

MA Chunlei, CHEN Liang

Research progress on isolation and cloning of functional genes in tea plants

© Higher Education Press and Springer-Verlag 2007

Abstract Tea, which has many sanitarian functions, is one of the most popular non-alcoholic soft and healthy beverages in the world. In many countries, as well as in China, tea (*Camellia sinensis*) is an important cash crop. It has great value as a source of secondary metabolic products. Molecular biology of tea plants has been one of the most active and kinetic research fields of tea science for the last decade. Isolation and cloning of important functional genes of tea plants have a critical significance on elucidating the molecular mechanism of high quality, yield and resistance, as well as genetic manipulating via biotechnological approaches for tea plants. In this paper, we introduced the research progress on the isolation and cloning of functional genes in tea plants. In addition, the brief prospect on the research of functional genes of tea plants in the near future is also given out.

Keywords tea plant (*Camellia sinensis*), functional gene, isolation and cloning

1 Introduction

Tea is one of the most important woody economic crops in China. By 2004, the acreage of tea plantation had exceeded 943 km², yielding 861 kt of made tea, all of which occupied the first position of the world. And a total production of 282 kt was exported, ranking the third of the world (<http://faostat.fao.org>). It is estimated that the annual production value is 40 billion Chinese Yuan which contributes greatly to the rural economy of mountainous areas. Tea has a great value as a source of secondary metabolic products, such as tea polyphenols, catechins, caffeine, theanine, and saponin, etc. And the same as many other plants, its growth and

development are integrative phenomena of genes expressed on different time and stages. The isolating and cloning of functional genes of potential value and studying their reflection mechanism systematically will play a crucial role in the genetic improvement of tea plants. Though, the molecular biology and biotechnology of tea plants were just initiated in 1992, in which year the first gene was cloned. However, many research achievements have been made thereafter in the research of functional gene isolation and cloning of tea plants to date.

2 Functional gene isolation and cloning

Approximate 25 full-length genes cloned from tea plants have been deposited in the GenBank so far (Table 1). Most of them are important enzymes related to tea plants' secondary metabolism, quality and stress tolerance, such as enzymes involved in polyphenols (catechins) and caffeine biosynthesis, floral aroma formation or the disease and defence.

2.1 Flavonoid biosynthesis related genes

There are lots of polyphenolic compounds in tea plants. About 15%–35% of the dry weight of tea is composed of polyphenols, of which catechins are the most abundant (about 80%). Catechins are the most valuable polyphenolic compounds in tea plants, which not only selectively inhibit the growth of cancer, but also have obvious curative effects on enhancing blood vessel obdurability, reducing blood sugar, blood fattiness, blood pressure, and defending against atheromatosis. Many important genes involved in the catechin biosynthesis pathway have been isolated, cloned and characterized so far, such as phenylalanine ammonia-lyase (*PAL*), chalcone synthase (*CHS*), flavanone 3-hydroxylase (*F3H*), flavonoid 3',5'-hydroxylase (*F3'5'H*), dihydroflavonol 4-reductase (*DFR*), anthocyanidin synthase (*ANS*) and anthocyanidin reductase (*ANR*). *PAL* catalyzes the deamination of L-phenylalanine yielding trans-cinnamic and NH₄⁺, which is the first step in phenyl-propanoid synthesis leading to catechins production. All flavonoids are derived

Translated from *Molecular Plant Breeding*, 2006, 4(3S): 16–22 [译自: 分子植物育种]

MA Chunlei, CHEN Liang (✉)
Lab for Tea Germplasm, Genetic Improvement and Molecular Biology,
Tea Research Institute, Chinese Academy of Agricultural Sciences,
Hangzhou 310008, China
E-mail: liangchen@mail.tricaas.com

Table 1 Full length genes cloned from tea plants deposited in the GenBank (<http://www.ncbi.nlm.nih.gov/GenBank/>)

Gene	GenBank accession	Length /bp	Author	Year
Flavonoid 3',5'-Hydroxylase (<i>F3'5'H</i>)	DQ194358	1 533	Rani et al.	2005
4-coumaroyl CoA ligase	DQ194356	1 767	Rani et al.	2005
Anthocyanidin reductase	AY641729	1 233	Singh et al.	2005
Trans-cinnamate 4-hydroxylase (<i>C4H</i>)	AY641731	1 352	Singh et al.	2005
Glutamine synthase (<i>GS</i>)	AB115184	1 508	Tanaka and Taniguchi	2005
	AB115183	1 595		
Ammonium transporter (<i>AMT</i>)	AB117640	1 697	Taniguchi and Tanaka	2005
	AB114913	1 702		
Anthocyanidin synthase (<i>ANS</i>)	AY830416	1 169	Singh et al.	2004
Leucoanthocyanidin reductase (<i>LCR</i>)	AY169404	1 350	Park et al.	2004
Delta-6-desaturase (<i>D6DES</i>)	AY169402	1 501	Park et al.	2004
QM-like protein	AY641733	969	Lal et al.	2004
photosystem II protein D1	AY665295	1 678	Hu et al.	2004
	AY741464	1 685		
Caffeine synthase (<i>CS</i>)	AB031281	1 405	Kato et al.	2004
β -glucosidase	AF537127	1 475	Li et al.	2004
Polyphenol oxidase (<i>PPO</i>)	AY659975	1 826	Raizada et al.	2004
Chalcone synthase (<i>CHS</i>)	AY656677	1 289	Singh et al.	2004
Flavanone 3-hydroxylase (<i>F3H</i>)	AY641730	1 365	Singh et al.	2004
Dihydroflavonol 4-reductase (<i>DFR</i>)	AY648027	1 413	Singh et al.	2004
β -primeverosidase	AB088027	1 729	Ijima et al.	2003
Violaxanthin de-epoxidase (<i>VDE</i>)	AF462269	1 562	Wei et al.	2002
Maturase (<i>matK</i>)	AF380077	2 035	Prince and Parks	2002
n-methyltransferase	AX138777	1 427	Mizuno et al.	2001
S-adenosylmethionine synthase (<i>SAM</i>)	AB041534	1 303	Feng and Liang	2000
5.8S ribosomal RNA	AF315492	653	Tang et al.	2000
PR-1 like protein	AB015047	707	Tomimoto et al.	1998
Phenylalanine ammonia-lyase (<i>PAL</i>)	D26596	2 330	Matsumoto et al.	1994

Note: If the same gene was deposited several times, only the latest accession and authors were listed in the table.

from the flavanone (2S)-naringenin which is formed from 4-coumaroyl-CoA and three units of malonyl-CoA by the consecutive activities of CHS and chalcone isomerase (CHI). Chalcone synthase is another key enzyme of the flavonoid pathway in plants, which has been purified from many plants. It catalyzes the formation of chalcone as the first stable interim production. And several other important enzymes, i.e. F3H, F3'5'H, LAR, ANS, DFR and so on, catalyze at the middle and later stages of the catechin biosynthesis process.

PAL, *CHS* and *DFR* of tea plants were all cloned by screening the cDNA library. Matsumoto et al. (1994) constructed the first cDNA library of tea plants using young tea leaves of the cultivar 'Yabukita' which is the most widely cultivated in Japan. This cDNA library established a foundation for isolating and cloning novel functional genes of tea plants. First, they screened the cDNA library using the labeled rice *PAL* cDNA as a probe, and two positive clones containing a short insert were obtained. The insert DNA was used as a probe to obtain another clone with a longer insert. After hybridization and detection, the *PAL* gene of tea plants was obtained finally. The full-length gene contained 2 344 nucleotides including a poly-A tail, and the open reading frame (ORF) consisted of 2 142 bp. A comparison of the deduced amino acid sequence from *PAL* cDNA of tea plants with those of other plants showed that the tea plant has a

higher homology to dicotyledons than to monocotyledons and gymnosperms, i.e. 87.3%, 84.3%, and 82.2% homologous with those of peas, poplars and sweet potatoes, respectively. Then a short-length probe, about 280 bp including the 3' untranslated sequence, detected three DNA fragments of different lengths, named A, B and D fragments, which were inherited according to the Mendelian monogenic ratio. Therefore, *PAL* genes identifiable by A, B and D fragments are multiple alleles.

Takeuchi et al. (1994a) used a parsley *CHS* probe labeled with [α - 32 P] dCTP to screen the cDNA library of tea plants. After four rounds of plaque hybridization and restriction analysis, three positive clones VEG13, VEG37 and VEG41 were obtained. The three clones all had an ORF of the same length, 1 167 bp, which encoded a deduced amino acid sequence of 389 residues. The ORF in VEG13, VEG37 and VEG41 showed a high degree of similarity to the sequence of the parsley *CHS*, namely, 85%, 85% and 84% identity at the amino acid level, respectively. The translated regions of the three *CHS* genes were very similar, but the 5'- and 3'-untranslated regions of them were rather dissimilar. Furthermore, they (Takeuchi et al., 1995) reported that the mRNA levels of *CHS* and *PAL* decreased in the shaded tea leaves. This result suggested that shading would reduce the expression of the *CHS* and *PAL*. Recently, some other crucial genes involved in flavonoid biosynthesis; for instance, *LAR*, *C4H*,

ANS and *F3H* had also been cloned (Table 1). These studies will possibly play an important role in manipulation of flavonoid biosynthesis with biotechnological methods in the future.

Polyphenol oxidase (PPO) is another important enzyme in tea plants, especially for made tea quality. High PPO activity is necessary for the fermentation process of black tea, and it should be deactivated promptly in the green tea making process. It is difficult to regulate the activity of PPO through conventional methods. So, if there is a way to adjust it using genetic engineering techniques, this will have an important theoretical significance and practical value. The *PPO* of tea plants was first cloned by Zhao et al. (2001). The conserve sequences of previously published *PPO* genes were used to design the degenerate primers and reverse transcription by using the nest-PCR method. Obtained *PPO* of tea plants consists of 1 006 bp and encodes a protein of 335 amino acids, which has a high homology with those found in other plants, especially copper binding regions. According to phylogenetic tree analysis, the *PPO* of tea plants can be clustered in the same group with most other woody plants (Zhao et al., 2001). Presently, the full-length cDNA of *PPO* consisting of 1 826 bp has been deposited to the GenBank database.

2.2 Purine biosynthesis related genes

Purine alkaloids, mainly caffeine, as well as theobromine and theophylline, are flavor chemicals and rich in tea plants. And almost 99% of total caffeine exists in tea leaves (2%–5% of the dry weight). The benefits of drinking tea, including excitation, detoxification, diuresis and so on have crucial relationships with caffeine in tea (Li et al., 2004a). Up to now, two key genes of caffeine biosynthesis, S-adenosylmethionine synthase gene (*SAM*) and caffeine synthase gene (*CS*), have been cloned.

S-adenosylmethionine synthase catalyzes the conversion of methionine to S-adenosylmethionine. It is the exclusive methyl donor of caffeine biosynthesis in tea plants. In addition, it serves as a methyl donor in many transmethylation reactions involving a wide range of acceptor molecules, and precursor of ethylene and polyamine biosynthesis in plants. Therefore, the isolation of the *SAM* established a good foundation for further study on stress physiology, senescence physiology and caffeine biosynthesis in tea plants. Feng and Liang (2001) synthesized the first strand of cDNA with the total RNA extracted from tea leaves, then the principal fragment, 3'-end fragment and 5'-end fragment of *SAM* were amplified with the first strand cDNA as template and three pair primers by the PCR method. Complete *SAM* gene sequence was obtained by BLAST comparison of the three fragments and splicing according to the overlapping regions. The complete cDNA sequence was 1 303 bp in length with an opening reading frame encoding 394 amino acids. The cDNA sequence showed a significant homology to the *SAM* genes from other plants.

There are only three N-methyltransferases in tea plants, 7-N-methyltransferase (7-NMT), 3-N-methyltransferase (3-NMT) and theobromine N-methyltransferase (1-NMT), respectively. Due to many properties of 3-NMT and 1-NMT are homologous, some researchers regard them as one enzyme, called caffeine synthase (CS). Kato et al. (1999) extracted caffeine synthase from young tea leaves for the first time, and a systematical research on its properties was conducted. Subsequently, they used the rapid amplification of the cDNA ends (RACE) technique with degenerate gene-specific primers based on the amino terminal sequence of caffeine synthase to obtain the *CS* gene finally. The isolated cDNA, termed TCS 1, which consisted of 1 438 bp, contained a 1 310 bp OFR and encoded a protein of 369 amino acids. Importantly, the deduced amino acid sequence of TCS 1 shared 41.2% sequence homology with the salicylic acid O-methyltransferase. When cDNA was expressed in *Escherichia coli*, the substrate specificity of the recombinant enzyme was very similar to that of the endogenous enzyme purified from tea leaves. The cloning of the *CS* gene was an important advance towards the development of transgenic caffeine deficient *C. sinensis* and *Coffea arabica* plants through antisense messenger RNA technology or by gene silencing (Kato et al., 2000).

2.3 Floral aroma formation related genes

Floral tea aroma is one of the most important factors to determine the character and quality of each made tea, especially oolong tea and black tea. It is not only influenced by the conditions of different climates and soils but also the manufacturing process. More and more attention is paid to the study on the technology of enhancing tea aroma, and most of floral aroma compounds are produced by endogenous enzymes during the tea manufacturing process of withering, rolling, and fermentation. The β -glucosidases and β -primeverosides are two of the most important endogenous enzymes for the tea floral aroma.

The β -glucosidases belongs to hydrolase, which almost naturally exists in all the plants. Takeo et al. (1981) found that the synthesis of terpenol components (linalool and geraniol) related to the tea aroma was restrained when Hg^{2+} and delta-gluconolactone, two kinds of inhibitors of β -glucosidases, were added into the leaf slurry of tea. But when the β -glucosidases was added into the heat treated slurry of tea, linalool and geraniol could be detected again. This result demonstrated the existence of monoterpene alcohols in the form of polyglycoside in tea plants. Decades after that, a lot of glycosidic components as aroma precursors were isolated and identified from green tea, oolong tea and fresh leaves of tea plants, respectively. And a new approach of tea aroma component formation that β -glucosidases and β -primeverosides took part in the hydrolyzation of polyglycoside and released the floral aroma was basically established. Li et al. (2004b) cloned the complete cDNA sequence of the β -glucosidase of tea plants. Its full-length was 1 475 bp, and

shared 40%–60% similarity to the corresponding parts of β -glucosidase genes from other plants in nucleotide sequence. Its secondary structure contained 14.33% α -helical conformation, 25.43% β -sheet conformation and many functional domains of amino acid. The *E. coli* expression product of this gene was a 63 kD recombinant protein, which possessed a normal bioactivity and could catalyze the dehydration of the glycosidic bond. This research established a foundation for the production of β -glucosidase through microbial engineering with a lower price.

The complete cDNA sequence of another similar important gene, β -primeverosidase genes, was isolated by Mizutani et al. (2002). The β -primeverosidase from tea plants is a unique disaccharide-specific glycosidase, which hydrolyzes aroma precursors of β -primeverosides to liberate various aroma compounds, and the enzyme is deeply related with the floral aroma formation in oolong tea and black tea during the manufacturing process. Mizutani et al. (2002) purified the β -primeverosidase from the fresh leaves of green tea cultivar 'Yabukita', and determined its partial amino acid sequences. The β -primeverosidase cDNA has been isolated from a cDNA library of 'Yabukita' using degenerate oligonucleotide primers. The cDNA insert encodes a polypeptide consisting of an N-terminal signal peptide of 28 amino acid residues and a 479-amino acid mature protein. The β -primeverosidase protein sequence was 50%–60% identical to β -glucosidases from various plants and was classified in a family 1 glycosyl hydrolase. The mature form of the β -primeverosidase expressed in *E. coli* was able to hydrolyze β -primeverosides to liberate a primeverose unit and aglycons, but did not act on the 2-phenylethyl β -D-glucopyranoside. These results indicated that the β -primeverosidase selectively recognized the β -primeverosides as substrates and specifically hydrolyzed the β -glycosidic bond between the disaccharide and the aglycons (Mizutani et al., 2002). Meanwhile, the function of β -primeverosidase in biodefense in tea plants was also worth studying.

An absolute quantification method for gene expression in tea plants using real-time PCR analysis was established, and the spatio-temporal expression patterns of the β -primeverosidase and β -glucosidase genes in different leaves of cv. Longjing 43 young shoot were determined using this method (Zhao et al., 2006). The highest β -primeverosidase expression was in the second leaf of the 'five and a bud' shoot. On the other hand, the fourth leaf of the 'five and a bud' shoot had the highest copy number of β -glucosidase mRNA. So it was concluded that the expression level and pattern of these genes vary in different leaf positions.

2.4 Theanine biosynthesis related genes

Theanine, an exclusive amino acid, almost exists in all the organs and tissues of tea plants, which is one of the primary substances influencing the taste of green tea. Plenty of researches demonstrated that theanine was first synthesized in the roots of tea plants, and then accumulated to the tender

shoots where the content of theanine was the highest. It has a lot of medical functions. Sugiyama and Sadzuka (1999) found the combination of theanine with doxorubicin could significantly inhibit hepatic metastasis of M5076 ovarian sarcoma and suppress the increase of liver weight. Further investigation suggested that theanine increased not only the antitumor activity on primary tumors but also the metastasis-suppressive efficacy of doxorubicin. Up to now, only one gene related to theanine biosynthesis was cloned, namely glutamine synthase gene (*GS*). It is a key enzyme which has an important function in the assimilation of ammonia generated by numerous plant processes and has many isoenzymes in higher plants. According to these isoenzymes present in different subcellular compartments, they are divided into cytosolic GS (*GS1*) and chloroplast GS (*GS2*). And the distinct, cell-specific patterns of expression conferred by the promoters for chloroplast *GS2* and cytosolic *GS1* indicate that the corresponding GS isoforms perform separate metabolic functions (Edwards et al., 1990).

2.5 Energy metabolism related genes

The process of green plants is to utilize their specific pigment chlorophyll to absorb solar energy, convert carbon dioxide and water into organic substance containing the energy, and release oxygen as a byproduct of photosynthesis. It is arguably the most important biochemical pathway, since nearly all organisms depend on it. Two genes related to photosynthesis have been cloned in tea plants, photosystem II protein D1 and violaxanthin de-epoxidase genes (*VDE*).

Solar energy is the foundation of photosynthesis of higher plants, but excessive energy can result in photo-oxidative damage to photosynthetic apparatus as well as to a range of other essential cell components. There are many mechanisms to protect photosynthetic apparatus against the adverse effects of excessive light in higher plants, and the xanthophyll cycle-dependent energy dissipation is one of the important mechanisms. Now the VDE has been best studied. When photosynthetic organisms absorb more light than that can be utilized through photosynthesis, violaxanthin will be converted rapidly via the intermediate antheraxanthin to zeaxanthin by catalysis of VDE, and this reaction can be reversed by catalysis of zeaxanthin epoxidase (*ZE*) under low light levels. The recent researches show that zeaxanthin can dissipate excitation energy exceeded in photosynthetic apparatus in the form of heat radiation, which protects the photosynthetic apparatus against damage. Therefore, VDE was believed to be a key enzyme in the xanthophyll cycle-dependent photo-protective mechanism. Wei et al. (2003) designed a pair of degenerate primers according to the high conserved amino acid sequences in the GenBank. Then RT-PCR, 3'/5'RACE and full-length PCR were used to isolate the full-length cDNA of *VDE* gene from young leaves of tea plants. Analysis of the cloned complete cDNA, which has 1 632 bp, showed that it encompassed an ORF with 1 422 bp encoding 473 amino acid residues. The alignment of the deduced amino acid of mature

protein with other high plants showed a high identity, for instance 83.5%, 82.7%, 82.1% and 83.6%, with *Arabidopsis thaliana*, *Lactuca sativa*, *Spinacia oleracea* and *Nicotiana tabacum*, respectively. Meanwhile, the lipocalin signature of *VDE* was found in the Swiss-Prot protein database. Subsequently, glycine and tryptophan, the most conserved sites in lipocalin signature, were site-mutated to leucine and tyrosine, respectively, by using over-lap extension. Experiment results showed the bioactivity of glycine-leucine mutant or tryptophan-tyrosine mutant was very low. It was demonstrated that the lipocalin signature was one of the most active domains in *VDE* (Wei et al., 2004).

2.6 Stress tolerance related genes

With the increase of world population, enhancing the yield of crops is becoming more and more urgent, and an important way is to decrease the loss caused by pests and diseases. It is estimated that the annual loss of total outputs of crops globally is up to 14%. Pests with all kinds of pathogens not only have a direct harm to crops but also make some indirect damage simultaneously. For a long time, chemical pesticides were the main means of pest prevention, but it resulted in the pollution of environments and water resources. Biological insecticides came in our eyes in this time. Now *Bacillus thuringiensis* (Bt) biological agent has become an important part of pesticides in the world, accounting for over 90% of the biological insecticide market, and the cysteine proteinase inhibitors (cystatin) is one of them. Wang et al. (2005) designed a pair of degenerate primers according to the conserved region among the known plant cystatins. A unique cDNA fragment was amplified by RT-PCR of total ribonucleic acid (RNA) extracted from the fresh leaves of tea plants (*C. sinensis* cv Longjing 43). A full-length cDNA of the cystatin gene was obtained by 3'/5' RACE. The cDNA sequence of the isolated 627 bp clone contained an ORF encoding a polypeptide of 101 amino acid residues with a predictable molecular mass of 11 kD. The deduced amino acid sequence contained the motif QXVXG conserved among most members of the cystatin superfamily. The blast search against the GenBank database indicated that its amino acid sequence had 54%–77% identity to all previously reported cystatins. Cloning of this gene will be helpful for the resistance to pests in tea plants.

2.7 Cloning of other genes

Microtubules are one of the components of the cytoskeleton, which are the polymers of α -tubulin and β -tubulin dimers and generally exist in all eukaryotic cells. Microtubules serve as structural components within cells and are involved in many cellular processes including mitosis, cytokinesis, and vesicular transport. It can also assist in regulating the sediment direction of microfilament on cell walls, which may control the growth of cells and affect the growth of the entire plant finally. The genes coding tubulin in some higher plants

have been cloned. Takeuchi et al. (1994b) first cloned a 505 bp cDNA of β -tubulin, without anti-primer sequence, from the cDNA library of 'Yabukita' using specific primer PCR. The putative amino acid sequence had a high identity to β -tubulin genes from other plants. 5.8S, 16S, and 26S rRNA are the main components of ribosome, and they play a crucial role in the research of plant origin and evolution. Ribulose-1,5-biphosphate-carboxylase (Rubisco) is a key enzyme in energy metabolism, which catalyzes the fixation of carbon dioxide in a calvin cycle pathway. Ammonium transporter (AMT) has main functions in the nitrogen absorption of plant roots. The cloning of *AMT* will be helpful for regulating its expressional level in tea leaves by biotechnology, which will make nitrogenous fertilizer utilized reasonably.

Furthermore, some other genes have been cloned recently from the tea plant (Table 1), and all of them will make it easier to study the physiological metabolism, development and quality of the tea plant on a molecular level.

2.8 Expressed sequence tag (EST) strategy isolating function genes

Except for these classic gene cloning methods, recently, with the dramatic improvement in DNA-sequencing technology, EST sequencing of cDNAs prepared from tissues with specific metabolic activities has provided a cost-effective and rapid alternative route toward the isolation of novel functional genes. As of April 2006, National Center for Biotechnology Information (NCBI) has approximately 35 550 000 entries of plant ESTs in its dbEST database, and its amount is increasing with an average of over one million entries every month. When constructing a library, we should do the best to choose the full-length cDNA, so that it could be isolated through the corresponding clone. Then comparing the ESTs obtained from the specific plant to the public database would rapidly determine the function of each gene.

Chen et al. (2005) constructed two cDNA libraries of the tea plant (*C. sinensis* cv. Longjing 43 and Anji Baicha), and then they randomly sequenced and analyzed a total of 4 320 cDNA clones from the cDNA library of tender shoots of Longjing 43. From this library, they obtained a total of 1 684 high-quality EST sequences (averaged 478 bp in length), among which 1 200 ESTs had a significant homology with the sequences in the NCBI non-redundant protein database by BlastX analysis. However, 1 008 ESTs of the known or putative functions could be assigned to 304 functional genes and they were grouped to highly abundant (10%), moderate abundant (30%) and low abundant (60%) expression, respectively. The database-matched ESTs were further classified into 12 putative cellular roles based on the functional categories established for Arabidopsis, i.e. metabolism-related category (corresponding 8.3% of total BlastX matched ESTs), energy (26.4%), cell growth and division (3.8%), transcription (3.9%), protein synthesis (17.9%), protein destination and storage (1.7%), transporters (4.7%), cell structures (10.7%), signal transduction (5.4%), disease and defence

(9.2%), secondary metabolism (4.8%), and unclear classification (3.4%). On the other hand, Park et al. (2004) analyzed the genes involved in the secondary metabolism in tea plants using suppression subtractive hybridization. A total of 588 cDNA clones from a subtractive cDNA library were randomly picked, sequenced, and analyzed. High-quality sequences were isolated for 508 of these clones. The BlastX comparisons indicated that about 8.7% of the clones encoded enzymes involved in the secondary metabolism, with a particularly high abundance of flavonoid-metabolism proteins (5.1%). And they believed the subtractive cDNA library and EST database described could represent a valuable resource for the future research aiming at improving economically important secondary metabolic characteristics in crops.

3 Prospects

For a long time, geneticists always study the organism's genotypes according to its observable phenotypes and analyze complex phenotypes to help hypothesize how individual genes function. However, along with the development of technology, the knowledge about genes becomes more and more profound. So in order to directly investigate the subtle structure, function and regulation mechanism of interesting genes, it is necessary to isolate them first. Up to now, the genomic information and gene expression patterns of tea plants have never been studied in detail. In the genome of an organism, only about 2% of the total bases make up the protein-coding portions of genes (Schuler, 1997), and the expressed sequence tag just studies the transcription products of genes correctly. They represent a snapshot of genes expressed in a given tissue and/or at a given developmental stage. To sequence the functional portions using the EST strategy is believed to be a more effective way for discovering new functional genes than the whole genome. According to the derivation of mRNA, the sequences of ESTs can be used as an indirect means of compiling gene expression profiles. This will be very helpful for finding some specific genes expressed at a particular stage or in a particular tissue. So, the large-scale generation and analysis of ESTs is an economical, rapid and highly efficient approach for discovering novel functional genes and elucidating gene expression profiles for the species whose genome is little understood, like the tea plant.

Over the past few years, with the maturity of the vector-mediated method and direct gene transfer technique, plant transformation researches have obtained a dramatic development. Many transformed plants modified for various economic traits have been cultivated cosmically; for instance, intenerate resistant transgenic tomatoes, insect resistant transgenic cotton and some disease resistant rice and soybeans. The *Agrobacterium*-mediated genetic transformation technique has become one of the commonest and maturest methods in the study of plant genetic engineering. However, due to a lower sensitivity of the tea plant to *Agrobacterium*, to

solve the problem of the lower efficiency of transformation is still a challenge. Therefore, Wu et al. (2005) optimized the particle bombardment procedure first, and then tested and compared several transformation methods, including *Agrobacterium*-mediated transformation, particle bombardment, and the combination of the two methods. Finally a higher transformation rate was obtained through the combination of *Agrobacterium*-mediated transformation and particle bombardment. Although the research of functional gene isolation and cloning had made great progress in tea plant in recent years, it was still behindhand compared with that of rice, maize or some other model plants. To our best knowledge so far, only one transgenic tea plant obtained by the *Agrobacterium*-mediated transformation of somatic embryos was reported (Mondal et al., 2001). Because tea belongs to the long life cycle, self-incompatibility and high inbreeding depression woody crops, which is limited by the development of genetic transformation techniques and the lack of suitable methods for the adventitious regeneration directly from explants, the study of the genetic improvement of tea plants becomes very difficult. So attaching importance to the isolation and cloning of key functional genes of tea plants, and simultaneously, looking for suitable methods for tea genetic transformation and tissue culture will become an important part of tea molecular biology in the future.

Acknowledgements This work was supported in part by the Zhejiang Provincial Key Science and Technology Project (No. 2004C22033) and the Scientific Research Foundation for the Returned Overseas Chinese Scholars, State Education Ministry of China (No. 2005-383, 2005-134).

References

- Chen L, Zhao L P, Gao Q K (2005). Generation and analysis of expressed sequence tags from the tender shoot cDNA library of tea plant (*Camellia sinensis*). *Plant Science*, 168: 359–363
- Edwards J W, Walker E L, Coruzzi G M (1990). Cell-specific expression in transgenic plants reveals nonoverlapping roles for chloroplast and cytosolic glutamine synthetase. *Proceedings of the National Academy of Sciences of the United States of America*, 87: 3459–3463
- Feng Y F, Liang Y R (2001). Cloning and sequencing of S-adenosylmethionine synthetase gene in tea plant. *Journal of Tea Science*, 21(1): 21–25 (in Chinese)
- Kato M, Mizuno K, Crozier A, Fujimura T, Ashihara A (2000). Caffeine synthase gene from tea leaves. *Nature*, 406: 956–957
- Kato M, Mizuno K, Fujimura T, Iwama M, Irie M, Crozier A, Ashihara H (1999). Purification and characterization of caffeine synthase from tea leaves. *Plant Physiology*, 120: 579–586
- Li Y H, Jiang C J, Wan X C (2004a). Study on the expression of caffeine synthase gene mRNA in tea plant. *Journal of Tea Science*, 24(1): 23–28 (in Chinese)
- Li Y H, Jiang C J, Yang S L, Yu Y B (2004b). β -glucosidase cDNA cloning in the tea (*Camellia sinensis*) and its prokaryotic expression. *Journal of Agricultural Biotechnology*, 12(6): 625–629 (in Chinese)
- Matsumoto S, Takeuchi A, Hayatsu M (1994). Molecular cloning of phenylalanine ammonia-lyase cDNA and classification of varieties and cultivars of tea plants (*Camellia sinensis*) using the tea *PAL* cDNA probe. *Theoretical and Applied Genetics*, 89: 671–675

- Mizutani M, Nakanishi H, Ema J, Ma S J, Noguchi E, Inohara-Ochiai M, Fukuchi-Mizutani M, Nakao M, Sakata K (2002). Cloning of β -primeverosidase from tea leaves, a key enzyme in tea aroma formation. *Plant Physiology*, 130: 2164–2176
- Mondal T K, Bhattacharya A, Ahuja P S, Chand P (2001). Transgenic tea [*Camellia sinensis* (L.) O. Kuntze cv. Kangral Jat] plants obtained by *Agrobacterium*-mediated transformation of somatic embryos. *Plant Cell Reporters*, 20(8): 712–720
- Park J S, Kim J B, Hahn B S, Kim K H, Ha S H, Kim J B, Kim Y H (2004). EST analysis of genes involved in secondary metabolism in *Camellia sinensis* (tea), using suppression subtractive hybridization. *Plant Science*, 166: 953–961
- Schuler G D (1997). Pieces of the puzzle: Expressed sequence tags and the catalog of human genes. *Journal of Molecular Medicine*, 75: 694–698
- Sugiyama T, Sadzuka Y (1999). Combination of theanine with doxorubicin inhibits hepatic metastasis of M5076 ovarian sarcoma. *Clinical Cancer Research*, 5(2): 413–416
- Takeo T (1981). Production of in linalool and geraniol by hydrolytic breakdown of bound forms in disrupted tea shoots. *Phytochemistry*, 120(9): 2145–2147
- Takeuchi A, Matsumoto S, Hayatsu M (1994a). Chalcone synthase from *Camellia sinensis* isolation of the cDNAs and the organ-specific and sugar-responsive expression of the genes. *Plant Cell Physiology*, 35(7): 1011–1018
- Takeuchi A, Matsumoto S, Hayatsu M (1994b). Amplification of β -tubulin cDNA from *Camellia sinensis* by PCR. *Bull Natl Res Inst Veg Ornament Plants & Tea, Japan* (B), 7: 13–20
- Takeuchi A, Matsumoto S, Hayatsu M (1995). Effects of shading treatment on the expression of the genes for chalcone synthase and phenylalanine ammonia-lyase in tea plant (*Camellia sinensis*). *Bull Natl Res Inst Veg Ornament Plants & Tea, Japan* (B), 8: 1–9
- Wang C X, Li Y Y, Jiang C J, Yu Y B (2005). Molecular cloning and sequence analysis on cDNA of cystatin gene from tea leaves. *Journal of Tea Science*, 25(3): 177–182 (in Chinese)
- Wei C L, Jiang C J, Tao H Z, Wan X C (2003). Cloning and bioinformatics analysis of sequence signature of violaxanthin de-epoxidase cDNA in tea plant (*Camellia sinensis* (L.) O. Kuntze). *Journal of Nanjing Agricultural University*, 26(1): 14–19 (in Chinese)
- Wei C L, Jiang C J, Tao H Z, Wan X C (2004). Site-directed mutation of violaxanthin de-epoxidase from tea plant (*Camellia sinensis*) *in vitro* and expression bio-activity assay of the mutants. *Chinese Journal of Biochemistry and Molecular Biology*, 20(1): 73–78 (in Chinese)
- Wu S, Liang Y R, Lu J L, Li H Y (2005). Combination of particle bombardment-mediated and *Agrobacterium*-mediated transformation methods in tea plant. *Journal of Tea Science*, 25(4): 255–264 (in Chinese)
- Zhao D, Liu Z S, Xi B (2001). Cloning and alignment of polyphenol oxidase cDNA of tea plant. *Journal of Tea Science*, 21(2): 94–98 (in Chinese)
- Zhao L P, Chen L, Wang X C, Yao M Z (2006). Quantitative detection of β -glucosidase and β -primeverosidase gene expressions in different leaves of tea plant (*Camellia sinensis*) by real-time PCR analysis. *Journal of Tea Science*, 26(1): 11–16 (in Chinese)