Construction of the Seed-Coat cDNA Microarray and Screening of Differentially Expressed Genes in Barley

Jin-Song PANG*, Meng-Yuan HE, and Bao LIU

Laboratory of Molecular Epigenetics, Institute of Genetics and Cytology, Northeast Normal University, Changchun 130024, China

Abstract
Some barley mutants can synthesize neither anthocyanins nor proanthocyanidins in the seed coat, which is related to several genes in locus Ant13, but the exact model of action remains unknown. We used the cDNA microarray technology with barley transcription-deficient mutant (ant13-152) that does not synthesize proanthocyanidins as the tester, and its wild type genotype (Triumph) as the driver, to study this question. Six-thousand and forty-eight clones from the wild type Morex testa+pericarp cDNA library were amplified using PCR, and the DNA fragments were spotted on commercial amino-modified glass slide as microarray. The mRNAs from the developing seed coat (8–15 days) of both the mutant and the wild-type barley plants were isolated, and labeled respectively with Cy3-dUTP and Cy5-dUTP when reversely transcribed to cDNAs. The labeled cDNAs were used as probes, mixed at the same molar concentration, and hybridized with the DNA fragments on the slide. Seventy clones exhibiting marked differential expression (ratio>4) were identified from the microarray. All the 25 cDNA clones that showed an over-expression in wild type in comparison to the mutant ant13-152 were sequenced. It was found that most of these over-expressing clones were transcription/translation and hordein-associated genes. These results have laid a solid material basis for further elucidation of the metabolic pathway in proanthocyanidin synthesis in barley and likely other plants.

Key words  barley; seed coat; proanthocyanidin; cDNA microarray

Proanthocyanidins are dimeric or polymeric condensation products of the flavonoids, including catechin, epicatechin or gallicatechin with leucocyanidin, leucopelargonidin or leucodelphinidin [1]. They are prominent colorless compounds, and are found widely existed in the bark of trees, leaves, fruits, flowers and seed coats. They have many natural functions, such as antioxidant properties [2] and insect resistance [3]. In forage, they can bind and precipitate dietary proteins, thus protect the animals from rumen bloat [4] and protect against bacterial deamination in the rumen [5]. There are more than 70 naturally occurring flavan-3-ols reported, with catechin, epicatechin and gallicatechin are particularly widespread in many plant species. Proanthocyanidins have been reported to present in ferns, conifers, monocots and dicots [6].

The proanthocyanidins in the testa layer of barley are involved in plant defenses against biological and environmental stresses. Unlike other compounds as phytoalexins that are often responsible for pathogen attack around the site of infection [7], the proanthocyanidins are constitutively synthesized. They play an important role in resisting the infections to Fusarium. In vitro infection tests showed that most of the proanthocyanidin-free mutants were more sensitive to Fusarium than their wild type mother varieties, except ant18-159 which accumulating dihydroquercetin in the testa layer as a strong inhibitor [8].

In barley, proanthocyanidins are exclusively synthesized in the seed coat of developing grains from 8 to 30 days after anthesis. They would cause beer chill-haze if they were not removed by chemical stabilizers. Some of the induced proanthocyanidin-free barley mutants are unable
to produce anthocyanins and/or proanthocyanidins. The enzymes responsible for these mutants have been localized to the different steps in the flavonoid pathway. The synthesis of proanthocyanidins in barley is controlled by the Ant genes. Many ant mutants have been induced and identified, which are found to be blocked at different steps of the flavonoid pathway. For example, ant 13, ant 17 and ant 18 mutations can block both proanthocyanidin and anthocyanin syntheses, mutants in ant 19 are blocked only in the proanthocyanidin synthesis, whereas mutants in ant 1 to ant 12 and ant 14 to ant 16 are blocked only in anthocyanin synthesis [9]. Several genes are silenced in the ant 13 mutant, which prevents the gene transcription which is responsible for precursors malonyl-CoA and p-coumaroyl-CoA to (+)-2,3-trans-3,4-cis-leucocyanidin. Mutants of ant genes are good materials for studying the enzymes converting dihydroquercetin to leucoan-
cthocyanidin, for the biosynthesis of dihydroflavonol-4-
reductase must have been blocked. Labeled leucocyanidin thocyanidin, for the biosynthesis of dihydroflavonol-4-
reductase must have been blocked. Labeled leucocyanidin feeding test indicated that the ant 13 gene encodes a transcription factor required both for the pathway forming leucocyanidin and for the last two steps synthesizing catechin and the procyanidin dimer B-3.

There are different genes in the steps producing the key enzymes responsible for the biosynthesis, such as ant 13, ant 17, ant 18, etc.. The mutant line ant13 is most remarkable as it fails to synthesize both anthocyanins and proanthocyanidins. The ant 13 mutant alleles have been shown to prevent the transcription of several genes in the flavonoid pathway from the precursors malonyl-CoA and p-coumaroyl-CoA to (+)-2,3-trans-3,4-cis-leucocyanidin. Although large amount of work has been done on the barley flavonoid pathway, the process of converting leucocyanidin to proanthocyanidin is still largely unknown [10]. A mutant ant13-152 is proved to be a transcription deficient mutant. This indicates that the ant 13 gene encodes a transcription factor required both for the pathway forming leucocyanidin and for the last two steps synthesizing catechin and the procyanidin dimer B-3.

The cDNA microarray technology is a powerful novel approach for high-throughput screening of differentially expressed genes between a mutant and its wild type [11,12]. Briefly, this technique involves the following steps. (1) PCR-amplified DNA fragments are spotted on glass slide at high density, creating a microarray containing thousands or even more than tens of thousands spots. (2) Two or more sources of mRNA are isolated, and labeled with different fluorescence dyes during cDNA synthesis or downstream modifications. (3) The probes are mixed and competitive hybridizations performed with the PCR amplified DNA fragments fabricated on the slide. (4) The relative expression levels are determined by the kinetics of hybridization based on the ratio with which each probe hybridized to an individual array element. (5) After confocal scanning and data processing, differential expressed genes’ cDNA spots can be found on microarray slide. (6) The differentially expressed genes can be acquired by retrieving back to the DNA library [13,14].

This paper aimed to establish a high-throughput technical platform based on the microarray technology, and to identify the genes responsible for encoding the key enzymes in the last steps of the flavonoid biosynthetic pathway in barley.

Materials and Methods

Plants

The wild type barley cultivar Morex is used to construct a barley seed coat-specific cDNA library. The wild type barley cultivar Triumph and its mutant ant13-152 are used as tester and driver respectively to identify the gene expression differences. All the plants were planted in green house at 22–25 °C, with 16 h light/8 h darkness light cycle. Immature heads were harvested 7–14 days after anthesis.

Construction of barley testa+pericarp cDNA library

4.6 g of immature testa+pericarp from barley wild type Morex were used for total RNA extraction, according to Tesniere & Vayda protocol [15] with some modification. The mRNA was isolated from 1 mg Morex testa+pericarp total RNA with PolyATtract® mRNA isolation system (Promega), following the standard protocol. The cDNA synthesis, packing and plating were carried out with the ZAP-cDNA gigapack III gold cloning kit (Stratagene). Primary λ phage library was constructed by ligating 20 ng of cDNA and 1 µg of vector, packed with Stratagene gigapack III gold packaging extract twice.

PCR amplification of cDNA library and products clean-up

5 µl out of 50 µl bacterial lysate of each clone were used as template in PCR amplifications. pfu DNA polymerase and M13 primers were used in all PCR reactions. The PCR program was: 94 °C 2 min; then 94 °C 30 s, 50 °C 30 s, 72 °C 2 min for 35 cycles; 72 °C extension for 8 min. Millipore 96-well multiscreen filter plates (Millipore MAFB NOB 10) were used to purify the PCR products. Dissolve the DNA with 40 µl 3× SSC.
Microarray printing and post-processing

Products of PCR amplified cDNA clones, as well as 3 known gene fragments (chalcone synthase, dihydroflavonol-4-reductase and the Rubisco small subunit) as positive controls and plasmid pUC19 as the negative control, were spotted on the substrate. DNA fragments were fixed at 240 mJ·cm⁻² UV energy with Stratelinker, and baked at 80 °C for 80 min. Before hybridizing with probes, the slides were treated with blocking buffer.

Probe preparation

Total RNA was extracted from seed coat (10–14 days old) of wild type barley (H. vulgare cv. Triumph) and its mutant (ant13-152) by the modified pine tree protocol [16]. The mRNA was isolated by two rounds of poly(A) selection using Oligotex resin (Qiagen) batch protocol from the total RNA. During reverse transcription, Cy3-dUTP and Cy5-dUTP were incorporated into the cDNAs. Microcon YM30 filter was used to purify the probes. Dye swap was applied to avoid incorporation bias.

Hybridization and post-hybridization processing

Mix the concentrated probes with 9.8 µl 10× SSC, 2.4 µl tRNA (2 µg/µl) and 8.4 µl 1% SDS. The probes were then heat denatured and spun cool. The supernatant was applied on the microarray and hybridized at 60 °C overnight. After hybridization, the slide was washed with washing solutions and spun dry.

Subtraction hybridization with dihydroflavonol-4-reductase probes

The dihydroflavonol-4-reductase (Dfr) gene is highly expressed in the wild type barley seed coat while maturation. In order to avoid the interference of Dfr like genes, Cy3-dUTP labeled Dfr gene fragment was prepared with PCR incorporation: the template was clone KFP158 with full length barley Dfr gene insert, primers were M13fwd and Dfr-D7 (5'-GCATGCAAGGAGGCCGGCAC-3'). The PCR program is 94 °C 30 s; then 35 cycles, 94 °C 30 s, 50 °C 30 s, 72 °C 30 s; 72 °C 5 min. After amplification, purify the products once with Millipore microcon YM-30 filter and measure the yield with spectrophotometer. Hybridize the Dfr-probes with microarray.

Data acquisition, normalization and analysis

After hybridization, the microarrays were scanned with ScanArray 4000XL scanner. All the data collection and analysis were done with QuantArray. The raw data were normalized with Lowess algorithm. The mean value for each spot from different experiments was accepted as the standard value. In order to simplify the procedure, all spots with ratio between 0.4 and 2.5 were regarded as yellow, spots with a ratio higher than 2.5 were regarded as green, and spots with a ratio lower than 0.4 were regarded as red (Fig. 1). The spots were retrieved back to the original cDNA library, and clones specifically expressed in wild type were sequenced.

Results

Characterization of the barley seed coat (testa+ pericarp) cDNA library

The lambda ZAP yield is 5.0×10⁶ pfu in 1000 µl SM buffer (3.75&1.25). Totally, 6048 clones were picked up by the Qpix-II automatic clone picker.

Subtraction with Dfr gene

The Cy3-dUTP labeled 1.3 kb Dfr gene fragment was hybridized with the slide. The ratio of signal/noise for each spot was calculated, and the spots with more than 10 folds S/N to mean value were marked as highly homologous to Dfr gene. Two-hundred and sixty-eight PCR fragments were identified in this way (data not shown but available upon request). In the early studies, the Dfr gene has been found to express exclusively in wild type barley [17]. Nevertheless, this gene is known to be not involved in the condensing of anthocyanin to proanthocyanidin process [9]. Therefore, all clones hybridizing to the Dfr probe (Fig. 2) were eliminated from further analysis.

Data acquisition

Twenty-five wild type-specific expressed cDNA clones and 45 mutant-specific expressed cDNA clones were identified through the experiment.

Sequencing of the clones with differential expression in the wild type versus the mutant

It is supposed that all the genes involved in the flavonoid pathway are expressed in wild type barley, whereas some of them failed to express in the ant13 mutant. To find out what genes are specifically expressed in the wild type barley, and which are likely involved in anthocyanins and proanthocyanidin biosynthesis, all the 25 wild type highly expressed clones were sequenced. It was found that some of the clones contained several kinds of genes with known functions, including barley B3-hordein, barley 60S ribosomal L12, barley pyruvate orthophosphate dikinase, but
levels will significantly decrease the biosynthesis in the testa/pericarp. Clone 11 is the barley mRNA for putative calmodulin binding protein. It is not previously found in barley, but the homology search has annotated its potential function. Clone 12 is a homologue to rice epsilon1-COP (87% identity), which has the protein transporter activity. Clone 13 is weakly similar to apyrase, whose function is unknown. Clone 14 encodes a BEL1-related homeotic protein. Clone 16 is poly(A)-binding protein II-like. Clone 17 is weakly similar to MADS box transcription factor, which may possess DNA-binding property. The sequence of clone 18 is found in several barley EST/cDNA libraries, but its biological property and molecular function are not clear. The function of clone 19 is unknown. Clone 20 is an unnamed gene encoding a protein with tyrosine/serine/threonine phosphatase activity. Clone 21 is an iron-deficiency induced gene, and also encodes a homologoue to the rice submergence-induced nickel-binding protein 2A. Clone 22 is cinnamoyl-CoA reductase. Clone 23 encodes a hypothetical protein which has weakly similarity to rice phosphoinositide phosphatase. Clone 24 encodes an Acyl-CoA binding protein which is very popular in many barley EST/cDNA libraries. Clone 25 is a legumin-like protein gene, the product of which serves the nutrient reservoir activity.

Discussion

Among the genes specifically expressed in wild type barley, some possess well-known functions such as the storage protein B3-hordein and legumin genes, transcription/translation genes and energy metabolism genes. But
the functions of some other genes are not well characterized, and these genes are probably involved in the flavonoid pathway. The biosynthesis of anthocyanin and proanthocyanidin are blocked because of loss-of-function mutation of critical gene(s). In the past two decades, more than 100 barley cDNA libraries have been constructed and a large number of clones sequenced, which may explain why most of the identified sequences in this study are not new ones. But for many known sequences, their functions are not understood or not well characterized. Further analysis needs to be taken to explore the role of these genes in flavonoid pathway.

In the \textit{ant13}-152, it is regarded that mutagenesis occurs at a transcriptional regulator gene which controls the downstream condensing reactions. The enzymes have not been isolated and identified yet. In this study, we tried to identify the regulator(s) controlling the condense of anthocyanin to proanthocyanidin, and the functional genes encoding the enzymes involved in barley flavonoid pathway. Although some genes highly expressed in wild type barley have been identified, it is difficult to isolate new genes for following reasons. First, new genes may be expressed at a low level, and function through a signal magnifying system, and their presences are undetectable for the low sensitivity of cDNA microarray. Second, new genes may be highly homologous to \textit{Dfr} gene, so that they have been subtracted while hybridizing with \textit{Dfr} gene fragment, for long DNA fragment has low specificity. Third, the differential expression of the genes may be not controlled by putative regulators, but caused by other unknown factors: (i) a mutation in its promoter region makes the dihydroflavonol-4-reductase gene unable to express, so there will be a lack of leucoanthocyanidin as precursor of anthocyanin and proanthocyanidins; (ii) a

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known none-transcriptional regulatory factor mutant makes the condensation enzymes fail to be expressed; (iii) there is no any sequential change occurred in the mutant ant13-152, instead, some epigenetic changes such as DNA methylation and/or heterochromatinization result in the differential expression between the wild type and the mutant barley plant.

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