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Invited Review Article

Peanut (*Arachis hypogaea* L.) Omics and Biotechnology in China

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Abstract

Peanut is one of the most important crops in the world, both for vegetative oil and as a protein source. Biotechnology approaches provide promising ways to increase peanut productivity, either through improved seed quality or stress resistance. These approaches require the identification of genes that control important agronomical traits, the understanding of gene regulation and metabolic pathways, as well as ways of delivering genes or small RNAs into peanut plants. Because of these requirements, extensive studies have focused on peanut functional genomics and biotechnology, and have made great strides during the past decades. This review summarized the advances in peanut omics and biotechnology in China.

Keywords: Peanut, functional genomics, EST sequencing, Expression profiling, miRNA, Molecular marker

Introduction

Peanut (*Arachis hypogaea* L.) is one of the most important oil crops in the world. Unlike their diploid wild-type relatives, which are genetically diverse and have experienced selection for accumulation of stress resistant genes during adaptation to different harsh environments, allotetraploid cultivated peanuts (AABB, 2n＝4X＝40) have very limited genetic diversity revealed by molecular maker analysis (Halward et al., 1991; Kochert et al., 1996; Subramania et al., 2000; Gimenes et al., 2002). Differences in ploidy, combined with the self-pollinating nature of these plants, block genetic exchange between cultivated and wild-type species. Therefore, gene engineering is of key importance for improving stress resistance of peanut. There are several other problems that negatively influence peanut consumption. The presence of allergens Ara h1 to Ara h 8 in peanut storage proteins substantially limits the population of consumers, especially in western countries (de Leon et al., 2007). In addition, the low ratio of oleic acid to linoleic acid (O/L) shortens the shelf time of peanut oil. The O/L ratios of many widely grown Chinese cultivars are much lower compared with O/L ratios of cultivars from the USA and many other countries. Artificial hydrogenation is required in some cases during factory processing of vegetative oils to make fatty acid chains more saturated and stable. However, hydrogenated oils contain higher levels of trans-fat, which is harmful to human health. Genetic modification provides a promising alternative for peanut germplasm innovation and breeding aimed at improving oil and protein quality, and increasing stress and disease tolerances. These requirements have raised the interest of scientists to work on peanut functional genomics, proteomics, molecular marker development and application, molecular biology and other biotechnology-related fields, with the goals of obtaining genes, markers, non-coding RNAs and promoters, and developing techniques for gene engineering. In recent years, these research areas have achieved great success throughout the world, especially in the United States, China, India and Brazil; peanut biotechnology still lags behind advances made in some other major crops such as rice, maize, cotton and soybean. Recently, two reviews summarized advances that improve peanut resistance to *Aspergillus flavus* infection and aflatoxin accumulation, as well as developments in peanut genomics in China (Liao et al., 2009; Liang et al., 2009a). In this article we focus more broadly on the advances in peanut omics and biotechnology investigations in China. The following four research areas are emphasized in this review: (1) functional genomics, including EST sequencing and gene cloning, gene expression and regulation, (2) molecular marker development and application, (3) peanut in vitro regeneration and gene transformation, and (4) proteomics. Most of the data we discuss are from the research groups of Chinese Academy of Agricultural Sciences, Shandong Academy of Agricultural Sciences, Guangdong Academy of Agricultural Sciences, Sun Yat-Sen University, Shandong Agricultural University, Henan Academy of Agricultural Sciences, Guangxi Academy of Agricultural Sciences, Fujian Agricultural and Forestry University, and several other universities or research institutes.

1. Peanut functional genomics

(1) EST sequencing

Due to the large size of the peanut genome (2800 Mb), it remains unsequenced because of both financially and technical

challenges. In order to identify new genes, useful promoters and to understand key metabolic pathways for biotechnology-based modifications, peanut functional genomics has becoming increasingly prominent in recent years. Because of the flexibility for gaining sequence information, expressed sequence tag (EST) approaches have been employed by several groups, using both cultivated and wild type peanut, and have generated more than one hundred sixty thousand ESTs worldwide (GenBank, December, 2010, http://www.ncbi.nlm.nih.gov/nucest?term=Arachis). Several Chinese research groups have carried out peanut EST projects using cultivated peanut since 2005. Huang's laboratory in Sun Yat-Sen University constructed a peanut seed cDNA library and sequenced a few thousands ESTs with the aim of cloning genes encoding seed storage proteins (Wang et al., 2005). These sequences represented the earliest EST sequence information from peanut. Subsequently, our laboratory carried out EST sequencing using a full-length cDNA library from immature seed to clone genes encoding storage proteins and fatty acid metabolic enzymes (Wang et al., 2006; Bi et al., 2010). We also hope to clone genes with special expression patterns, for example, seed or developmental stage-specific expression, because the promoters of these genes have potential applications in gene engineering research. So far, we have obtained 17,000 ESTs from this library, about 10,000 of which have been deposited in the GenBank database (Bi et al., 2010). To gain a better understanding of the high oleic acid peanut variety E12, a cDNA library was constructed and more than 12 thousand ESTs were sequenced (Refer Wang et al., 2009). This large number of EST sequences provides valuable information for gene cloning, especially for genes encoding key enzymes for fatty acid synthesis and seed storage proteins. Using a bacterial wilt resistance peanut, Liao's group constructed two cDNA libraries from normal leaves and leaves challenged with pathogens. They focused on identification of genes that are differentially expressed between normal and pathogen-infected plants. More than 25 thousand ESTs were sequenced from these two cDNA libraries (Huang et al., 2008). Aflatoxin contamination is a serious problem in peanut production. Because aflatoxin is highly toxic and it is difficult to remove contaminated peanuts from healthy ones, breeding of peanut varieties with higher tolerance to fungi is a crucial focus of both conventional and biotechnological research. Detailed advances in this field in China have been reviewed recently (Liao et al., 2009). More than 20 thousand ESTs have been sequenced from a peanut pod cDNA library, aiming to understand the mechanisms of fungal infection and accumulation of aflatoxin in peanuts and to discover genes that may play roles in *A. flavus* infection resistance (Liang unpublished data). For similar reasons, a peanut seed coat cDNA library also was constructed, and a few thousand ESTs were sequenced (Li et al., 2008).

2. Gene cloning

Genes involved in oil metabolism

An important goal for biotechnology studies on crops is the improvement of yield and quality. For peanut, oil content, oil quality and storage protein composition are major issues for quality improvement, and genes controlling these important agronomic traits have been the focus of peanut gene cloning. The first enzyme complex for de novo fatty acid synthesis is acetyl-CoA carboxylase (ACCase), which catalyzes adenosine triphosphate (ATP)-dependent carboxylation of acetyl-CoA to form malonyl-CoA. The multisubnuit form of ACCase is composed of four components: biotin carboxyl carrier protein (BCCP), biotin carboxylase (BC), and the α-subunit and β-subunit of carboxyltransferase (CT). Genes encoding these four subunits were cloned from a Chinese cultivated peanut Luhua-14. Genomic DNA of these four subunits then was cloned using gene-specific primers designed based on cDNA sequence information (Li et al., 2010a). In most plants, there is another ACCase, the multi-functional ACCase, which is a large multifunctional polypeptide with three structural domains of BCCP, BC, and CT (Schulte et al. 1994; Shorrosh et al. 1994). The multi-functional ACCase gene was also cloned from peanut. Its cDNA contains a 6,783-bp ORF encoding a protein of 2,260 amino acids with a predicted molecular weight of 252.2 kDa and a pI of 6.283 (Li et al., 2010a). In addition, BCCP genes from wild type peanut including *Arachis duranensis, Arachis rigonii, arachis batizocoi,* and *Arachis hoehnei* were cloned. Sequence analysis showed that BCCPs from both cultivated and wild type peanut are highly conserved (Li et al., 2009a).

The plant fatty acid biosynthesis pathway includes another large enzyme complex, the type II fatty acid synthase (II FAS) complex. The II FAS complex is composed of an acyl carrier protein (ACP), malonyl-CoA:ACP transacylase, β-ketoacyl-ACP synthase (I, II, III), β-ketoacyl-ACP reductase, β-hydroxyacyl-ACP dehydrase and enoyl-ACP reductase. All these genes were cloned from peanut using cDNA library construction and EST sequencing, together with homology cloning and 5'and 3' RACE (Li et al., 2009b). Sequence alignments revealed that primary structures of peanut type II FAS enzymes are highly conserved with sequences from other higher plants and catalytic residues are strictly conserved between *Escherichia coli* and higher plants (Li et al., 2009b). ACP is a central cofactor for de novo fatty acid synthesis, carrying the nascent acyl chains during the synthesis of acyl groups. Five different types of ACP genes were cloned from peanut (Li et al., 2010b). The sequences of these ACPs contained a strictly conserved Ser residue in the Asp-Ser-Leu (DSL) motif, which is an important characteristic of ACPs in both plants and bacteria; however, the N-terminal and C-terminal sequences of these ACPs vary significantly, and distinct 5' UTRs also were observed. Three of these five ACPs were predicted to be localized to the chloroplast, while the other two were predicted to be mitochondra localized. Genomic sequence comparison revealed that the chloroplast and mitochondria ACPs have very different intron-exon organization (Li et al., 2010b).

The introduction of double bonds into fatty acid chains at different positions is needed to produce varied unsaturated oils, and this requires the activity of several desaturases. The △12-fatty acid desaturase catalyzes the formation of linoleic acid from oleic acid by introducing a double bond at the delta 12 position. The oleic acid/linoleic acid (O/L) is a key determinant for oil and nutritional quality, and reducing △12-fatty acid desaturase activity by antisense or RNAi strategies would produce oil with higher O/L ratio desired by breeders. Several studies have focused on cloning the △12 fatty acid desaturase gene from peanut (Zhang et al., 2007; Pan et al., 2007; Xie et al., 2007; Yin et al., 2009; Zhang et al., 2008). The activity of this enzyme was confirmed by expressing the peanut △12-fatty acid desaturase cDNA in a yeast system (Zhang et al., 2007). An RNAi vector was constructed and transformed into peanut (Zhang et al., 2007; Yin et al., 2008). FAD2 genes from the commonly cultivated peanut and a high oleate mutant that contains >80% of oleate and 2% of linoleate in seed oil were compared, and found that the high oleate phenotype was cause by a single nucleotide insertion (Yu et al., 2008). The same result was previously observed in the F435 mutant (Lopez et al., 2000; Lopez et al., 2002).

In maturing seeds triacylglycerol (TAG) is synthesized through the Kennedy pathway, which requires the activity of several enzymes, including three acyltransferases glycerol-3-phosphate acyltransferase (GPAT), lysophosphatidic acid acyltransferase （LPAAT） and diacylglycerol acyltransferase (DGAT). These enzymes are critical determinants both for oil content and composition (Jain et al., 2000; Zou et al., 1997; Zheng et al., 2008). Our lab cloned the putative peanut GPAT gene, which shows high sequence similarity (91%) to Castor (*Ricinus communis* L.) GPAT (Wang and Xia unpublished data). Full length cDNA of DGAT2 and DGAT3 were cloned from peanut by Bi's group in Shandong Academy of Agricultural Sciences. Transgenic tobacco over expressing peanut DGAT gene showed a significantly altered oil content and fatty acid composition (Bi unpublished data). There are many other genes that are not direct components of fatty acid or TAG biosynthesis, but significantly affect oil content or influence fatty acid biosynthesis pathways. TAG is stored in oil bodies in seeds. Oleosins are major components of oil bodies and play critical roles for determining oil body size and seed oil content in *Arabidopsis* and *Brassica napus* (Siloto et al., 2006; Hu et al., 2009). We obtained 284 ESTs that show high similarity to oleosin gene sequences from other plants. These ESTs could form six contigs that encode six subfamilies of peanut oleosins. Based on their predicted molecular weights they were named as AhOLEO-16.9, AhOLEO-17.7, AhOLEO-18.6, AhOLEO-22, AhOLEO-18.4 and AhOLEO-14.3 (Wang and Zhao, unpublished data). LEC1 is a transcription factor that plays regulatory roles both in embryo development and material accumulation, for example, lipid synthesis and accumulation (Lotan et al., 1998; Stone et al., 2001; Mu et al., 2008). Two members of the LEC1 family gene were cloned from Luhua-14, *AhLEC1A* and *AhLEC1B*; both contain the strictly conserved B domain that characterizes LEC1. Twenty eight nucleotide differences were observed between ORFs of these two *LEC1* sequences. Peanut *LEC1s* were shown to be highly expressed in developing seeds, but not detected in root, stem, leaf and flower (Li et al., 2009).

Seed storage protein genes

Seed storage protein genes are also a focus of gene cloning from peanut. For example, peanut arachin, conarachin, coglutin-like proteins were cloned from a cultivated peanut Shanyou-523 seed cDNA library (Wang et al., 2005; Li et al., 2005; Yan et al., 2005). Peanut allergy is a serious health problem in western countries due to the presence of numerous allergens in peanut seeds. Ara h1 gene was cloned from Shanyou-523 (Wang et al., 2005). Our laboratory cloned eight peanut allergen (Ara h 1 to Ara h 8) genes from Luhua-14 and obtained sequence information for biotechnology-based silencing of these genes (Wang et al., 2008). High sequence conservation was found between a particular allergen from different cultivars; however, significant differences were detected in some allergens between different cultivars. Some mutations occurred in the epitope region, which probably affects IgE binding activity and consequently influences the relative allergenicity of different isoforms. For example, Ara h3 contains four epitopes and mutations were found in all of them; in epitope 4 alone, more than 50% of the amino acids were variable (Wang and Xia unpublished data). Lea proteins are a group of proteins that accumulate abundantly during late stages of seed development. We obtained 271 ESTs representing 8 different groups of lea protein-coding genes including the extensively characterized dehydrin gene from peanut. Each group contains 1-6 different members and the full length cDNAs of 19 members have been cloned (Su et al., 2010; Su and Wang unpublished data). All these genes were detected at high expression levels in peanut seeds. Lea 2 (dehydrin), Lea3-1, Lea3-2, Lea6-1 and Lea7-1 were also detected in vegetative tissues and Lea3-4 was found to be highly expressed in flowers (Su and Wang unpublished data).

Stress and disease related genes

Gene expression profiling can provide useful information for cloning stress response or pathogen-induced genes. EST data indicate that many genes are up-regulated after *A. flavus* infection. Based on these EST sequences, as well as results from RT-PCR, RACE and genome walking, PR-10 gene and pathogenesis-induced protein (PIP) genes have been cloned from peanut (Xie et al., 2009 a,b). Resveratrol plays key roles in plant resistance to UV radiation and fungal infection. Resveratrol synthase is the last enzyme in the resveratrol synthesis pathway, and the gene from peanut has been cloned and analyzed Expression analysis indicated that this gene was specially expressed in peanut root and could be induced by UV treatment (Zhou et al., 2008; Han et al., 2010). Lipid transfer proteins (LTP) which were reported to be involved in disease resistance in plants also have been cloned from peanut (Zhao et al., 2009). Metallothionein could play roles in heavy metal detoxification and soil recovery, and several metallothionein genes have been cloned from peanut. The expression of these genes in unstressed plants and plants grown under heavy metal medium was analyzed, and their function in improving heavy metal resistance was detected by transgenic Arabidopsis over-expressing the peanut metallothionein gene (Quan et al., 2007). Several genes encoding transcription factors associated with stress response, including the NAC and DREB genes, were cloned from peanut (Shao et al., 2008; Liu and Li, 2009; Zhang et al., 2009). Peanut *DREB* was shown to be constitutively expressed in root, stem, leaf, flower and seed, and strongly induced by low temperature and drought; however, its expression was not affected by ABA and salt treatment (Zhang et al., 2009). Further investigation of the expression and regulation of the peanut NAC gene, as well as its ability to confer stress tolerance, would provide useful information. A peanut pericarp- or testa-specific gene has been cloned to obtain a tissue specific promoter that could be used in gene engineering for disease resistance (Zhang et al., 2010).

(3) Gene expression and regulation

Microarray analysis is a powerful tool for global gene expression profiling (Girke et al. 2000; Casson et al. 2005). Because a commercial peanut genechip is not available, high-throughput gene expression analysis in peanut currently is very limited. To fill this gap, two USA research groups designed peanut microarrays to address different biological questions, for example, to characterize *Aspergillus parasiticus* infection-induced changes in gene expression and to profile gene expression in different tissues of peanut (Luo et al. 2005; Payton et al. 2009). In China, several research groups are currently working on genechip-based expression profiling studies. Our laboratory made a cDNA microarray to analyze differential gene expression among peanut tissues and organs. Gene expression patterns were also analyzed during peanut seed development (Bi et al., 2010). Liao's laboratory together with other research groups are in the process of making an oligo-nucleotide genechip using all peanut sequences available in the EST database, along with a large number of their own ESTs that are currently not in the database (Personal communication). These ESTs are from the following different sources: (1) more than 160 K from the EST database, including

about 90 K *Arachis hypogaea* ESTs, 35 K *Arachis duranensis* ESTs, 32 K *Arachis ipaensis* ESTs, 6 K *Arachis stenosperma* ESTs from different tissues under normal and stressed condition, or when challenged by pathogens (http://www.ncbi.nlm.nih.gov/nucest?term=Arachis%20EST); (2) about 25 K ESTs from cDNA libraries constructed using bacterial wilt resistance peanut leaf, before and after bacterial challenge (Huang et al., 2008; Liao unpublished data). Because of the lack of a peanut genechip, soybean genechips also have been used to analyze differential gene expression of peanut varieties with high resistance or high sensitivity to *A. flavus* infection (Shan et al., 2007). Besides microarray based high throughput gene expression analysis, the expression and regulation of individual genes also has been investigated in peanut. Nucleosome remodeling and histone modifications are important mechanisms for transcriptional regulation. Oleosins are major oil body proteins that specifically accumulate at a high level during the late embryo maturation. Concomitant changes in chromatin structures of two peanut oleosin genes, were examined in relation to transcriptional activity. The results showed that histone eviction from the proximal promoters and coding regions is associated with high expression levels of oleosin genes during late embryo maturation. Moreover, basal expression of oleosins in early maturation of embryos is accompanied by an increase of histone H3 acetylation and decrease of histone H3K9me2 modification (Li et al., 2009). MicroRNAs (miRNAs), a large group of small RNAs in plants and animals, were first found to play key roles in gene regulation in *C. elegans* (Lee et al., 1993; Zhang et al., 2006).

MicroRNAs are transcribed by RNA polymerase II from non-protein coding genes, which most frequently are located in intergenic regions of the genome and contain their own promoters (Chen, 2004; Kim 2005; Moss et al., 2002). The expression of genes with a wide range of functions are regulated by miRNAs. Abnormal miRNA expression affects plant development (Schwartz et al., 1994; Ray et al., 1996; Park et al., 2002; Vazquez et al., 2004; Lu et al., 2000; Zhang et al., 2007) and adaptation to environment variation (Sunkar et al., 2004; Liu et al., 2008; Zhang et al., 2005). Understanding the functional and regulatory roles of miRNAs could open a new window for crop improvement in overall yield, quality and stress or disease tolerance. Taking advantage of high throughput sequencing, combined with bioinformatics analyses, the authors' laboratory discovered 89 peanut miRNAs belonging to 14 new miRNA families and 22 conserved miRNA families from a widely grown Chinese cultivars (Zhao et al., 2010; http://www.mirbase.org/cgi-bin/mirnasummary.pl?org=ahy). Two studies predicted 13 conserved peanut miRNAs through bioinformatics analysis of peanut ESTs and genomic survey sequences (Pan et al., 2010; Zhang et al., 2006). The predicted targets of peanut miRNAs comprise a wide range of biological processes, including several transcription factors (Zhao et al., 2010; Pan et al., 2010). These results provide the basis for peanut miRNA research both on gene and miRNA-based improvement of peanut quality and resistance to environmental stress.

2. Molecular marker development and application

Several studies have focused on general marker development especially polymorphic markers from peanut. Many other studies have emphasized the application of different type of molecular markers, such as construction of peanut linkage maps, analyses of genetic diversity and affinity, distinguishing different cultivars or subspecies, or tagging important agronomic traits, for example, disease resistance using specific markers.

(1) Marker development

DNA markers have significant advantages to compare with protein or phenotypic markers. Compared with major crops such as maize, wheat, soybean, rice and even cotton, peanut has a very limited number of molecular markers available, which hinders the progress of molecular-based breeding. Several research groups are working on peanut marker development. Wang et al. (2007) established a highly simplified peanut SSR discovery protocol and they identified 119 SSRs using this method. Liang's laboratory and their collaborator developed large number of EST-SSR markers from peanut EST sequences (Guo et al., 2009; Liang et al., 2009b; Liang et al., 2009c). From 780 SSR-containing ESTs, 881 SSRs were identified, from which 251 primer pairs could generate amplification products. Interestingly, they found 26 and 221 SSRs exhibited polymorphism in cultivated and wild type peanut, respectively (Liang et al., 2009b). Because of the limited number of peanut ESTs available in databases, the same group tried to develop SSR markers from the huge number of soybean ESTs and to determine whether these markers could be used in peanut. The results showed that 12.4-15.7% of the soybean SSRs could be amplified successfully in peanut (Hong et al., 2010a). Our group discovered 841 EST-SSRs from immature seed EST sequences; Thirty three SSRs were selected for polymorphism analysis and most of them could amplify polymorphic bands among 25 wild type peanuts. However, very limited diversity could be detected in more than 70 cultivated peanuts using these SSRs (Song et al., 2010). Wang and colleagues identified 3104 SSRs from more than 80 K peanut ESTs downloaded from GenBank and 12 K ESTs from a high oleic acid containing peanut cDNA library (Wang et al., 2009). Markers developed from these studies contributed a large portion of peanut molecular markers currently available in China. There are many other studies focusing on identification of markers that may associate with a specific trait.

Peanut diseases such as peanut rust, late leaf spot, bacterial wilt and root knot nematodes cause serious defects in growth, while *Aspergillus flavus* infection and toxin accumulation contaminate peanut products. Identification of disease resistance loci and innovation of highly resistant germplasms is always a major focus of peanut research. Several studies in China have reported attempts to discover molecular markers linked to the resistance trait. For example, two SSR markers with the genetic distance of 4.42 cM and 7.40 cM to a root knot nematode resistance trait were discovered by analysis of an F2 population derived from Huayu-22 and D099 (Wang et al., 2008). Through analysis of germplasms with varied levels of bacterial wilt resistance, AFLP and SSR markers linked to the resistance phenotype were identified (Jiang et al., 2007a). By crossing the bacterial wilt resistant line Yuanza 9102 with the susceptible line Chico, recombinant inbred lines (RILs) were developed through a single seeded descent method. Combining polymorphic DNA markers with evaluation results of bacterial wilt resistance in F6 and F7 populations, two SSR markers were identified that associated with bacterial wilt resistance. However, the distances between these markers and the resistance loci was very large (10.9 cM, 13.8 cM) (Jiang et al., 2007b). An F2 segregation population derived from Yuanza 9102 (a rust susceptible line) and ICGV86699 (a rust resistant variety) was employed to screen AFLP markers linked to rust resistance loci. Two AFLP markers were found to associate with the resistant loci (Hou et al., 2007). By analysis of an F2 population derived from the cross of ICGV 86699 (resistant to late leaf spot) and Zhonghua-5 (susceptible to late leaf spot) three AFLP markers that linked to late leaf spot resistance trait

were identified (Xia et al., 2007). A RILs population derived from the bacterial wilt resistant variety Yuanza 9102 and the susceptible variety Zhonghua-5 was used to discover markers linked to disease resistance trait (Jiang et al., 2003). By analyzing this RILs population two AFLP markers linked to the resistance trait were identified (Ren et al., 2008). Chen et al. (2008) evaluated the susceptibility of 79 wild type peanuts to bacterial wilt and found that nearly 20% of the accessions tested showed high resistance. The relationship of these wild peanuts to the cultivated peanut was accessed by SSR. These results would be very useful for marker assisted selection during the peanut breeding. Most of these studies were carried out by Liao's group in Chinese Academy of Agricultural Sciences.

Identification of markers linked to *Aspergillus flavus* infection was also a focus of peanut molecular maker investigations, and AFLP and SCAR markers were identified (Lei et al., 2005; Lei et al., 2006). Hong et al. (2009b) reported the identification of five SSR markers that are highly associated with resistance to *Aspergillus flavus* infection. One of the markers, pPGSseq19D9, could distinguish all resistant cultivars from susceptible ones. For more detailed information about *Aspergillus flavus* infection and related biotechnological studies in this area, please refer to two recent review articles (Liao et al., 2009; Liang et al., 2009a).

(2) Genetic diversity analysis using DNA markers

Several experiments were designed to analyze the genetic diversity of peanut with different types of molecular markers. Ye et al. (1999) analyzed genetic variation using 20 RAPD primers and found 132 polymorphic bands, accounting for more than 70% of total amplified RAPDs. From these results, 12 different peanut cultivars could be distinguished as different groups. AFLP fingerprinting analysis showed that considerable diversity exists between peanut cultivars from different area of China (Chen et al., 2003). The genetic diversity of 31 peanut germplasms with various level of bacterial wilt resistance was analyzed using AFLPs, and the results showed that pairwise distances among 31 genotypes ranged from 0.06-0.57. The bacterial wilt susceptible Chico genotype was highly dissimilar from all other resistant genotypes (Jiang et al., 2007b). It was reported that SSR markers could detect more polymorphisms in cultivated peanut than other types of makers (Hopkins et al., 1999). SSR markers have been used widely to analyze peanut genetic diversity in China. Han et al., (2004) reported SSR polymorphisms among peanuts with diverse origins and even among different market types. Four SSR markers could differentiate 21 out of the 24 peanut genotypes tested. Another study was carried out using 34 SSR markers to analyze the genetic diversity of 96 peanut accessions belonging to 4 botanical varieties (Tang et al., 2007). About 50% of these SSR primers could amplify polymorphic bands from these accessions. The results showed that peanuts belonging to each of the four botanic types can be further distinguished as sub-groups (Tang et al., 2007). A similar analysis using 110 SSR primers was performed using 28 accessions of cultivated peanut including var. *fastigiata*, var. *hirsuta*, var. *hypogaea* and var. *vulgaris*. More than 40% of these primers could amplify polymorphic bands from these peanuts. These SSR makers could divide these peanut into different groups, which agreed with classification results based on morphological characteristics (Hong et al., 2008). Jiang and colleagues investigated genetic variation among bacterial wilt resistance and susceptible genotypes with SSRs, and revealed genetic distances among these peanuts ranging from 0.12 to 0.94, which was larger than what was reported from AFLP results (Jiang et al., 2007a). Inter-simple sequence repeat (ISSR) also has been used to investigate genetic diversity. Yin and colleagues analyzed genetic diversity from 24 peanut germplasms; however, the genetic variation detected was not significant between most of the germplasms tested. Compared with widely grown peanut varieties, the native varieties showed more genetic variation (Yin et al., 2010). These results provide useful information for utilizing peanut germplasm in future breeding programs.

(3) Construction of linkage maps and QTL/gene analysis

Attempts by the peanut research community to make a fine genetic map started nearly two decades ago but progressed slowly. Low levels of polymorphisms in peanut at the DNA level negatively impacted progress in linkage map construction, especially with cultivated peanut varities. Because only a few peanut maps initially were reported worldwide, I would like to provide a brief introduction of these early maps before summarizing recent advances in this area in China. Using an F2 population from a cross between two AA genome wild type *Arachis* (*A. stenosperma* and *A. cardenasii*), the first peanut linkage map which contained 117 RFLP markers was constructed (Halward et al., 1993). Later, an improved RFLP map containing 370 markers was constructed using a backcrossed population, in which the donor parent was a synthetic amphidiploid [*A. batizocoi K9484* x (*A. cardenasii GKP10017* x *A. diogoi GKP10602*)]. This map covered a total of 2210 cM with an average distance between markers of less than 6 cM (Burow et al., 2001). The third peanut linkage map was constructed using an interspecific diploid backcrossed population [*A. stenosperma* x (*A. stenosperma* x *A. cardenasii*)], which contained 167 RAPD markers and 39 RFLP markers (Garcia et al., 2005). The fourth peanut linkage map (F2 population of *A. duranensis* and *A. stenosperma*) contained 170 SSR markers and covered 1230.89 cM with an average distance of 7.24 cM between adjacent markers (Moretzsohn et al., 2005).

Only very recently, two maps from cultivated peanut were reported independently by an Indian and a Chinese group (Varshney et al., 2009; Hong et al., 2010b). Varshney et al. (2009) used a mapping population derived from two cultivars, TAG24 and ICGV86031 to produce a map containing 135 SSR markers, which mapped to 22 linkage groups. The small number of polymorphic markers available for cultivated peanut is a major limiting factor for constructing a map with a higher marker density. In order to integrate more makers in the map, Liang's group constructed 3 mapping population by using one female parent and three different male parents (Hong et al. 2010b). They developed 3 maps independently using polymorphic markers, and then joined these maps based on 93 common loci using JoinMap. This composite peanut map contained 175 SSR markers with an average distance of 5.8 cM between markers (Hong et al., 2010b). So far, this is the most detailed map reported for cultivated peanut. The same research group reported another map using only one mapping population, which contained 108 SSR markers in 20 linkage groups covering a total of 568 cM, and with an average of 6.45 cM between adjacent markers (Hong et al., 2009a). Jiang et al. (2007a) developed a recombined inbred line and constructed a linkage map containing 29 markers in eight linkage groups and covering 603.9 cM (Jiang et al., 2007a). One important role for genetic maps is to facilitate genes and QTLs cloning. Although a fine scale genetic map is lacking in peanut, based on the polymorphic DNA markers and current maps available, genes and QTLs controlling important traits could be analyzed. Twelve peanut traits including pod mass, oil content, protein

content, number of mature pod, number of branches, number of fruit branches, height of main axis, stem diameter, leaf length, leaf width, leaf length/width ratio and resistance to *Aspergillus flavus* invasion have been analyzed and found that they were associated with specific DNA markers (Liang et al., 2009a). The same group found that one SSR marker (PM93) was linked to peanut testa color. The genetic distance between the marker and the gene was about 5.4 cM (Hong et al., 2007).

For two major reasons, the limited number of molecular markers available and the low level of genetic diversity between different peanut cultivars, we still have long way to go to construct a fine genetic map. Consequently, effective discovery of genes through map-based cloning remains a great challenge. Therefore, development of molecular markers that are closely linked to important agronomic traits, or new DNA markers that are polymorphic among different cultivars, will be of key significance for fine map construction, gene localization, and marker-assisted selection in peanut.

3. In vitro regeneration and gene engineering

(1) In vitro regeneration

Establishment of a high efficiency *in vitro* regeneration system is a prerequisite for gene engineering based peanut improvement. Many studies have reported the establishment of peanut regeneration systems using different explants and medium compositions (Brar et al., 1994; Cheng et al., 1997; Li et al. 1997; Yang et al., 1998; Magbanua et al., 2000; Sharma and Anjaiah 2000; He and Bin, 2003; Matand and Prakash, 2007; Shan et al., 2009). Extensive studies on peanut *in vitro* culture and plant regeneration have been reported in China. These studies have addressed different issues involved in plant tissue culture, for instance, basal medium composition, types and concentrations of plant growth regulators, and type of explants as well as the culture conditions. In addition to a suitable medium composition, selection of proper explants is a key factor that influences the regeneration rate. Several types of explants such as cotyledon, hypocotyls, epicotyl and embryonic true leaf were investigated in peanut regeneration studies.

Due to the convenience of handling mature seeds, hypocotyls or epicotyls from mature seeds have been selected as explants in several studies (He and Bin, 2003; Shan et al., 2009). Briefly, mature seeds were sterilized and germinated in 1/2 Murashige and Skoog medium (Murashige and Skoog, 1962) for 2-4 days. Two cotyledons, the shoot meristem and the primary root tip were removed, leaving only the hypocotyl and epicotyl for regeneration in shoot induction medium. Multiple shoots formed at the epicotyl incision, in most cases more than 10 shoots from each epicotyl. The regenerated shoots from the epicotyl were strong and easily survived after root induction and transplant to soil (Zhuang et al., 1999; Liang et al., 2004; Zhang et al., 2006; Xu et al., 2006; Lei et al., 2009; Shan et al., 2009). The embryonic leaflet is more juvenile than other explants, so it easy to differentiate and dedifferentiate. 2,4-D were used for somatic embryo induction from leaflets. Different varieties showed different frequencies of embryogenesis (Li et al., 2005). The shoot induction rate reached 81.5% in suitable medium, but regeneration rates showed significant differences between two genotypes (Li et al., 2008). Normally, explants were collected from sterilized dry seeds or 4 day geminated peanut seedlings by removing the cotyledons and hypocotyls (Li et al., 2008). Cotyledons have been used as peanut regeneration explants in the 1990s (Li et al., 1992). Recently Gao and colleagues established an efficient regeneration system in the peanut variety Huayu 21-24 using cotyledons as explants (Gao et al., 2007).

(2) Gene transformation

Most peanut transformations reported in China have used the *Agrobacterium tumefeciens* mediated transformation method. Liang and colleagues optimized a peanut transformation method by adding tobacco or potato extract to the medium, together with the reduced form of glutathione and vitamins C and E, which significantly decreased the brownish of explants and increased regeneration rates (Liang et al., 2004). Peanut transformation efficiency was markedly improved by surfactants (MES, Methyl ester sulfonate), vacuum infiltration and double infection, and rates of positive GUS expression as high as 73.3% were achieved (Qiu et al., 2010). The cowpea trypsin inhibitor gene was transformed to peanut (Xu et al., 2003; Zhuang et al., 2003) and an increased insect tolerance of the transgenic plants was observed (Xu et al., 2003). Using embryonic leaflet from mature seed and embryonic meristem as explants, the γ-tocopherol methyl transferase gene was transformed into Luhua-11 and Fenghua-2, two widely used peanut cultivars (Liu et al., 2005). A similar method was used to transform chitinase and β-1,3-glucanase genes into peanut in order to improve disease resistance (Shan et al., 2003). However, whether the transgene changed the phenotype of the transgenic plants was not reported. To reduce the content of linoleic acid and increase the stability of peanut oil, an FAD2 gene RNAi construct was transformed into peanut (Zhang et al., 2007; Huang et al., 2008; Yin et al., 2009). Seeds from the transgenic plants showed an increased O/L ratio (Huang et al., 2008). Several major peanut seed storage proteins are allergens, for example, Arah 1 and Arah 2, which can cause serious allergic reactions in a large portion of the human population. It is extremely importance to reduce the content of these allergens in peanut. In the United States two research groups have shown that RNA interference can successfully repress the accumulation of these allergens, without causing detectable changes in viability and morphology of transgenic seeds (Chu et al., 2008; Dodo et al., 2008). These studies demonstrate an efficient method for hypoallergenic crop creation through genetic modification. Nevertheless, there is no report of peanut allergen repression by gene engineering in China, despite the fact that many allergen genes have been cloned from Chinese cultivars (Wang et al., 2008).

In some cases, it is difficult for transgenic peanuts to generate a number of health roots and, therefore, the survival rate when transgenic plants transferred to soil. Furthermore, due to poor root regeneration the growth of transgenic plants in soil is slow. Transgenic plants often flower when plant size is quite small and generate small number of seeds. To overcome these problems, our lab tried to integrate grafting into the peanut regeneration system, using germinated seedlings as stock and regenerated shoots as scions. Using this method, root induction is not necessary. Because of the healthy root system of the stock, grafted plants grow much better than controls (transgenic plants transferred to soil after root induction) (Li et al., 2009). The small sizes of both scion and rootstock make it possible to keep newly grafted plants in a controlled condition at low light intensity and high humidity, and survival rate as high as 97-100% was achieved (Li et al., 2009).

4. Peanut proteomics

Proteomics has advantages compared to genomics and transcriptomics, considering that many genes in the genome are inactive, and even genes that are actively transcribed may not be translated into functional proteins. Proteomics allows us to study relationships between the functional molecules and plant

phenotypes more specifically. High resolution two-dimensional electrophoresis (2-DE) and highly sensitive mass spectrometry (MS) are the two major technologies that make proteomics a powerful tool for studying organisms globally at the protein level. Because of its higher costs and relatively low throughput compared with genechip analysis, application of proteomics to peanut is very limited in China. A Chinese research group collaborated with USDA-ARS to carry out promising studies of peanut storage protein profiling through proteomics. They analyzed storage proteins from 12 different genotypes of cultivated peanut from runner market (*Arachis hypogaea ssp hypogaea*) and Spanish-bunch market types (*Arachis hypogaea ssp. fastigiata*) and discovered protein markers that are able to distinguish these subspecies (Liang et al., 2006). Proteomics study of imbedded peanut seeds discovered proteins that may play roles in aflatoxin contamination resistance (Wang et al., 2008). In addition, other research groups have reported preliminary studies of peanut proteomics, including establishment of a peanut 2-DE method and identification of proteins that could play roles in peanut embryogenesis (Zhang et al., 2005; Zhang et al., 2007; Shao et al., 2010).

5. Prospectives

Not only peanut is an important oil crop, it is also an important source of protein around the world. Soybean is the major source of vegetable protein in China; however, the total production of soybean in China is far from the actual national requirement for this crop. Consequently, a huge amount of soybean has to be imported each year. Peanut proteins are typically by-products of peanut oil production and can compensate for the deficit in soybean proteins if utilized properly. However, the protein content of peanut is lower than that of soybean. In addition, peanut proteins exhibit some disadvantages that negatively affect the consumption: for examples, lower levels of methionine and tryptophan, poor gel formation quality and the presence of multiple allergens. Functional genomics and biotechnological related approaches would play more and more important roles in the future for improvement of peanut protein content/quality, oil content/quality as well as abiotic/biotic stress tolerance. Therefore, it is crucial to understand the molecular mechanisms underlying seed storage protein production, oil accumulation and regulation of stress tolerance and metabolic pathways through functional genomics, proteomics and other biotechnological related studies. Very recently, the international peanut community came to an agreement to initiate whole genome sequencing of peanut. The completion of this project and the available of the whole genomic sequence data will surely help open a new era in peanut research.

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