA). Acid hydrolyzed plant fractions collected were dried by rotary evaporation at 30 ˚C prior to LC-MS. The dried plant fraction and standard procyanidin B2 fraction were re-suspended in 10 µl of hexafluoro-2-propanol, vortexed, spun down, and sonicated at room temperature for 5 min. A 1:10 dilution was made with HPLC-grade water to analyze the fraction in LC-ESI-MS. Instrumentation for LC-ESI-MS required a Nano-LC reverse phase C18 300 Å column (100 µm x 150 mm). The mobile phase consisted of acetonitrile in water, with 0.1% formic acid in a gradient program from 5-100% over 30 minutes.

Using LC-ESI-MS that confirmed the molecular mass was identical in a given koa stem extract, a fraction was collected at the same retention time as the procyanidin B2 standard in HPLC. Similarly, an HPLC fraction was collected from the hydrolyzed procyanidin B2 standard at the same retention time as the un-hydrolyzed standard. These peak collections were dried by rotary evaporation at 30 ˚C and made up using methods aforementioned.

3.3.4 Statistical analysis

The distribution of total tannin and PA content in one koa family expressing either green (O‘ahu, MW-99 #37) or red hue (Maui, MLPF-26 #20) was done in a randomized complete block design at Maunawili station of HARC. Two-way ANOVA and t-tests were conducted, statistical analyses reported at the appropriate results section, and completed using GraphPad Prism® 7 software.

CHAPTER 4

RESULTS

Characterization of PAs as a biomarker for wood quality in the native timber-wood tree *Acacia koa*

4.1 Identification and isolation of PA biosynthesis genes

The biosynthesis of proanthocyanidins (PAs) was proposed in koa according to legume species such as *G. max*, *M. truncatula*, and woody tree *Populus trichocarpa*. The complete coding sequences for DFR and ANS genes as well as the partial coding sequences for ANR and LAR genes in koa were identified from the transcriptome sequences in the NCBI Transcriptome Shotgun Assembly (Table 2).

Table 1 Transcriptome sequences identified for the four PA biosynthesis genes. PA biosynthesis genes including DFR, ANS, ANR, and LAR were identified from koa in NCBI Transcriptome Shotgun Assembly (TSA). Protein sequence alignment of two legume species *Medicago truncatula* and *Glycine max* as well as a woody species *Populus trichocarpa* are aligned to koa.

Gene	Transcriptome ID	Transcriptome length		Complete/partial coding sequence		Species	% Identity	GenBank/ Reference ID
		ORF	peptide	ORF	peptide			
DFR	GBYE01008771.1	1285 bp	346 aa	1038 bp	346 aa	*M. truncatula* *G. max* *P. trichocarpa*	46 % 34 % 75 %	AES79932.1 EU380189.1 XP_002300759.1
ANS	GBYE01004356.1	1454 bp	361 aa	1090 bp	361 aa	*M. truncatula* *G. max* *P. trichocarpa*	82 % 83 % 79 %	ABU40983.1 NP_001239794.1 XP_002304452.1
ANR	GBYE01006661.1	1290 bp	335 aa	788 bp	237 aa	*M. truncatula* *G. max* *P. trichocarpa*	75 % 76 % 76 %	AAN77735.1 AEM23932.1 XP_002305639.1
LAR	GBYE01018431.1	1482 bp	378 aa	1087 bp	307 aa	*M. truncatula* *G. max* *P. trichocarpa*	72 % 70 % 61 %	AES62081.1 AEM23933.1 XP_002305639.1

4.2 Gene expression

4.2.1 Old compared to young stem tissue from koa tree

To begin testing gene expression involved in PA biosynthesis, a comparison of old to young stem tissue was done from the same koa tree at Maunawili station which was used for total tannin and PA content analysis also. The newly formed young stem tissue was sampled at the tip of a tree branch whereas the established old stem tissue was

sampled at a point of the tree branch closest to the tree trunk. qRT-PCR analysis showed DFR transcription was 6-fold in old stem tissue compared to the young. ANS transcription was upregulated 3.6-fold, ANR upregulated 3-fold, and LAR had higher upregulation in the old compared to young tissue (Fig. 9).

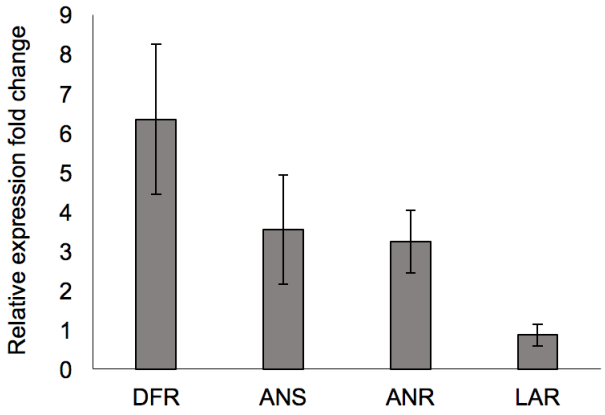
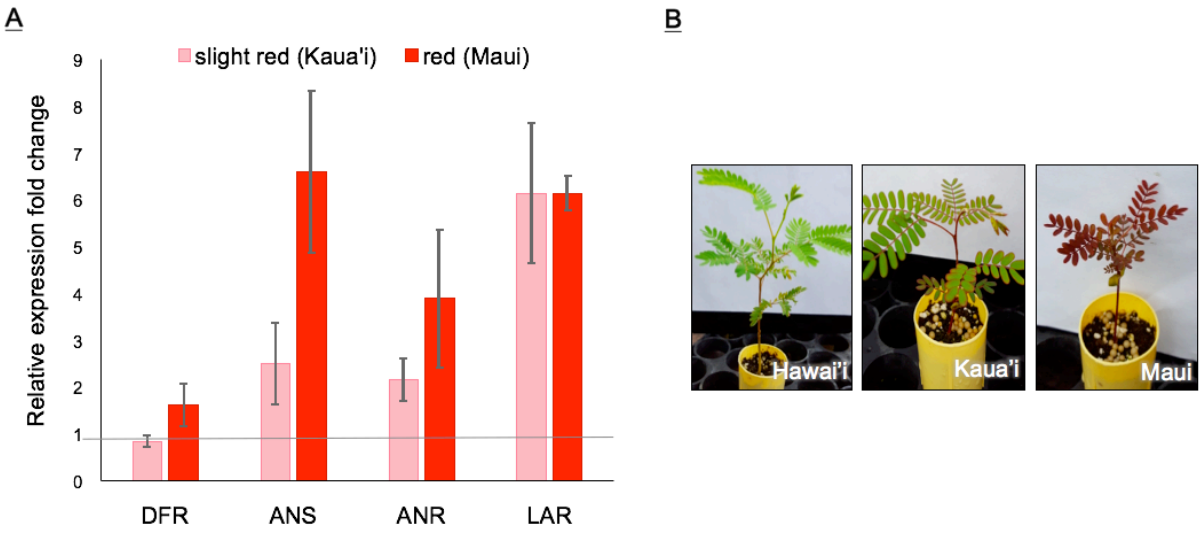


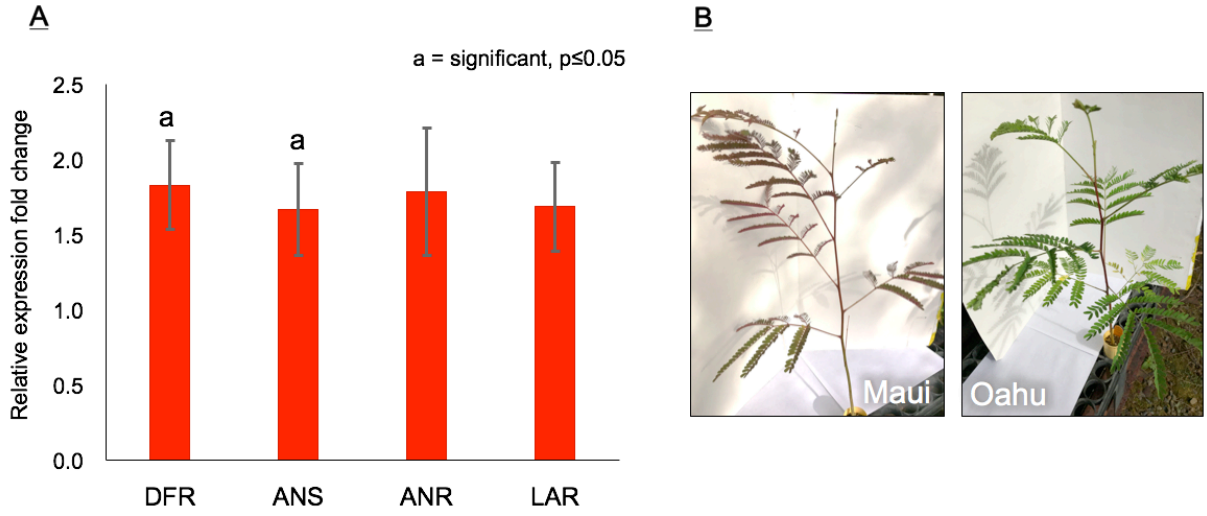
Fig. 9 PA biosynthesis gene expression of old compared to young stem tissue of a koa tree. The newly formed young stem tissue was sampled at the tip of a tree branch whereas the established old stem tissue was sampled at a point of the tree branch closest to the tree trunk. All four PA biosynthesis genes showed higher levels of transcription in the old compared to the young stem tissue. To normalize gene expression of old compared to young stem tissue, reference gene ubiquitin was used. Ubiquitin was selected based on the lowest inter- and intra-variance among the six reference genes tested (actin, tubulin, ubiquitin, ef1-α, 18SrRNA, and 5.8SrRNA). *Error bars* indicate ± SE (n=3), with no significant differences.

4.2.2 Red-hued compared to green-hued koa at one- and three-month-old

Green-hued koa (Hawai‘i, K-27) was used as a baseline for comparison with the slight red-hued koa (Kaua’i, LE) and the red-hued koa (Maui, S-1) (Fig. 10b). All four PA biosynthesis genes were significantly upregulated in the slight red-hued koa compared to the green-hued koa. ANS and ANR were upregulated ≥ 2- fold while LAR was upregulated 6-fold (Fig. 10a). Comparison of the red-hued koa to the green-hued koa showed an upregulation of all four PA biosynthesis genes as well. DFR was upregulated 2-fold, ANR upregulated 4-fold, LAR upregulated 6-fold, and highest upregulation was 7-fold in ANS (Fig. 10a). PA biosynthesis gene expression was further analyzed at a three-month seedling stage to compare red-hued koa to green-hued koa (Fig. 11b). Six seedlings of red-hued koa from Maui (MLPF-26 #20) and six seedlings of green-hued koa from O‘ahu (MW-99 #37) were tested. All four PA biosynthesis genes were upregulated almost 2-fold each (Fig. 11a).



Figs. 10a and 10b PA biosynthesis gene expression of slight red-hued and red-hued koa compared to green-hued koa seedlings at one month. Slight red-hued koa from Kaua'i (LE), and red-hued koa from Maui (S-1) were compared to green-hued koa from Hawai'i (K-27) at one month. Both slight red-hued and red-hued koa showed an upregulation of all four PA biosynthesis genes: DFR, ANS, ANR, and LAR compared to green-hued koa. A geomean of the reference genes actin and tubulin was used to normalize gene expression for slight red-hued koa compared to green-hued koa. A geomean of the reference genes ubiquitin and tubulin was used to normalize gene expression for red-hued koa compared to green-hued koa. The reference genes were selected as described for Fig. 9. *Error bars* indicate ± SE (n=3), with no significant differences.



Figs. 11a and 11b: PA biosynthesis gene expression in red-hued koa compared to green-hued koa at three months. Red-hued koa (Maui, MLPF-26 #20) was compared to green-hued koa (Oʻahu, MW-99 #37) at three months. Red-hued koa seedlings showed an upregulation of all four PA biosynthesis genes: DFR, ANS, ANR, and LAR compared to green-hued koa at three months also. A geomean of the reference genes actin and ubiquitin was used to normalize gene expression for red-hued koa compared to green-hued koa. *Error bars* indicate ± SE (n=6), with significant difference in DFR and ANS gene expression of red-hued koa compared to green-hued koa.

4.3 Quantification of total tannin and PA content in koa tissues

4.3.1 Koa tree at Maunawili station of HARC

From a mature koa tree at the Maunawili station of Hawai‘i Agricultural Research Center, various sections of the koa tree were sampled to determine total tannin content. True leaflet, phyllode, stem, and bark of the koa tree were analyzed for total tannin content. Total tannin contents were approximately 4 mg in bark, 5 mg in stem, 9 mg in leaf, and 13 mg in phyllode (mg TAE in 100 g DW) (Fig. 12).

In another experiment, young and old tissue samples of leaf, stem, and root of the same tree as above were assessed (Fig 13). For stem, the older tissues have a thicker diameter than the younger and located closer to the trunk. The young stem tissues have a thinner diameter and further away from the trunk. Similarly, young leaflets were sampled based on their texture; younger tissues are softer and lighter in color. The older root tissues are thicker and closer to the taproot while younger root tissues are thinner and located away from the taproot. Total tannin content in was highest in old established root at 24.4 mg TAE, while young tissues of leaf and stem were 12.7 mg TAE and 5.1 mg TAE in 100 g DW, respectively.

4.3.2 Seedlings at one month expressing green to red hues

Three koa families: Hawai’i (K-27), Kaua’i (LE), and Maui (S-1) expressed green to red hues that were compared for total tannin and PA contents at one month (Figs. 14a and 14b). Leaf and stem were analyzed among the families that showed similar amounts in total tannin and PA content. Green-hued koa (Hawai’i, K-27) had the highest total tannin and PA contents in the stem tissue, 0.38 mg TAE and 0.2 mg TAE in 100 g DW, respectively (Fig. 14a). Leaf tissue of red-hued koa (Maui, S-1) had the highest total tannin and PA content, 1 mg LCE and 0.33 mg LCE in 100 g DW, respectively (Fig. 15).

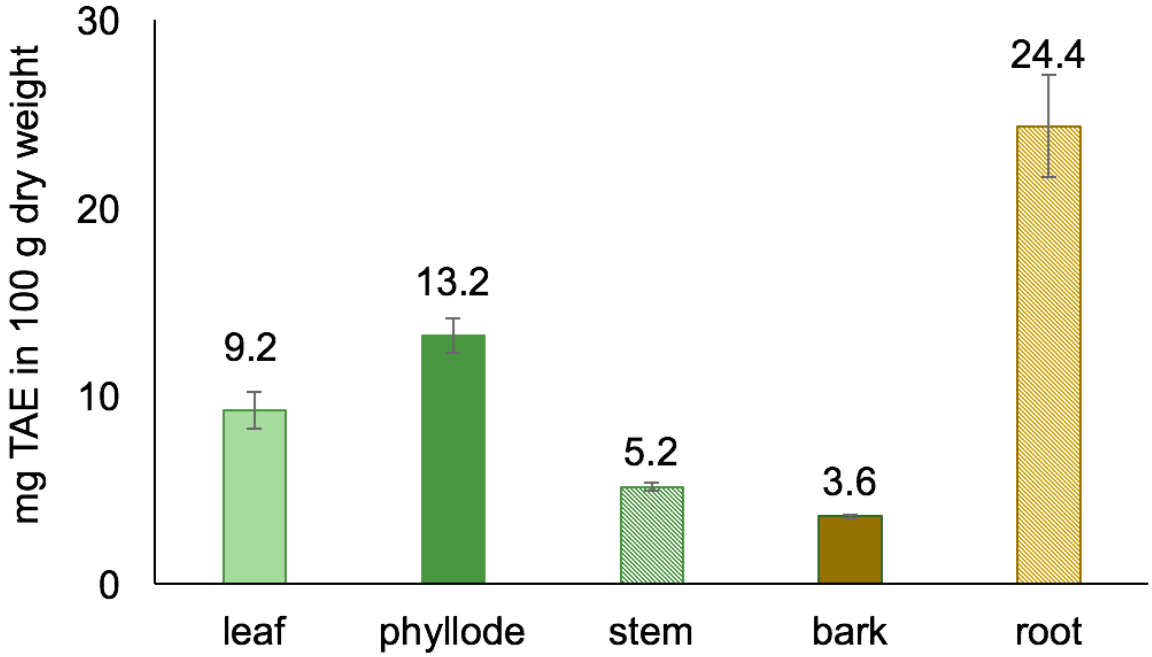


Fig. 12 Total tannin content in the koa tree at HARC. The highest concentration of total tannin was in the established root whereas the lowest concentration was in the bark. One-way ANOVA determined differences among tissue categories (n=3 for all except root, n=6, *P<0.0001*).

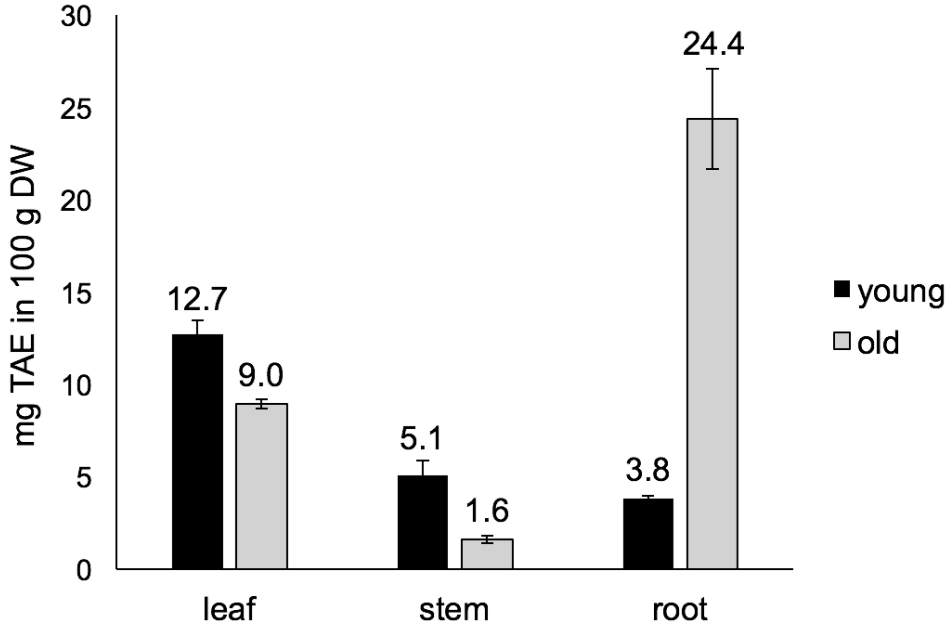


Fig. 13 Comparison of aged plant tissues of koa tree at HARC. Young photosynthetic tissues leaflet and stem showed higher total tannin content than old, whereas root tissues showed higher content in the old tissues and highest of all tissues analyzed.

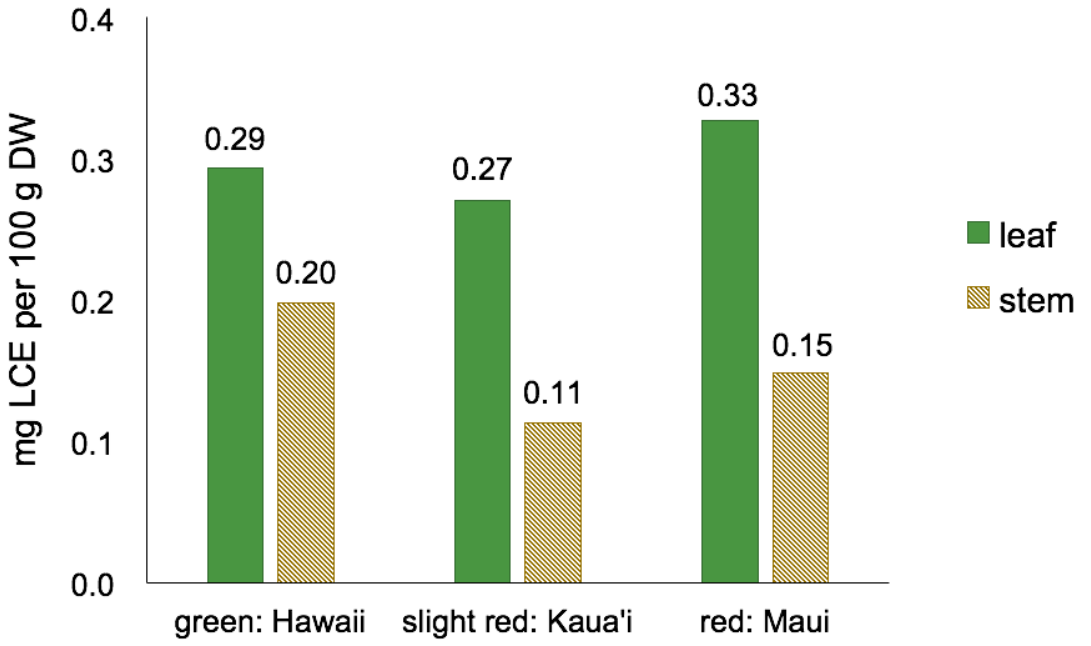


Fig. 14 Total tannin content at one month in tree foliage of koa families expressing green to red hues. Total tannin content was compared among red to green-hued koa seedlings from different islands. Red-hued koa from Maui (S-1) at one month had the highest total tannin content in leaf. The total tannin content among families was not significantly different (n=20).

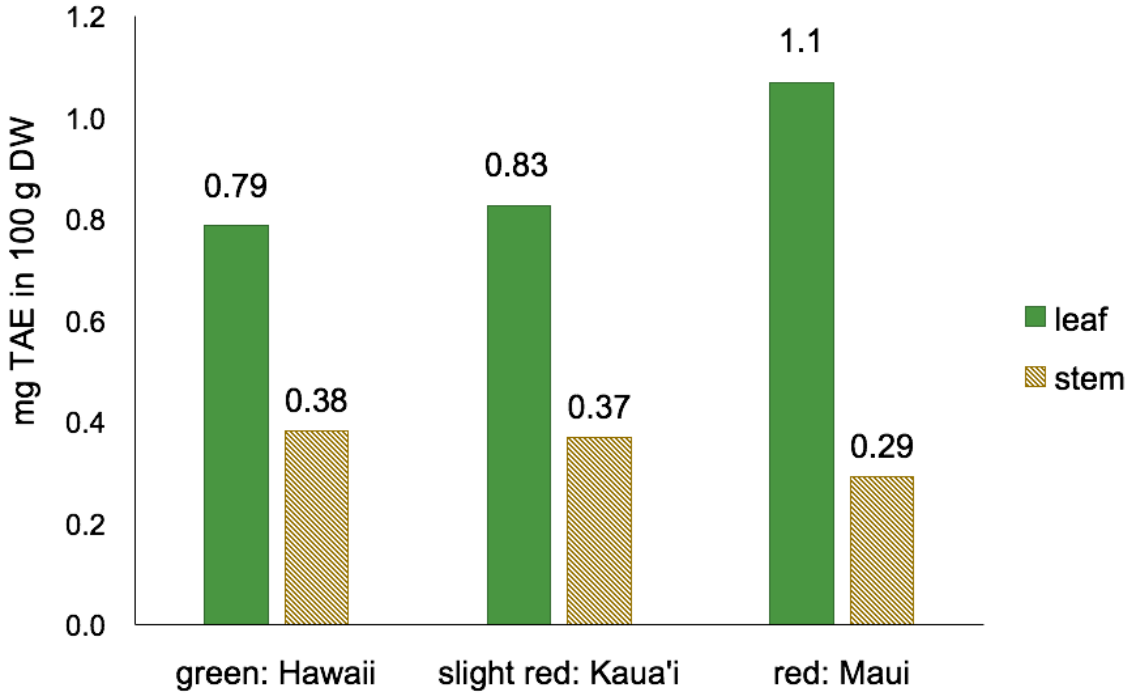


Fig. 15 PA content at one month in tree foliage of koa families expressing green to red hues. PA content was compared among red to green-hued koa seedlings from different islands. Red-hued koa from Maui (S-1) at one month had the highest PA content in leaf. The PA content among families were not significantly different (n=20).

4.3.3 Distribution of total tannin and PA content within a koa family

Given the heterozygous and heterogeneous nature of koa, the distribution of total tannin and PA contents were determined in two koa families expressing either a green hue (O‘ahu, MW-99 #37) or red hue (Maui, MLPF-26 #20) at three months. It was expected that seedlings from both families would show high variation among seedlings for total tannin and PA content. Quantification of total tannin and PA content was determined in the leaflet, stem, and root (Table 2). Box and whisker plot analysis of the total tannin content showed highest variation among the tissue types including leaflet, stem, and root (Fig. 16, Table 2). Highest within variation was seen in leaflet and stem of both red and green-hued koa, whereas lowest within variation was shown in the root. Another box and whisker plot analysis showed PA content among the red-hued koa and green-hued koa seedlings had significant variation among tissue types (Fig. 17). Aside from the PA content variation of tissue types, there was an opposing trend of lowest to highest PA content dependent on the hue expression of the family being analyzed. Red-hued koa seedlings had highest PA content in root, less in stem, and lowest in leaflet. On the other hand, green-hued koa seedlings had highest PA content in leaflet, less in stem, and lowest in root (Fig. 17, Table 2).

Table 2 Total tannin and PA content compared within a koa family of different tissue types

Hue (Family)	Tissue type	Total tannin		PA	
		Range	Average ± SEM	Range	Average ± SEM
Red – Maui (MLPF-26 #20)	leaf	2.3 – 4.8	3.7 ± 0.2	0.28 – 1.8	0.8 ± 0.1
	stem	0.33 – 1.5	0.86 ± 0.1	0.1 – 2.9	1.3 ± 0.2
	root	0.05 – 0.33	0.16 ± 0.02	0.36 – 4.4	2 ± 0.4
Green – Oahu (MW-99 #37)	leaf	2.6 – 7.8	5 ± 0.4	0.75 – 5	2.6 ± 0.3
	stem	0.4 – 1.8	0.75 ± 0.08	0.2 – 3.8	1.2 ± 0.2
	root	0.05 – 0.2	0.1 ± 0.01	0.4 – 2.3	1 ± 0.1

Values are represented as mg TAE in 100 g DW for total tannin content and mg LCE in 100 g DW for PA content. Population sampled indicates n=14 for red-hued koa (Maui, MLPF-26 #20) and n=18 for green-hued koa (O‘ahu, MW-99 #37).

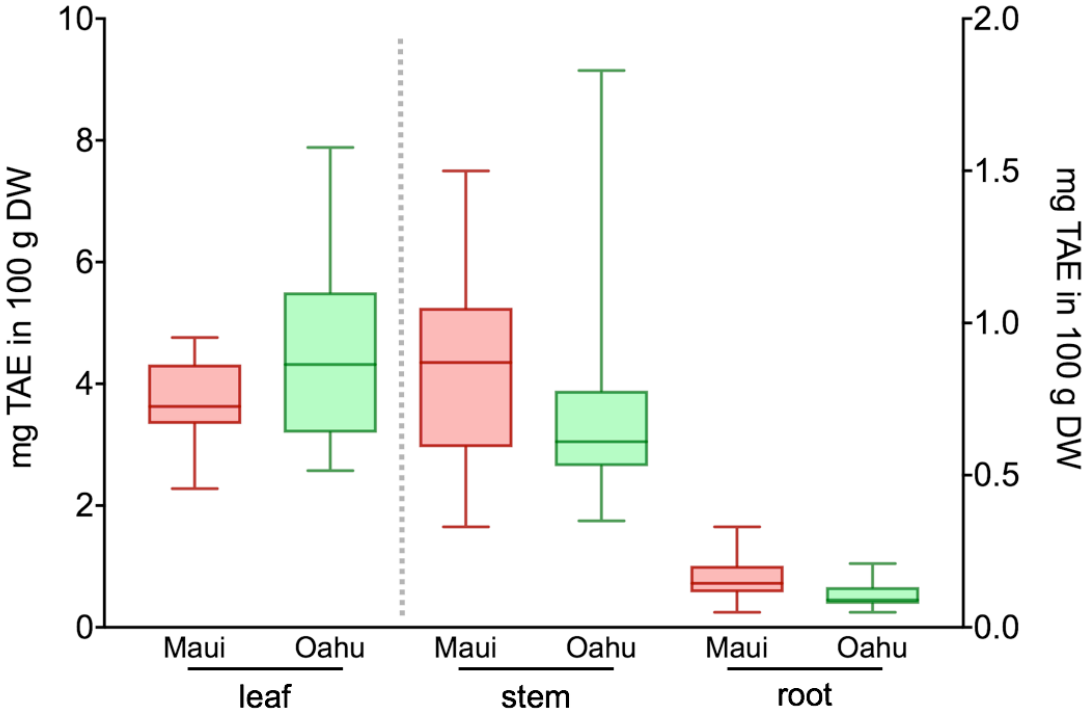


Fig. 16 Total tannin distribution among three-month-old green-hued and red-hued koa seedlings. Red-hued koa seedlings (Maui, MPLF-26 #20, n=14) and green-hued koa seedlings (Oʻahu, MW-99 #37, n=18) were analyzed for total tannin content (mg TAE in 100 g DW). The variation was most significant among tissue types of both red-hued and green-hued koa at 2 – 8 mg TAE in leaf, 0.3 – 2 mg TAE in stem, and root at 0.05 – 0.33 mg TAE in 100 g DW. The total tannin distribution was significantly different among tissue types and families (Two-way ANOVA, $P \leq 0.0005$).

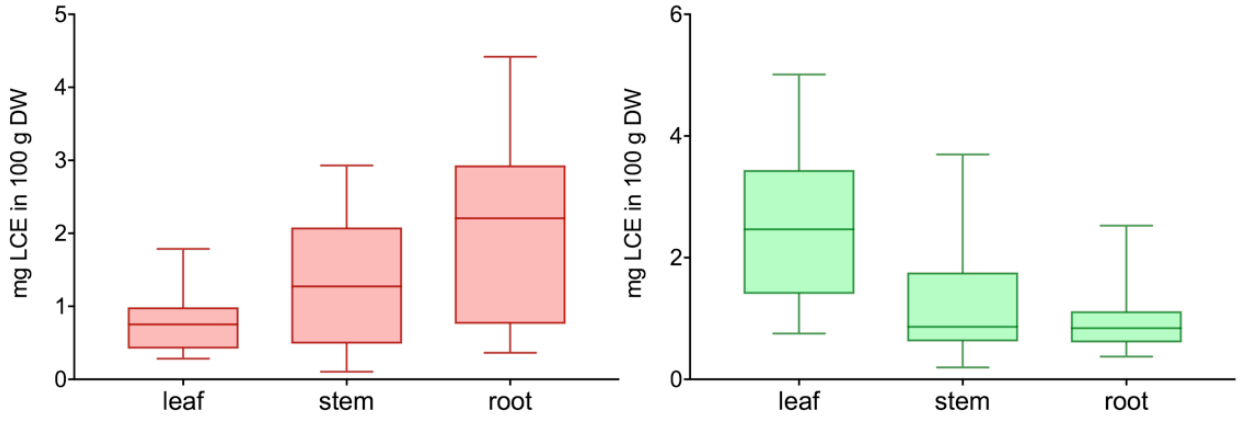


Fig. 17 PA distribution among three-month-old green-hued koa or red-hued koa seedlings. Red-hued koa seedlings (Maui, MPLF-26 #20, n=14) and green-hued koa seedlings (Oʻahu, MW-99 #37, n=18) were analyzed for PA content (mg LCE in 100 g dried plant). Significant variation among tissue types was observed in PA content similar to total tannin content. The PA content in tissue types of either green-hued or red-hued koa showed an opposing trend of highest to lowest PA content. Red-hued koa seedlings had PA content that ranged from 0.3 – 2 mg LCE in leaf, 0.1 – 3 mg LCE in stem, and 0.4 – 4 mg LCE in root. Green-hued koa seedlings had PA content that ranged from 0.4 – 2 mg LCE in root, 0.2 – 4 mg LCE in stem, and 0.8 – 5 mg LCE in leaf. The PA distribution was significant different among koa seedlings in a family (Two-way ANOVA, $P \leq 0.01$).

Two-way ANOVA showed the distribution of total tannin and PA content among koa seedlings in both red and green-hued koa families was analyzed (Table 3). The highest total variation for total tannin content was among tissue category (n=14 for red-hued, 18 for green-hued koa, $P<0.0001$). The highest total variation for PA content was among family catergory (n=14 for red-hued, 18 for green-hued koa, $P\leq0.0002$).

Table 3 Two-way ANOVA of total tannin and PA distribution in red- and green-hued koa seedlings at three months. ** signifies highest variation among factors tested.

Total tannin content		
Source of Variation	% of total variation	P value
Interaction	7.977	<0.0001
family	7.456	<0.0001
tissue type	** 89.58	<0.0001
PA content		
Source of Variation	% of total variation	P value
Interaction	12.9	0.9816
family	** 50.97	0.0002
tissue type	7.002	0.0161

4.4 LC-ESI-MS analysis of monomeric and oligomeric PA structures in koa

4.4.1 Characterization of PA monomer (-)-epicatechin

Proanthocyanidins are categorized by the types of extension units that oligomerize in different conformations (Gu et al. 2003; Xie and Dixon, 2005). In the bark of a woody pine species (*Pinus spp*.), procyanidin B2 was identified as the type of PA (de la Luz Cadiz-Guerra et al. 2014). Therefore, PA monomer (-)-epicatechin and PA dimer procyanidin B2 standards were compared with koa stem extracts. In HPLC analysis, the (-)-epicatechin monomer was identified, whereas procyanidin B2 dimer was not observed in the same koa stem extract (Fig. 18). Hydrolyzed extracts of koa exhibited a peak of low concentration at the same retention time as procyanidin B2, which was 12.1-12.2 min (Fig. 19). A peak for (-)-epicatechin was not identified in the hydrolyzed koa plant extract at a visible level. This may be due to the relatively higher limit of detection in HPLC analysis. Another possibility of not characterizing the monomer in koa plant extracts could be that only the oligomer form was present. Hydrolyzed standards and koa plant extracts were analyzed under the same HPLC gradient

program. Stem tissue of a green-hued koa seedling from Hawai'i (Pii-138) showed a significant peak at a similar retention time of 12.1 min as procyanidin B2 standard. Within the same plant sample, no peak was observed for monomer (-)-epicatechin.

4.4.2 LC-ESI-MS

In HPLC analysis of the koa plant stem extract (above) a significant peak was collected at the same retention time of un-hydrolyzed standard procyanidin B2. Similarly, an HPLC peak from the hydrolyzed procyanidin B2 standard was collected at the same retention time as the un-hydrolyzed standard. Using LC-ESI-MS, procyanidin B2 dimer of the hydrolyzed koa stem fraction and standard were analyzed. Both samples resulted with a significant peak at 10.1 min in LC. The peaks identified in plant and standard corresponded identically with a $[MH]^+$ at 579.3 and 579.4 Da, respectively (Figs. 20a and 20b, Table 4).

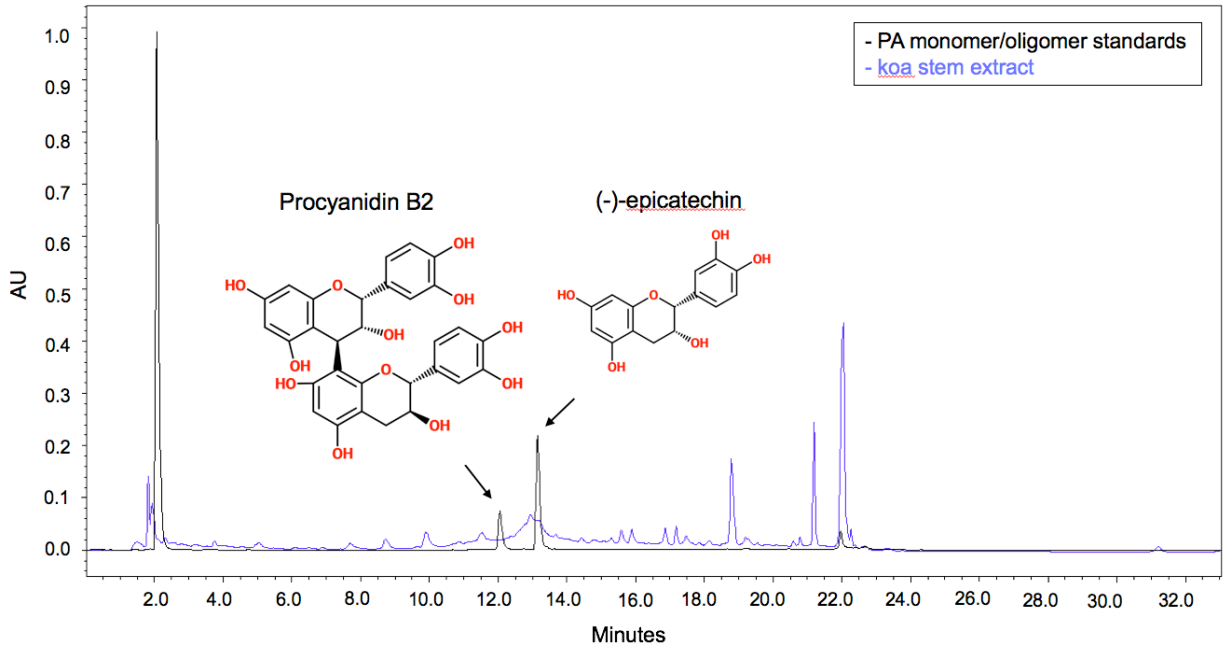


Fig. 18 Identification of (-)-epicatechin in koa. PA monomer (-)-epicatechin was identified in HPLC using a (-)-epicatechin standard (in black) to compare to the koa extract (in blue).

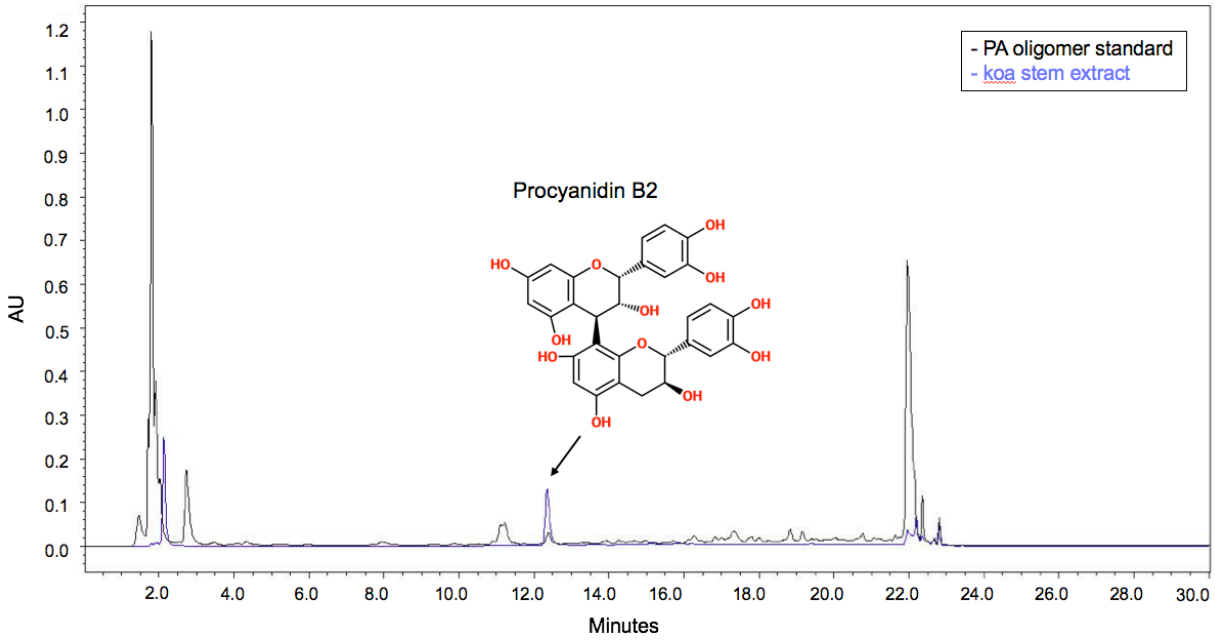


Fig. 19 HPLC of koa stem tissue after acid hydrolysis. Once koa stem tissue was exposed to acid hydrolysis, a deglycosylation of the flavonoids occurred. The parent flavonoid molecule was identified by a similar retention time in the stem extract to the standard of procyanidin B2.

Table 4 LC-ESI-MS data of procyanidin B2 identification in koa stem extract compared to standard

Proposed compound	Retention time (min)	Molecular Formula	molecular mass [M]	$[MH]^+$
Procyanidin B2	10.1	$C_{30}H_{26}O_{12}$	578.5 Da	579.4 Da

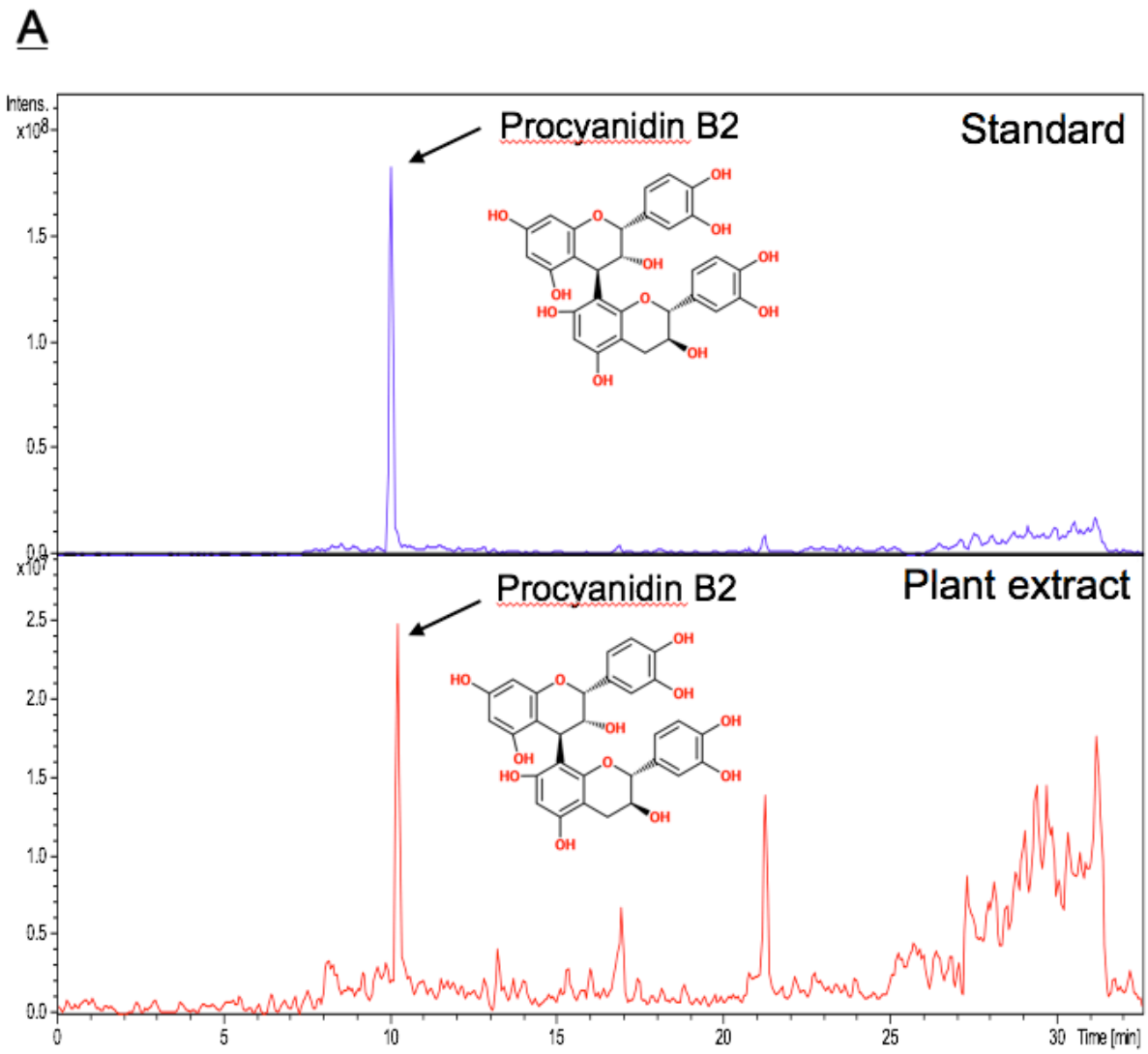


Fig. 20a LC-ESI-MS of procyanidin B2 in koa. A similar retention time was observed in the koa stem extract as the procyanidin B2 standard in LC.

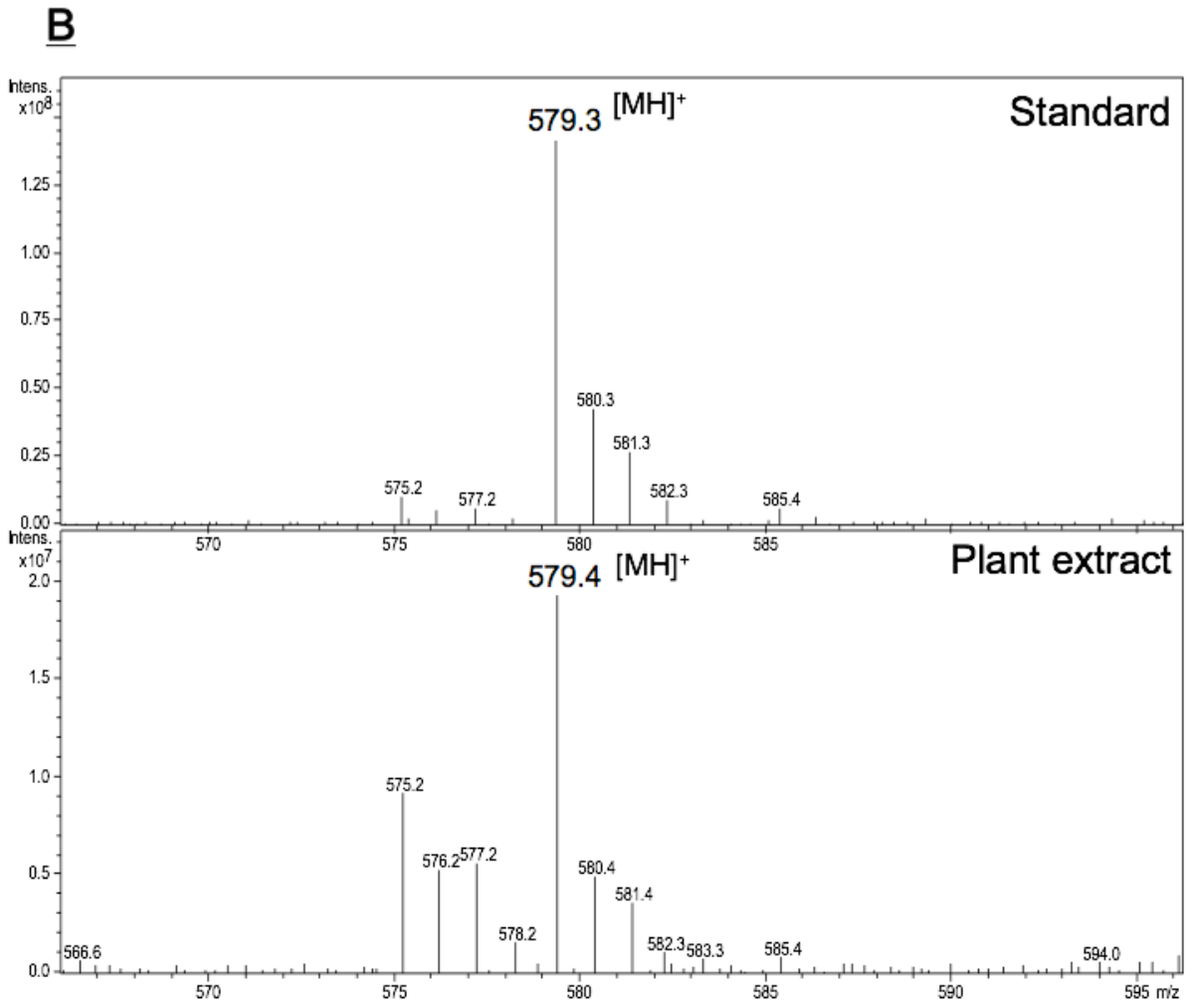


Fig. 20b LC-ESI-MS of procyanidin B2 in koa. The LC data in Fig 4.9a was confirmed by ESI-MS which showed identical monoisotopic masses $[MH]^+$ for the koa stem extract and standard at 579.4 and 579.3 Da, respectively. The fragmented products identified in the stem extract are shown identically to the standard. These products are indicative of being induced by the mass spectrometer and further show independent identities to confirm that procyanidin B2 is found in koa.

CHAPTER 5

DISCUSSION & CONCLUSION

The present study gives insight into the biosynthesis of PAs in koa. The PA biosynthesis genes DFR, ANS, ANR, and LAR were identified and isolated from a koa transcriptome database. These genes were identified on the basis of their homologies with PA biosynthesis genes of other species including *M. truncatula*, *G*. *max*, and *P. trichocarpa*. As previously shown in these species, PA biosynthesis involves DFR, ANS, ANR, and LAR as the leading steps to PA biosynthesis. In *A. thaliana* and *M. truncatula*, the steps of flavonoid and PA biosynthesis are well-defined, with ANR as the first committed step in PA biosynthesis (Xie et al., 2003). In leguminous *G. max*, it has been shown that the PA dimer procyanidin B2 is an intermediate in biosynthesis of oligomeric PAs (Kovinich et al., 2012). Poplar (*Populus spp.*) is a woody tree species, for which the PA biosynthesis pathway has been outlined. One of the first molecular studies of PA biosynthesis pathway in trees was conducted by Peters and Constabel (2002) in trembling aspen (*P. tremuloides*). They observed the effects of increased DFR levels on PA biosynthesis. Huang et al. (2012) observed that LAR overexpression increased PA levels in *P. trichocarpa*. Collectively, in several legume and woody tree species, the key steps of the flavonoid and PA biosynthesis pathway have been identified, although how the PAs oligomerize from PA monomers/dimers has not been established. In the present study, the four PA biosynthesis genes identified by PCR amplification and visualization by gel electrophoresis confirm the presence of the genes in koa. In addition, the PA biosynthesis gene expression analysis of koa seedlings at different ages indicated their involvement in the synthesis of PAs in koa.

Koa tissues of the tree located at HARC, including leaf, stem, phyllode, bark, and root, showed differential total tannin content. We could make inferences about the PA content of these tissues because PA content is generally positively correlated to total tannin content. Highest total tannin content was observed in established and woody root of the tree. In a study of tannin content in pines (*Pinus banksiana Lamb*), root tissue of pine was divided into three regions: youngest (most apical) as the “white zone”,

adjacent to this is the “condensed tannin zone” that appears tan or brown macroscopically, and adjacent to the condensed tannin zone (basipetal) is the “cork zone”. This was based on the distinctive internal structures, which would have consequences for ion and water uptake (McKenzie and Peterson, 1995a,b). Similarly, the PA content in the roots of a mature koa tree may also result in a difference of PA levels dependent on position of the root sampled with respect to the tree trunk. There could also be a distinction of three different zones in the root for koa trees. Sampling in the condensed tannin zone, if identified similarly to that in pine, strengthens PAs as a biomarker to identify the composition of the heartwood without compromising the health of the tree.

From the same koa tree at HARC, total tannin content was compared between distances along a branch to indicate aged material – young vs. old, closest to the tip of the branch vs. closest to the tree trunk. Among photosynthetic tissues, young leaf and young stem contained higher total tannin content than older leaf and stem tissues. In contrast, the old, established root of the koa tree had higher total tannin content compared to the young, newly-formed root. Gene expression analysis comparing old stem to young stem tissues from the koa tree, showed an upregulation of the PA biosynthesis genes in the older tissues. DFR, signifying the first step of PA biosynthesis, showed the highest increase in gene expression in the old stem tissue compared to the young, while LAR signifying the last step showed the least increase. This was contrary to our expectations; we expected higher gene expression in the young tissues compared to the old tissues. These anomalies of lack of correlation between total tannin content and gene expression can be explained as follows. Although, young stem tissues were shown to have higher total tannin content than the old stem, these tannins could have been other types aside from PAs such as ellagitannins and/or gallotannins. Another significant option is the upregulation of the PA biosynthesis genes in old stem may be important for other molecular pathways to maintain homeostasis of the woody tree. DFR is involved in the synthesis of anthocyanins that are important for pigmentation to attract pollinating animals (Koes et al., 2005). DFR is also known to synthesize flavan-4-ols, which will produce phlobaphenes that are complex products of

PAs. These phlobaphenes may play a role in plant disease resistance due to its presence in the cell wall. These results aided in the characterization of total tannin and PA amounts in a koa tree that gave rationale for studying PA synthesis and content in koa.

Seedlings of some koa families show reddish hue in the leaflet. The hue tends to slowly disappear as the seedling matures. Red hues are also often seen in the young shoots of some adult trees. A goal of this study was to correlate the expression (qRT-PCR analysis) of the PA biosynthesis genes with quantities of total tannin and PA contents among koa seedlings from different islands, which expressed variations of green to red hues. It was hypothesized that the red hue of certain koa families (Kaua'i and Maui) had more PA content, and therefore more color. Accordingly, the green hue of koa seedlings (Hawai‘i and O‘ahu) would indicate less PA content and therefore less color. It was of interest to determine whether there are correlations between red-hued koa and elevated PA biosynthesis gene expression and between green-hued koa and reduced PA biosynthesis gene expression.

The comparison of slight-red and red-hued koa to green-hued koa in one-month-old seedlings, showed upregulation of all four PA biosynthesis genes. This was further analyzed by conducting the same analysis in two different sets of koa families expressing red hue and green hue in three-month-old seedlings. The gene expression of the red-hued koa was also upregulated ~ 2-fold in comparison to the green-hued koa. This suggests that there is a positive relationship of PA biosynthesis gene expression with the PA amounts synthesized. Generally, PA contents were positively correlated with total tannin contents in the leaflets and stems of red-hued, slight red-hued, and green-hued koa seedlings. Leaflets of red-hued koa seedlings had the highest total tannin and PA contents, supporting the hypothesis that red-hue expression results in more tannin/PA amounts. This is consistent with the observation that the expression of the PA biosynthesis genes corresponded with the total tannin and PA contents in the red and green-hued koa seedlings.

Koa is a tetraploid species with an outcross-breeding system and therefore has a highly variable nature. The distribution of total tannin and PA contents in both red-hued and green-hued koa seedlings was determined to observe any variability within each koa family. In total tannin distribution, the highest variation was observed among tissue types rather than among seedlings of a koa family. A general observation showed that the three-month-old koa seedlings have more total tannin and PA contents than the one-month-old seedlings, showing a ten-fold increase from 0.2 - 1 mg TAE to 2 - 5 mg TAE in 100 g DW. Although, the two red-hued koa families tested for one- and three-month-old are different, the genotypes of the varieties may be the same due to similar island origins. This data suggests PA content accumulates in koa seedlings during development. Most literature for other woody tree species such as *Acacia spp.* and *Casuarina equisetfolia* quantified PA contents in ≥ 5-month-old seedlings at 50 – 350 mg g^{-1} DW (Hattas and Julkunen-Titto, 2012; Zhang et al., 2011). A recent study in 3- to 4-month-old silver birch (*Betula pendula*) seedlings quantified leaf PA content ranging from ≤ 20 - 80 mg g^{-1} DW whereas the stem PA content ranged from ≤ 10 - 20 mg g^{-1} DW (Kosonen et al., 2015). On the other hand, the shrubby plant creosotebush (*Larrea tridentata*) had the highest PA concentration in the photosynthetic tissues including leaves and green stems (Hyder et al., 2002). The green stem of creosotebush contained only ≤ 0.4 mg g^{-1} DW. The results in the present study using relatively young seedlings of koa are comparable to those described for creosotebush (Figs. 4.6a and 4.6b).

Koa wood research may benefit from further analysis of PA biosynthesis at the molecular level. A review by Zhao et al. (2010) defined the possible mechanisms of PA biosynthesis in the plant cell. One highlight is the synthesis of PA monomers catechin/epicatechin, which are compartmentalized into membrane vesicles, trafficked to the vacuole, stored and/or polymerized into PAs in the vacuole, which are then transferred to the plant cell wall. Brillouet et al. (2013) described “tannosomes”, which are chloroplast-derived organelles, as the site for PA polymerization. Their research used transmission electron microscopy to study ultrastructure and morphology of tannin accretions that are possibly derived from the chloroplasts. These studies have opened a

new avenue for plant phenolic research that may give further insight of PA biosynthesis and polymerization in the plant cell.

Given the present study is the first to characterize PAs in koa, it is essential to understand the features of the PA molecules as well as its composition. The results of HPLC and LC-ESI-MS analysis determined that koa has procyanidin B2 as the type of proanthocyanidin. After acid hydrolysis of the koa stem extracts, procyanidin B2 was identified in the koa stem extract by similar retention time (12.1-12.2 min) to the standard in HPLC analysis. These results show the importance of de-glycosylation prior to HPLC to isolate the parent flavonoid molecule. In LC-ESI-MS analysis, both fractions resulted at similar retention times (10.1 min). The observed molecular mass $[MH]^+$ for the procyanidin B2 sample was 579.4 Da whereas the observed molecular mass $[MH]^+$ for the standard was 579.3 Da. The calculated molecular mass for procyanidin B2 ($C_{30}H_{26}O_{12}$) is 578.5 Da, which is identical to the monoisotopic mass of procyanidin B2 in the koa stem extract and standard. Further studies are needed to validate the role of procyanidin B2 in koa that may suggest its involvement in wood quality.

The wood quality of koa in the study by Dudley and Yamasaki (2000) infers the general properties of the wood. Color and figure were highlighted while sampling koa trees to determine their wood properties. They sampled two koa trees at Keauhou Ranch on Hawai'i from two positions in each tree; a cross-section of heartwood and of branch. Both sections displayed the same color and figure of wood from each tree sampled. It was determined that branch sampling could be utilized for predicting wood properties, as it is a less invasive and accurate method. Making inferences of the color of heartwood based on the branch suggests the suitability of young wood for sampling. Seedlings also have some properties similar to growing branches. Therefore, it may be possible to predict the properties of the heartwood based on the biochemical properties of the seedlings. Thus, PA contents of the koa seedlings may serve as a biomarker for seedling selection for superior wood quality of a future mature tree.

PAs in other woody plants such as oak, poplar, and pine are shown to play a role in herbivore defense and microorganism disease resistance (Feeny, 1970; Witzell and Martin, 2008). Koa faces a fungal wilt infection caused by *Fusarium oxysporum* that leads to dieback symptoms in the mature tree. *Fusarium oxysporum* invades through the root tissue traversing up the xylem tissue where it produces tyloses or gum that will create a water blockage and drought stress in the tree (Baker et al., 2009; Ishihara et al., 2017). PAs were found to be increased in the seed coat of barley mutants to combat/prevent infection by *Fusarium spp.* (Skadhauge et al., 1997). Testing for PA content in koa seed coat may also give indication to disease-resistant koa seedlings depending on PA levels. Therefore, PAs may be used as a biomarker for disease resistance in future studies of koa.

CONCLUSION

The present study gives insight into the use of secondary metabolite, PA, as a biomarker for wood color of koa. PA biosynthesis genes have been identified to outline PA biosynthesis in koa. The results have suggested general positive correlations among the koa seedling hue, PA amount, and PA biosynthesis gene expression. Therefore, the results lead us to accept the hypothesis that the color difference of seedlings among koa families is due to differential production of PAs. In the future, it may be possible to use PAs as a biomarker for wood color, which will aid in rapid screening of progenies for improved wood quality in koa improvement programs.

APPENDIX

Summary: The purpose of this appendix is to present the entire supplementary tables pertaining to this research project that has not been included within the chapters.

Table S1 Primer sequences for the four PA biosynthesis genes isolation, sequencing, and qRT-PCR

Primer type	Forward primer (5' → 3')	Reverse primer (5' → 3')
Gene isolation		
AkDFR	TCCCTTATCTCCTTCCCCTTCATT	GCCAAGGCTATGGCGATGAA
AkANS	AGCGTTGGGTAGATGGGATGA	TCACTGCCCACTAAACGAGGG
AkANR	GACACTGGTTGGCGTAATGGAT	GGAGGAAGAGTAGCATCCCATAAT
AkLAR	GCCAAGGCTATTTCTCACCTG	GAGTAATTCAACACATGGCCGA
Sequencing		
AkDFR	same as gene isolation	same as gene isolation
AkANS	TGCACAGGGGCTTGGTGAAC	CGTGGCATCTGGCTCGAACC
AkANR	ACTCCACAGTCTCGTCGTAG	TGGTTTCGTCGCTTCTGAGC
AkLAR	AATTCAACACATGGCCGAGATG	CATGACTGTGTCCCCCGC
qRT-PCR		
AkDFR	GTCGCCACCCCTATGGATTT	GTTCCGGCAGAGGATGTGAA
AkANS	CGAAGACGTTGCCCATGTTG	CTAGTTCTGTGGCGGTGGTT
AkANR	AACCAGCAATCCAAGGCGTA	CCAAGTGGGTGGTTTTGCAG
AkLAR	AGAAGTTGGTGGGTGGTTGG	ACGATGATGGCTGCAAGTGA

Table S2 Total tannin and PA quantification at one month in three koa families from different islands

Hue (Family)	Tissue type	Total tannin	PA content
Red – Maui (S-1)	leaf	1.1 ± 0.07	0.33 ± 0.01
	stem	0.29	0.15
Slight red – Kaua'i (Lapa Exclosure)	leaf	0.83 ± 0.06	0.27 ± 0.02
	stem	0.37	0.11
Green – Hawai'i (Koala-27)	leaf	0.79 ± 0.04	0.29 ± 0.02
	stem	0.38	0.20

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