II.1 Walnuts

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1 Introduction

1.1 Walnut Botany

Walnuts belong to the genus *Juglans*, which includes the four sections *Juglans* (English/Persian walnut), *Rhysocaryon* (black walnuts, native to the Americas), *Cardiocaryon* (Japanese, Manchurian and Chinese walnuts, including heartnuts) and *Trachycaryon* (the butternut of eastern North America) (Manning 1978). *Juglans* is comprised of a single dichogamous monoecious species, *Juglans regia* L. The trees contain both male (catkins) and female (pistillate) flowers that display protandry (male maturing earlier than female) and protogyny (female maturing earlier than male) (Forde and Griggs 1972). This dichogamy promotes outcrossing. Thus, walnut production is dependent upon wind pollination and bloom overlap. Successful pollination of the pistillate flower leads to the formation of walnuts distinguished by a dehiscent hull that separates from the shell at maturity (Manning 1978).

1.2 Juglans regia

Native to central Asia, *J. regia* grows as a wild or semi-cultivated tree in a wide area from southeastern Europe and the Caucasus to Turkey and Iran, through southern portions of the former Soviet Union into China and the eastern Himalayas. Also referred to as the Persian walnut, it was prized by the Romans and was utilized in medieval Europe as an herbal medicine, particularly for brain and scalp ailments. *J. regia* has been cultivated for its nut crop for several thousand years and thus is the most horticulturally developed and widely cultivated of all the walnut species. It was probably introduced into European commerce and agriculture by the ancient Greeks. Its introduction into North America was more recent, where it has commonly been referred to as the English walnut to distinguish it from the American black walnut (Leslie and McGranahan 1998).

Grafting techniques, developed in France, allowed the first selection, development and propagation of cultivars. Many of these were introduced into

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Biotechnology in Agriculture and Forestry, Vol. 60
Transgenic Crops V (ed. by E.C. Pua and M.R. Davey)

California in the late 1800s where, seedling orchards derived from introduced Spanish and Chinese seed sources had been established previously. In ensuing years, selections of superior seedling trees found in orchards derived from these combined sources were propagated to form the basis of the California walnut industry (McGranahan and Leslie 1990; Forde and McGranahan 1996; Ramos 1998).

Successful implementation of grafting allowed not only the development of improved cultivars, but also a choice of rootstock. In much of the world, *J. regia* seedlings are used as rootstock, but in California the native black walnut species, *J. hindsii*, has been widely preferred for its enhanced vigor, salt tolerance and disease resistance. In the early 1900s Luther Burbank first observed the superior vigor of *J. hindsii* × *J. regia* hybrids that he named 'Paradox' (Whitson et al. 1914; Howard 1945). Most California walnut orchards are currently grown on either seedling Paradox or seedling *J. hindsii* rootstocks. The development of clonal rootstocks has been impeded by the difficulty of rooting walnut cuttings.

1.2.1 Breeding and Genetics

The major breeding objectives are to increase yield, quality and range of harvest dates, while decreasing the amount of chemical input required to control pests and diseases. For a recent review of *J. regia* breeding and genetics, see Dandekar et al. (2005).

1.2.2 Rootstocks

Nearly all walnut production in the United States, and increasingly worldwide, is derived from grafted scion varieties on seedling rootstocks. This reflects the difficulty of rooting walnut cuttings, which precluded, until recently, substantial use of either rooted scions or improved clonal rootstocks. Employment of rootstocks is driven by the need to deal with soil, environment, disease and pest problems (McGranahan and Catlin 1987). In California, *J. hindsii* (Northern California black walnut) was preferred for much of the first half of the 20th century as it is a native species adapted to several soil-related problems, including resistance to crown and root rot caused by *Armillaria mellea* (oak root fungus) and tolerance to waterlogging and drought (Smith et al. 1912). Today, the most popular rootstocks are the interspecific hybrids, the first of which was 'Paradox', a hybrid between *J. hindsii* and *J. regia* described in 1893 by Burbank (Whitson et al. 1914; Howard 1945).

The major objective for walnut rootstock breeding is vigor, in order to promote rapid growth of the scion under a variety of soil and environmental conditions and to establish rapidly a full-sized bearing canopy. Other objectives include resistance to diseases and pests, most notably *Phytophthora*, nematodes and crown gall, as well as tolerance to cherry leafroll virus and tolerance of

soil-related problems, including waterlogging and salt accumulation. There is interest in controlling tree size, but not at the cost of vigor.

1.2.3 Scions

The ideal walnut cultivar would be relatively late leafing to escape the rains that spread walnut blight (*Xanthomonas campestris* pv. *juglandis*), precocious (yielding more than 500 kg ha^{-1} in the fourth year) and vegetatively vigorous with bearing on both terminal and lateral shoots. It would have a low incidence of pistillate flower abscission and fruit drop and would not be alternate bearing. It would have high production capacity with low chemical input required. The harvest season would end in early October. The nutshell would be relatively smooth and well sealed and would make up no more the 50% of the nut weight. The nuts would fit the category of large or jumbo. The kernel would be plump and light colored, weighing about 7–8 g and be extracted easily in halves. The tree would be resistant to pests and diseases.

1.3 Juglans nigra

American black walnut (*J. nigra* L.) is a native hardwood species found throughout the eastern United States. Nearly all of this is in natural stands, in which less than 11% of the trees are walnut. Fewer than 14,000 acres are planted black walnut orchards (Shifley 2004). Although 2 million pounds per year of nutmeats are produced (Hammons et al. 2004), the trees also command high value for lumber and veneer. In the first half of the 20th century, the number of quality trees declined due to overharvesting of timber and clearing of land for agriculture. By the 1960s a program of tree planting had begun (Victory et al. 2004). Since then, this natural resource has been well managed. The number of trees, volume of lumber and quality has steadily increased over the last 40 years (Shifley 2004).

1.3.1 Breeding and Genetics

Breeding objectives include climatic adaptation, vigor and growth rate, form, fecundity and pest resistance. Because black walnut is grown for timber and veneer as well as nut production, trees that grow straight, with few lateral branches, and possess a large amount of heartwood with good color are very desirable (Woeste and McKenna 2004). Genetic improvement is accomplished primarily by testing of progeny derived from wild trees. By the early 1980s, walnut improvement programs had been established in 11 states in the US. However, it was found that significant interactions between environment and genotype greatly influenced the phenotypes of trees grown in the various climates, complicating the task of progeny evaluation (Victory et al. 2004).

Recent reviews of black walnut breeding have been presented by Tourjee and Gradziel (2001), Reid et al. (2004) and Woeste and McKenna (2004).

Walnut anthracnose, resulting from a fungal infection by *Gnomonia leptostyla*, is the only disease for which resistance is actively sought in breeding programs. Crosses were made between *J. nigra* and other walnut species including *J. regia*, *J. hindsii* and *J. ailantifolia* (Japanese walnut). Progeny testing indicated that *J. regia* and *J. ailantifolia* are potential sources of anthracnose resistance (Victory et al. 2004).

1.3.2 Propagation

Few viable seeds are available for propagation due to low production, predation and poor germination rates. Therefore, vegetative propagation is important to black walnut. Grafting has been employed, with a high proportion of successful grafts. More recently, tissue culture protocols have been developed (Victory et al. 2004).

2 Economic Importance

Worldwide, nearly 1.5 million metric tons of walnuts are produced annually. The top ten walnut producing and exporting countries and the value of the exports are listed in Tables 1 and 2. Within the United States, 100% of Persian walnut (*Juglans regia*) production is in California, where it is one of the top 20 agricultural commodities, with an annual production value of more than $350 million. (California Department of Food and Agriculture 2004).

Table 1. Leading walnut producing countries in the world [FAOSTAT Agricultural Production data, Crops Primary, Walnuts (updated 20 December 2004), http://faostat.fao.org/faostat/]

Country	Rank	Production (Mt)	Percentage of world production	Area harvested (ha)
China	1	415,000	27.96	185,000
United States	2	294,840	19.86	86,000
Iran	3	160,000	10.78	60,000
Turkey	4	125,000	8.42	59,000
Ukraine	5	68,000	4.58	28,000
Romania	6	37,000	2.49	2,000
India	7	31,000	2.09	30,500
France	8	30,000	2.02	15,000
Egypt	9	27,000	1.82	5,000
Serbia and Montenegro	10	23,600	1.59	13,200
All others		296,412	18.38	117,930
Total		1,484,252	100.00	623,630

Table 2. Leading walnut exporting countries in the world [FAOSTAT Agricultural and Food Trade data, Crops and Livestock Primary and Processed. Total of Shelled and Unshelled Walnuts (updated 7 December 2004), http://faostat.fao.org/faostat/]

Country	Rank	Exports (Mt)	Exports (× $1000)	Percentage of world exports
United States	1	83,253	215,674	41.06
Mexico	2	30,718	76,900	15.15
France	3	15,112	39,523	7.45
China	4	9,634	27,213	4.75
Moldova	5	9,564	23,070	4.72
Ukraine	6	9,297	18,864	4.59
Romania	7	8,913	19,809	4.40
Chile	8	7,182	22,908	3.54
India	9	6,301	21,330	3.11
Bulgaria	10	3,257	4,739	1.61
All Others		19,505	64,292	9.62
Total		202,736	534,322	100.00

3 Current Research and Development

3.1 Tissue Culture

3.1.1 Micropropagation

Walnuts have traditionally been propagated by grafting onto seedling rootstock. Micropropagation has been investigated for propagation of cultivars on their own roots, for production of selected rootstock clones, and for development of genetically engineered plants. Commercially, walnuts are micropropagated in only one laboratory in Spain (López 2001).

The first reports of micropropagation of Persian walnut are from the early 1980s (Chalupa 1981; Rodriguez 1982a, b; Somers et al. 1982; Caruso 1983; Cossio and Minolta 1983; Driver and Kuniyuki 1984). These techniques have been reviewed (McGranahan et al. 1987; Preece et al. 1989; Leslie and McGranahan 1992). DKW medium (Driver and Kuniyuki 1984) was developed specifically for walnut, but success has also been obtained on MS medium (Murashige and Skoog 1962). A comparison of different media for *J. regia* conducted by Saadat and Hennerty (2002) found that DKW was optimal when 2.2 g l^{-1} Phytagel was used as the gelling agent.

Walnuts are initiated into culture by introducing disinfested nodal segments of vigorous field- or glasshouse-grown shoots. Multiplication occurs through axillary shoot proliferation. Rapid transfer (two to five times per week) is essential after introduction into culture until discoloration of the medium is

no longer evident. Once established, cultures need relatively frequent transfer (two times per month) for optimum growth. It has been shown recently that the addition of 1 mM phloroglucinol to the multiplication media increases subsequent rooting (Leslie et al. 2006).

Techniques for rooting are still under investigation and rooting ability is clone specific. The most promising rooting technique utilizes a two-phase system originally developed by Jay-Allemand et al. (1992) and subsequently modified by Navatel and Bourrain (2001) and Vahdati et al. (2004). Roots are induced by placing shoots on MS medium containing auxin and at least 40 g l^{-1} sucrose for 6–8 days in the dark. Induced shoots are then transferred to a root development medium consisting of a mix of one-quarter-strength basal DKW medium and vermiculite (to improve aeration) and maintained in the light for 3–4 weeks until roots are visible. An alternative method is to treat unrooted microshoots with auxin, and root them in vermiculite in a fog chamber (Leslie et al. 2006). This technique results in a lower percentage of rooted shoots. However, those that were rooted using this procedure had very little callus and produced roots that were more fibrous than microshoots rooted in vitro.

Rooted shoots are planted in a well-drained potting soil and are acclimated by growth in a fog chamber for 2 weeks, followed by a week or two on a shaded glasshouse bench. However, the stress of acclimatization can lead to arrest of the apical meristem. Budbreak can be improved by application of 25 ml l^{-1} Promalin [a commercial product containing 1.8% gibberellic acid (a mixture of GA4 and GA7) and 1.8% benzylaminopurine (BAP, Valent Biosciences, Walnut Creek, California)] as a foliar spray to stimulate growth (Vahdati et al. 2004).

In a study comparing variations of this protocol on several different walnut cultivars, a correlation was found between the vigor of adult trees and their rooting ability. Although smaller microshoots can be produced more rapidly and more efficiently in vitro, longer microshoots appear to acclimatize better in the glasshouse, perhaps due to a greater internal reservoir of carbohydrates and a more lignified stem, which may lead to increased pathogen resistance (Vahdati et al. 2004).

Bisbis et al. (2003) also found a link between shoot lignification and root development, concluding that signals from the roots, as well as auxin, enhance lignin formation. This lignin was only found in xylem cells, and was correlated positively with the number of developing roots, beginning immediately after treatment with exogenous auxin (Kevers et al. 2004). This appears to trigger peroxidases that are involved in the process of building cell walls. Peroxidase activity had previously been shown to be a good marker for walnut rooting (Gaspar et al. 1992; Ripetti et al. 1994).

While the above protocol is generally effective, it is often found that media must be optimized for specific cultivars and clones. Dolcet-Sanjuan et al. (2004) investigated the influence of different factors on root formation of micropropagated walnut shoots from several genotypes, including *J. regia* and *J. nigra* × *J. regia* hybrids, and concluded that the ability to acclimatize was dependent both on genotype and the juvenility of the starting plant material. Microshoots

derived from embryos rooted much more easily than those originally started from adult plant material.

Shoots of black walnut have also been micropropagated using both liquid DKW (Pearson et al. 2001) and Long and Preece media (Pearson et al. 2000). It is important to note that the media composition generally must be optimized for each walnut species and cultivar for successful tissue culture.

3.1.2 Micrografting to Eliminate Viruses

No information is available regarding the elimination of viruses via micrografting in walnut.

3.1.3 Somatic Cell Genetics

Very little work has been carried out on the somatic cell genetics of walnuts. Intact protoplasts have been isolated from soft stem tissue of micropropagated shoots (McGranahan, unpublished data), but no additional work has been performed to induce the protoplasts to synthesize new cell walls or to utilize the protoplast-derived cells.

A tetraploid walnut cultivar, 'Mitsuru', derived by colchicine treatments, has been recently compared to the diploid 2X Mitsuru and analyzed for pollen characteristics (size, germination rate, fertility, etc.) (Yajima et al. 2003). The 2X Mitsuru is known as a Shinano cultivar and was derived from a cross between *J. regia* var. *orientis* Kitamura (Teuchi walnut) and the Persian walnut (*J. regia* L.). Shoots of Mitsuru were exposed to a 0.4% colchicine solution containing 1 ppm of NAA (α–naphthaleneacetic acid) to produce the tetraploid 4X Mitsuru walnuts. These were confirmed by chromosome analysis of shoot tips of the seedlings obtained from open- and self-pollinated plants (Yajima et al. 1997).

3.1.4 Somatic Embryogenesis

Development of embryos from asexual tissues has been a very useful tool in genetic improvement, particularly because tissues (i.e. leaf discs, protoplasts) commonly used in other plants have not been regenerated to plants in walnut. Somatic embryogenesis has been used to generate triploids (Tulecke and McGranahan 1988), intergeneric hybrids (McGranahan et al. 1986) and genetically transformed clones (McGranahan et al. 1988, 1990; Dandekar et al. 1989).

The techniques were developed for *J. regia* (Tulecke and McGranahan 1985) but have been applied to other species (Neuman et al. 1993). Immature cotyledonary explants harvested from developing nuts, cultured on conditioning medium for 2–4 weeks and then placed on basal DKW medium, will develop small white somatic embryos from single cells (Polito et al. 1989) on the explants after 8–16 weeks. These new embryos are repetitively embryogenic and, with monthly subculturing, cultures can be maintained for years.

For initiation and multiplication, embryos are maintained at room temperature in the dark. In the light, embryos turn green and a certain percentage will germinate. Germination frequency can be increased following desiccation over a saturated salt solution (Zn_2SO_4, NH_4NH_3, $MgCl_2$) until the embryos are white, with the consistency of popcorn, but not until they have turned brown. Embryos are then returned to DKW basal medium to germinate. Additional details of methods and progress in walnut embryogenesis are given in the reviews by Preece et al. (1995) and Tulecke et al. (1995).

A major challenge today in walnut tissue culture is to obtain embryogenesis or organogenesis from maternal tissue. This is important because embryos from zygotic tissue do not allow the exact genotype to be predicted, even if both parents are known. Therefore, the use of these embryos for genetic transformation can result in plants that can be brought into the breeding program in order to combine desirable traits. Efforts to generate somatic embryos from nucellus have been unsuccessful (Aly et al. 1992). Repetitively embryogenic cultures have been obtained from immature anther tissue, but only from the cultivar Chandler. Recently, a modified protocol allowed a somatic embryo line to be generated from immature anthers of a Paradox hybrid (McGranahan, unpublished data). This may provide a source of elite rootstock tissue for genetic transformation. The protocol consists of three stages. First, dissected anthers are placed in liquid flask culture in the dark for 7–8 weeks to induce callus production. Second, callus from the flask is transferred to semi-solid DKW medium with hormones for 2 weeks. Third, the callus is transferred to semi-solid DKW medium lacking hormones and subcultured at 2-week intervals.

3.1.5 Triploid Recovery from Endosperm

Walnut endosperm has been used to generate triploids ($3n = 48$) through somatic embryogenesis (Tulecke et al. 1988). Endosperm was cultured 4–12 weeks post-pollination using standard techniques for somatic embryogenesis. The cultivars Payne, Early Ehrhardt and Manregian produced repetitively embryogenic cultures. Triploids from endosperm of Manregian seed are maintained in the *Juglans* germplasm collection at the University of California, Davis. Trees flower and set nuts but embryos do not develop, so the shells are empty and very small.

3.1.6 Cryopreservation

Zygotic embryos, somatic embryos and pollen have been successfully stored under liquid nitrogen (LN). Walnut pollen with less than 7.5% moisture content survives cryostorage for at least 1 year (Luza and Polito 1988). Satisfactory moisture status is obtained by air-drying pollen for 24 h after anthesis. Zygotic embryo axes of *J. regia* survive LN storage after desiccation to 5–10% moisture content (Pence 1990). Somatic embryos survive when treated with 0.2 M

sucrose for 24 h and then desiccated to 30–40% moisture before LN storage (Setka 1994).

3.2 Transgenic Technology

Tree crops, such as walnut, are highly heterozygous and have very long generation times, which makes traditional breeding difficult. A large investment in both time and land is needed to grow seedlings to maturity in order to determine nut quality. It is therefore advantageous to be able to introduce specific traits into existing elite cultivars. These traits may come from within the germplasm of the genus or from other organisms. Desirable traits for walnut include disease and pest resistance, as well as good nut and timber quality. Herbicide tolerance would allow weeds to be controlled economically in nurseries and young orchards without damage to the trees.

3.2.1 Genetic Transformation

Genetic transformation is the process of asexually introducing DNA into plants, typically via *Agrobacterium tumefaciens*. Walnuts are susceptible to *A. tumefaciens* and were one of the first woody plants to be transformed and express foreign genes (Dandekar et al. 1988; McGranahan et al. 1988). Use of transgenics can allow existing elite cultivars to be improved without the long generation times required for traditional breeding. Transformation of rootstocks such as Paradox may allow desirable traits to be incorporated without changing the genetic makeup of the scion or nuts and without the potential for horizontal gene transfer.

3.2.2 Objectives

Plant transformation is useful for recalcitrant problems in walnut improvement, including resistance to diseases and pests. Codling moth is the key insect pest of walnut and chemical application is the main method of controlling this insect, because there is little genetic resistance in walnut germplasm that can be utilized. Crown gall is a serious problem for many fruit, nut and ornamental crops, greatly diminishing productivity. Walnuts are very susceptible to this disease. Losses are incurred from both contaminated nursery stock and infected orchard trees. Current prophylactic measures and mechanical removal of galls have not adequately controlled the problem.

3.2.3 Protocol

Walnut is transformed by inserting genes into embryogenic cultures, since *A. tumefaciens* readily infects young proliferating somatic embryos (McGrana-

han et al. 1988). Since new embryos develop from single epidermal cells (Polito et al. 1989), transformed cells produce entirely transformed embryos and chimeras are eliminated. Several independent transgenic lines can be obtained from a single embryo, indicating multiple infection sites on the surface of the walnut embryo (McGranahan et al. 1990). This feature makes the walnut transformation system very efficient. A detailed protocol for the transformation of walnut has been published (Dandekar et al. 1989; Leslie et al. 2006). The efficiency of selecting transformed embryos can be improved by the introduction of a scorable marker along with the desired transgene (McGranahan et al. 1990; Escobar et al. 2000).

Transgenic embryos are subsequently germinated to produce uniformly transformed plants. In 1989, this method was used to produce walnut trees that were the first transformed woody fruit or nut tree to be field tested. These trees bore nuts and the introduced genes were found to be both stably incorporated and inherited in a simple Mendelian fashion (McGranahan, unpublished data).

Transformation of black walnut (*J. nigra* L.) has also been reported recently (Bosela et al. 2004) through the use of somatic embryos. For black walnut the most desirable traits include herbicide resistance, control of flowering and the development of heartwood.

3.2.4 Regeneration

Most of the success in regenerating walnut from tissue explants has come from embryogenesis and not organogenesis. Walnuts seem to be highly recalcitrant to undergoing organogenesis and form shoots from callus or tissue explants. This has posed a problem in the use of transgenics to improve existing walnut cultivars and particularly rootstocks, since the elite rootstocks are interspecific hybrids and exhibit moderate to severe sterility. Research is currently ongoing to develop novel regeneration technologies, including the use of genes identified in Arabidopsis whose over-expression results in a high frequency of embryo formation from a wide range of tissues (Dandekar, unpublished data).

3.3 Molecular Genetics

Molecular genetics encompasses a broad suite of technologies for analysis of genes and their expressed products. Unfortunately, research at the molecular level in many horticultural tree crops has lagged, in large part due to the time and effort it takes to generate data. This is now changing, as new and more profound methods are available to investigate the 'gene space' of crops such as walnut. Genomic approaches that involve a non-biased data collection of genetic information are now available to the scientific community (Bent 2000; Weinstein 2002). Robotics is simplifying the analysis of thousands of genes, with the genetic data being analyzed by specific computer programs

and the useful data stored in public databases. These tools will dramatically improve the availability of genetic information for crops like walnut in the near future.

3.3.1 Gene Cloning

Gene cloning is an important endeavor because tree crops are likely to possess many unique genes that may not be discovered in other plants. However, little progress has been reported primarily due to the few walnut researchers worldwide and the availability of resources to fund these endeavors. One of the useful sources of information on cloned walnut genes is GenBank (NCBI), the public repository for DNA sequences. Currently, this database has over 5000 entries. Table 3 lists the number of GenBank entries by walnut species.

Genes specific to walnut currently being studied include those involved in tannin, naphthoquinone, unsaturated fatty acid and flavonoid biosynthesis. Several of the genes involved in the biosynthetic pathway of flavonoids have been identified (Beritogoli et al. 2002). Recently, homologues of two Arabidop-

Table 3. Walnut DNA sequence entries in GenBank (October 2006)

Species	Chloroplast genes	Ribosomal genes	Mitochondrial genes	Microsatellites	Nuclear encoded genes	Total entries
J. ailantifolia	6	2	0	0	0	8
J. australis	6	2	0	0	0	8
J. boliviana	1	1	0	0	0	2
J. californica	25	5	0	0	0	30
J. cathayensis	7	3	0	0	0	10
J. cinerea	7	3	0	0	0	10
J. guatemalensis	5	2	0	0	0	7
J. hindsii	34	6	0	0	0	40
J. hopeiensis	4	1	0	0	0	5
J. major	19	7	0	0	0	26
J. mandshurica	16	3	1	0	0	20
J. microcarpa	22	7	0	0	0	29
J. mollis	4	1	0	0	0	5
J. neotropica	7	1	0	0	0	8
J. nigra	26	9	1	39	10	85
J. nigra × *J. regia*	0	0	0	0	12	12
J. olanchana	5	2	0	0	0	7
J. regia	14	5	0	0	5040	5059
J. sigillata	5	1	0	0	0	6
J. sp. NSW 476481	1	0	0	0	0	1
Total	214	61	2	39	5062	5378

sis genes controlling floral transition and flower differentiation were cloned from microshoots of Early Mature (EM) walnuts (Breton et al. 2004). EM walnut trees can flower within 1 year of germination and develop into trees that are smaller, bushier and more cold-hardy than other *J. regia* genotypes. These traits may be useful in transgenic plants and traditional breeding programs.

A key determinate of walnut kernel quality is the oil content, about 90% of which is polyunsaturated. Of that, omega 3-fatty acid α-linolenic acid (ALA) comprises 25% (Greve et al. 1992). The presence of unsaturated fatty acids is an important factor in the rancidification of walnuts, in which these acids are oxidized, thus reducing the shelf life of walnut kernels (Greve et al. 1992). The omega-3 fatty acids have been shown to play an important role in growth and development, nutrition and disease prevention. Nutritional studies have demonstrated that walnut consumption can reduce the incidence of coronary heart disease. The genes encoding the various fatty acid desaturases involved in the synthesis of polyunsaturated fatty acids, including *fad 2* and *fad 3*, have been cloned from walnut (Dandekar, unpublished data).

3.3.2 Marker Assisted Selection

The selection of desired progeny in a breeding program can be facilitated by the use of molecular markers. Cloned genes can be excellent markers as long as they display some polymorphism. Additionally, molecular markers can be used in more traditional genetic strategies utilizing linkage mapping and map-based cloning. Molecular markers have improved the efficiency of linkage mapping, allowing identification of discrete DNA segments where genes of interest reside (Camilleri et al. 1998). Some mapping efforts are ongoing in black (Woeste et al. 2002) and Persian (Aradhya et al. 2001) walnuts. These mapping efforts utilize AFLP and microsatellite markers.

Microsatellite loci are being used to fingerprint walnut cultivars and, most recently, inter-simple sequence repeat markers (ISSR) have been used to determine the genetic relationships of closely related walnut cultivars (Potter et al. 2002a; Orel et al. 2003; Pollegioni et al. 2003, 2006; Dangl et al. 2005; Foroni et al. 2005). This is important to verify and document breeding programs, as well as to understand how pollen flows within and between orchards. Sequences from the intergenic regions of chloroplast and ribosomal genes have been used to investigate the phylogenetic relationships between *Juglans* species and cultivars (Potter et al. 2002b; Aradhya et al. 2006). Isozymes have also been used to determine genetic relationships between cultivars from different geographic locations (Ninot and Aleta 2003; Vyas et al. 2003). Marker assisted selection is currently in use to identify individuals resistant to cherry leaf roll virus among a backcross population of Persian × black walnut (Woeste et al. 1996a, b).

Black walnut population genetics studies have been conducted over the last 25 years with allozyme markers to determine the amount of genetic diversity within the populations comprising the species (Victory et al. 2004).

More recently, microsatellites (SSRs) have been identified (Woeste et al. 2002; Robichaud et al. 2006).

3.3.3 Functional Genomics

Enzymes in the phenylpropanoid pathway from phenylalanine lead to the biosynthesis of a range of natural products, including flavonoids. Genes for these enzymes, including the key enzyme chalcone synthase, have been investigated in walnuts. Walnuts expressing antisense chalcone synthase were found to be deficient in the accumulation of flavonoids, but interestingly, these deficient plants showed an increase in adventitious root formation (El Euch et al. 1998). These results contrast with other root initiation studies using walnut cotyledons in which adventitious rooting was observed to occur when the appearance of the lateral root primordia coincided with the expression of chalcone synthase at the same location (Ermel et al. 2000). The genes in the phenylpropanoid and flavonoid pathways were also studied in order to understand the accumulation of flavanols during heartwood formation in black walnut (Beritogoli et al. 2002). The authors concluded that flavanol synthesis was due to the increased transcriptional activity of genes in the phenylpropanoid pathway in black walnut sapwood cells that are undergoing the transition to heartwood.

Naphthoquinone metabolism has also been investigated and proteins involved in some of the steps have been identified. Naphthoquinones are important for plant defense and may also be involved in developmental processes (Duroux et al. 1998).

Oil biosynthesis in the embryo is a major metabolic pathway and some effort has been directed at functional characterization of two key steps in the biosynthesis of polyunsaturated fatty acids. Transgenic walnut embryos expressing antisense *fad 2* or sense *fad 3* have been developed (Dandekar, unpublished data) and some of the lines show alterations in the profile and composition of fatty acids. It is hypothesized that expression of antisense *fad 2* will suppress the interconversion of oleic to linoleic acid, leading to an increase in the accumulation of the monosaturated oleic acid. The expression of sense *fad 3* is aimed at overexpressing the enzyme involved in the conversion of linoleic acid to the omega 3 fatty acid linolinic acid. These studies will be useful in developing walnut lines with high oleic acid (monounsaturated fatty acid) content for stability and also lines with increased omega 3 fatty acids.

4 Practical Applications of Transgenic Plants

A major accomplishment has been the development of walnut trees expressing resistance to codling moth. Various insecticidal crystal proteins (ICP) from *Bacillus thuringiensis* were tested by incorporating the proteins into insect

diets (Vail et al. 1991). The crylA(c) protein was found to be the most effective. However, transformation of walnut using the bacterial gene failed due to lack of expression resulting from the codon bias of the bacterial gene sequence (Dandekar et al. 1994). A synthetic gene corrected this problem and high levels of codling moth mortality were observed when larvae were fed transgenic embryos (Dandekar et al. 1998). Laboratory tests have confirmed that high expressing genotypes are lethal to the codling moth larvae, and field trials are in progress (Vail et al. 1991; Dandekar et al. 1992, 1994, 1998; Leslie et al. 2001).

Recently, a gene silencing strategy was developed to produce resistance to crown gall disease in Arabidopsis and tomato, demonstrating for the first time the use of gene silencing to generate resistance to a major bacterial disease (Escobar et al. 2001). This approach has subsequently been successfully applied to walnuts (Escobar et al. 2002). Transgenic walnuts were highly resistant to galling. Resistant genotypes display an absence of macroscopic and microscopic indications of tumorigenesis after infection with a wide range of *A. tumefaciens* strains, indicating a broad-spectrum durable resistance (Escobar et al. 2003). These plants also provide a unique resource for examining fundamental questions about *Agrobacterium* biology and post-transcriptional gene silencing (PTGS) (Escobar et al. 2003).

Success has also been achieved in modifying tree architecture using the *rolABC* genes from *Agrobacterium rhizogenes*. Transgenic walnut trees expressing these genes show leaf curling, a marked reduction in internode length, and altered root architecture, but no increase in rootability (Vahdati et al. 2002).

Walnut is a tree crop whose nuts can be contaminated with aflatoxins, carcinogenic and teratogenic chemical compounds synthesized by members of the fungal genus *Aspergillus*. Many countries that import walnuts have set total aflatoxin action threshold levels at 4 ppb, significantly below the US Food and Drug Administration recommendation of 20 ppb. Because fungal infections often follow damage due to feeding of insects such as codling moth larvae, an important objective of breeding and genetic engineering is to develop lines that demonstrate insect resistance (Campbell et al. 2003). As discussed above, the engineering of walnuts to produce the insecticidal crylA(c) protein shows promise of reducing codling moth damage and subsequent fungal infection.

Recently, it was discovered that kernels of the 'Tulare' variety of Persian walnut are able to suppress the production of aflatoxin (Mahoney et al. 2003). Evidence points to the high concentration of gallic acid in the pellicle (seed coat) as the factor that inhibits aflatoxin generation. The gene encoding shikimate dehydrogenase (SDH), the enzyme responsible for gallic acid production, has been cloned. Somatic embryos of the cultivar 'Chandler' were transformed to overexpress the SDH gene, resulting in the production of a high concentration of gallic acid and increased inhibition of aflatoxin production (Muir, unpublished data).

5 Conclusions and Future Challenges

A combination of conventional, in vitro and molecular approaches has facilitated considerable progress in diverse aspects of walnut improvement. Traditional crossing has contributed to the development of scion cultivars with improved yield, quality, harvest timing and virus resistance and to rootstocks selected for vigor, virus tolerance and nematode resistance. Selection among these crosses has been made more efficient by the development of molecular markers for blackline resistance, cultivar identification and germplasm diversity. Genomic tools will have a profound impact on the genetics of crops like walnut as they will provide key genetic resources that will afford a greater precision in ongoing breeding efforts. Over the next decade, the genomes of many crops will be sequenced, including walnuts, providing a simple list of all of the walnut genes. Such information will greatly enable the development of novel diagnostics and therapeutic approaches, as well as make breeding more targeted.

Micropropagation and somatic embryogenesis techniques have enabled the development of improved rootstocks and the implementation of gene transformation. The latter has already been used to develop walnuts resistant to codling moth (the key insect pest) and crown gall disease (a widespread and commercially important rootstock problem). Disease and pest resistance are significant targets as they also reduce the use of pesticides and thus have a profound impact both on productivity and the environment. Progress to combat disease and pest problems in walnut will be aided by advances made in biotechnology applications in other plants. Functional genomics approaches will provide a much clearer understanding of the metabolic processes that make walnuts unique. Functional genomics will also give us a clearer understanding of walnut metabolism and physiology, presenting additional opportunities to develop disease and pest resistance and to improve wood structure and kernel quality traits in the future. Furthermore, it should be possible to maximize the relationship between walnut components and the potential health benefits for consumers of walnuts and walnut products.

5.1 Emerging Opportunities

In recent years, a large body of evidence has accumulated from studies that points to health benefits derived from incorporating walnuts into a healthy diet. Diseases that appear to be improved by the sufferer consuming walnuts include cardiovascular and coronary heart disease (Zambon et al. 2000; Morgan et al. 2002; Zibaeenezhad et al. 2003; Ros et al. 2004; Reiter et al. 2005), type 2 diabetes (Fukuda et al. 2004; Gillen et al. 2005), Alzheimer's disease (Chauhan et al. 2004), liver cytotoxicity (An et al. 2005), tooth decay (Jagtap and Karkera 2000) and acne (Qadan et al. 2005). Studies on the effects of walnuts and coronary heart disease have been reviewed by Feldman (2002). There is also

evidence that compounds contained in walnut may be useful in reducing the toxicity of broad-spectrum chemotherapy drugs (Haque et al. 2003).

The beneficial aspects of walnuts are derived from both their unique fatty acid composition and numerous products of secondary metabolism, including high levels of fiber, folate, polyphenolic compounds, tannins and the amino acid arginine (Ros et al. 2004). Although walnuts are high in total fat content, increased consumption of walnuts does not result in gains to body weight, because the resulting diet has a low proportion of saturated fat (Zambon et al. 2000; Gillen et al. 2005). The potential health benefits have led to research into how walnuts can be incorporated into other foods to increase their consumption. Recently, the addition of walnuts as filler to frankfurters was investigated (Colmenero et al. 2005).

The consumption of many nut species, including walnuts, can initiate an allergic reaction in susceptible persons. The major food allergen genes have been cloned, but elimination of the allergen proteins by conventional breeding is unlikely to be accomplished (Comstock et al. 2004). Therefore, this drawback to widespread walnut consumption could be addressed by genetic engineering to silence the genes involved. Walnut somatic embryos can be used as a model system to test many of these ideas as they are easily transformable and represent the edible portion of the walnut. Transgenic walnut somatic embryos could be cultured in vitro in bioreactors to produce valuable health-related products.

Walnuts may also be useful in phytoremediation programs, particularly where contaminated land could be planted for timber production. Recently, *J. regia* plants grown in lead-contaminated soil demonstrated the ability to accumulate high concentrations of lead in the root tissues, with little translocation to above-ground parts (Marmiroli et al. 2005). Genetic transformation could be used to enhance the range and capacity of walnut rootstocks.

Biotechnology can also directly benefit developing countries where walnuts are grown. For example, extraction of kernels results in a large mass of shells, which are used for combustion. Research is now showing that this biomass can be converted to pyrolytic oil, which can be further refined to produce transportation quality fuels of equal or higher quality to those derived from petroleum (Onay et al. 2004). Crushed walnut and almond shells can also be combined with activated carbon to produce high-efficiency and low-cost filters for drinking water (Ahmedna et al. 2004).

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