cDNA-AFLP analysis of transcript derived fragments during seed development in castor bean (Ricinus communis L.)

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ABSTRACT
Ricinoleic acid, one of the biggest components in castor oil, is an unusual fatty acid with numerous industrial applications. Despite much research on the oil content of Ricinus communis L., the genes encoding the oil biosynthesis function in the seed developmental stages are still poorly known. Here, we analyzed differentially expressed transcripts during seed development using cDNA-amplified fragment length polymorphism. Almost all ingredients significantly decreased along with seed maturation, except for ricinoleic acid, which increased greatly from 6.7% to 86.8%. A total of 534 distinguished fragments were differentially expressed in six development stages. Among these, 67 fragments showed high homology with known-function genes, and 26 sequences were successfully annotated with fatty acid synthesis or storage proteins in castor and other species. The results showed that fatty acid synthesis and accumulation in castor seeds displayed various temporal patterns. Different cDNA-fragment patterns were mainly found in later development stages, coinciding with the onset of oil synthesis.

Introduction
Castor bean (Ricinus communis L.), from the family Euphorbiaceae, is an important non-edible oilseed crop which being widely cultivated throughout tropical and sub-tropical regions with drought- and saline-tolerance properties. It has high commercial value for the chemical industry and is a feedstock for biodiesel production, as it is the only commercial source of ricinoleic acid, a hydroxylated fatty acid [1,2]. Based on its unique fatty acid composition and high seed oil content, castor has tremendous potential industrial applications [3]. Now, more than 3000 kinds of chemical products could be derived from castor oil using different chemical methods.

With the continuous development and innovation of modern science and technology, castor oil is regarded as a strategic material and is used in the production of sophisticated products. So far, the researches on the measurement of the oil content in castor seeds have reported high values from 40% to 60% [4–6]. Although researches about the biosynthesis process of castor oil started early, its related enzymes and metabolic pathways have been less extensively explored [7]. However, there are few studies on castor oil biosynthesis reported at the molecular level.

Generally, a direct approach to reveal the molecular basis of a biological system is to screen for differentially expressed genes and transcript derived fragments (TDFs) [8]. cDNA-amplified fragment length polymorphism (cDNA-AFLP) analysis is an AFLP-based transcript profiling method for identification of differentially expressed genes by purifying fragments from sequence gels and sequencing [9,10]. This method is an attractive technique for gene discovery on the basis of fragment detection and for temporal quantitative gene expression analysis [9]. From its establishment to the present time, this technology has been used in many plant species, including Arabidopsis thaliana [11], Vitis vinifera [12], Cicer arietinum L. [13] and Robinia pseudoacacia L. [14].
Previous researches showed variations in oil content and fatty acid composition of castor due to seed developmental stages [15]. However, the genes encoding oil biosynthesis have not been tested and identified yet. Here, we analyzed differentially expressed transcripts in castor during seed development using cDNA-AFLP analysis. Our findings provide an insight into genes involved in castor oil biosynthesis and serve as a basis for further and more detailed investigations for better understanding of the genetic and transcriptomic information of castor oil biosynthesis in plant cells.

Materials and methods

Plant materials

Castor seeds (accession 2129) were collected from the Academy of Agricultural Science in Tongliao, China. Then, mature and healthy seeds were cultivated in the farmland of Inner Mongolia University for the Nationalities, Tongliao. The fruits were harvested at 10, 20, 30, 40, 50 and 60 days by bagging after pollination. The morphological characteristics of castor in each development stage have been described in our previous paper [15]. The seeds were collected and stored at −80°C for subsequent experiments.

Measurement of fatty acid composition

The composition and relative percentage of fatty acids was calculated according to the corresponding chromatographic peaks. By applying the computer automatic and manual retrieval with NIST98 and Wiley of Mass Spectral Data, the fatty acid composition of castor oil was measured and analyzed [4]. The fatty acid composition and absolute content were measured as described in our previous reports [4,15].

cDNA-AFLP assay

Total RNA was extracted from seeds using TRNzol reagent according to the manufacturer’s protocol (TIANGEN, Beijing, China). RNA purity and integrity was checked via Nanophotometer (IMPLEN, CA, USA). The quantified RNA samples (1 μg) were used for double-strand cDNA synthesis using M-MLV RTase cDNA Synthesis Kit (TakaRa, Kyoto, Japan). The cDNA-AFLP protocol was adapted according to previous reports with minor modifications [16,17]. First, 150 ng of cDNA was double-digested by MseI (TakaRa, Kyoto, Japan) and EcoRI (TakaRa, Kyoto, Japan) at 37°C for 5 h, then ligated with oligonucleotide adaptors using T4 DNA ligase at 16°C for

| Development stage (days) | 100-seed weight (g) | Myristic acid (% | Palmitic acid (%) | Stearic acid (%) | Oleic acid (%) | Linoleic acid (%) | Linolenic acid (%) | Arachidic acid (%) | Arachidonlic acid (%) | Behenic acid (%) | Lignoceric acid (%) | Ricinoleic acid (%) |
|--------------------------|---------------------|------------------|------------------|-----------------|---------------|------------------|-------------------|---------------------|---------------------|-----------------|-------------------|---------------------|
| 10                       | 36.41 ± 0.03a       | 0.60 ± 0.03c     | 24.30 ± 0.23c    | 1.70 ± 0.07b    | 21.80 ± 0.58c  | 31.30 ± 0.15d    | 10.40 ± 0.17c      | 0.90 ± 0.03b       | 0.70 ± 0.06b       | 1.00 ± 0.03c     | 0.80 ± 0.06c       | 6.70 ± 0.06a       |
| 20                       | 45.01 ± 0.72b       | 0.20 ± 0.03b     | 9.50 ± 0.35b     | 1.20 ± 0.01a    | 7.40 ± 0.23b    | 19.10 ± 0.23c    | 4.10 ± 0.03b       | 0.20 ± 0.03a       | 0.50 ± 0.03a       | 0 ± 0a           | 1.10 ± 0.03a       | 0 ± 0a             |
| 30                       | 46.83 ± 0.03c       | 0 ± 0a           | 1.20 ± 0.07a     | 1.20 ± 0.06a    | 4.80 ± 0.24a    | 5.40 ± 0.23a     | 0.50 ± 0.03a       | 0 ± 0a             | 0 ± 0a             | 0 ± 0a           | 0 ± 0a             | 0 ± 0a             |
| 40                       | 48.31 ± 0.03c       | 0 ± 0a           | 1.20 ± 0.03a     | 1.20 ± 0.03a    | 4.80 ± 0.24a    | 5.60 ± 0.23a     | 0.50 ± 0.03a       | 0 ± 0a             | 0 ± 0a             | 0 ± 0a           | 0 ± 0a             | 0 ± 0a             |
| 50                       | 38.65 ± 0.04a       | 0 ± 0a           | 1.20 ± 0.07a     | 1.20 ± 0.06a    | 4.80 ± 0.24a    | 5.60 ± 0.23a     | 0.50 ± 0.03a       | 0 ± 0a             | 0 ± 0a             | 0 ± 0a           | 0 ± 0a             | 0 ± 0a             |
| 60                       | 38.41 ± 0.04a       | 0 ± 0a           | 1.20 ± 0.07a     | 1.20 ± 0.06a    | 4.80 ± 0.24a    | 5.60 ± 0.23a     | 0.50 ± 0.03a       | 0 ± 0a             | 0 ± 0a             | 0 ± 0a           | 0 ± 0a             | 0 ± 0a             |

Note: Same letters indicate statistically significant differences (p < 0.05) within each column.
overnight. The DNA amplification was conducted in a Thermal Cycler 480 (Perkin-Elmer, Norwalk, CA, USA). The ligated products were diluted in a ratio of 1:30 with ddH2O for pre-selective amplification. The pre-amplification reaction was carried out for initial denaturation at 94°C 30 s, followed by 30 cycles of amplification (94°C 30 s, 56°C 30 s and 72°C 1 min), then final extension at 72°C for 10 min. Next, the products were implemented in a 1:10 dilution for the selective amplification with 256 primer combinations. The selective amplification included a touch-down program: a scale-down of 0.7°C per cycle from 65°C. The final selective amplification products were loaded onto a 6% polyacrylamide gel containing urea and electrophoresed at 1500 V for 2.5 h, and then visualized using the silver-nitrate staining protocol according to Zhou et al. [17].

Isolation and sequencing of TDFs

The polymorphic TDFs were excised from the gel and soaked in distilled water. TDFs were re-amplified using the same primers as described for the selective amplification and cloned into a pMD18-T Vector (TakaRa, Kyoto, Japan). The sequencing of TDFs was performed by Sangon Biotech (Shanghai, China). Sequences of TDFs were analyzed for homology searching by Basic Local Alignment Search Tool in the National Center for Biotechnology Information (NCBI) databases.

Data analysis

Data are mean values with standard deviation (±SD) from three independent experiments. Statistical analysis was performed using SPSS 17.0 software package.
ricinoeate and oil synthesis. We obtained similar transcriptional activation of genes associated with the efficient accumulation of ricinoeate was related to seed development. Therefore, they thought that the expansion occurs during the mid-phase (26 days after pollination; and the endosperm tissue that the maturation of castor seeds needs about 60 industrial applications. Chen et al. [18] summarized castor oil, is an unusual fatty acid with numerous variations in fatty acid composition of castor bean in seed development stages

During the development stages of the castor bean plants, the 100-seed weight reached the highest value in 40 days, varying from 36.84 to 48.62 g. By gas chromatographic analyses, 11 major ingredients were detected in the fatty acid composition of the castor seeds, including myristic, palmitic, stearic, oleic, linolenic, arachidic, arachidonic, behenic, lignoceric acids (Table 1). Almost all ingredients showed high homology with known-function genes, which increased greatly from 6.7% to 86.8%. Interestingly, myristic, behenic and lignoceric acids were just detectable in the initial phase of seed development (10–20 days old seeds).

Ricinoleic acid, one of the biggest components in castor oil, is an unusual fatty acid with numerous industrial applications. Chen et al. [18] summarized that the maturation of castor seeds needs about 60 days after pollination; and the endosperm tissue expansion occurs during the mid-phase (26–40 days). Importantly, they detected ricinoleate after 26 days of seed development. Therefore, they thought that the efficient accumulation of ricinoleate was related to transcriptional activation of genes associated with the ricinoleate and oil synthesis. We obtained similar findings: the content of ricinoleic acid reached the highest value at about 30 days after pollination, and differentially expressed transcripts were significantly induced at later seed development stages, coinciding with the onset of oil synthesis.

cDNA-AFLP analysis

Out of 256 primer combinations, 24 primer pairs successfully produced high intensity, reproducible and polymorphic fragments. A total of 534 clear bands were differentially expressed in six seed development stages, with an average of 22.6 fragments per primer. Based on the presence, absence or differential intensity in the gels, 266 bands were identified as TDFs with unequivocally differential expression, and their sizes ranged from 50 to 2000 bp (Figure 1). These results indicated that TDFs could modify and change along with seed development.

TDFs identification and homology analysis

Among 266 TDFs, 143 fragments were successfully cloned and sequenced. Among these, 67 sequences showed high homology with known-function genes, and 26 sequences were successfully annotated to fatty acid synthesis or storage protein pathways in castor and other species (Table 2). Nucleotide sequence homology demonstrated similarity to genes with the following function categories: cysteine protease, ATP synthase, serine/threonine protein kinase, RNA-binding

Table 2. Sequencing results of transcript derived fragments at different seed development stages.

| No. | Accession number | Genbank homologs | Gene source | E value | Similarity |
|-----|------------------|-------------------|-------------|---------|------------|
| 7   | XM_002531888.1   | Conserved hypothetrical protein, mRNA | Ricinus communis | 3e–27  | 100        |
| 21  | XM_002510124.2   | Cysteine protease, putative | R. communis | 1e–26  | 94         |
| 24  | XM_002529729.1   | Proline-rich protein DC2.15 precursor | R. communis | 2e–43  | 100        |
| 30  | XM_004513749.1   | Cicer arietinum ATP synthase subunit beta 2-li | C. arietinum | 2e–09  | 100        |
| 34  | XM_002532234.1   | Conserved hypothetrical protein, mRNA | R. communis | 5e–07  | 97         |
| 38  | XM_002534007.1   | dTDP-glucose 4-6-dehydratase, putative, mRNA | R. communis | 2e–17  | 100        |
| 178 | XM_002511972.1   | Serine/threonine protein kinase, putative, mRNA | R. communis | 1e–47  | 99         |
| 185 | XM_01237522.1    | PREDICTED: serine/threonine-protein kinase | Jatropha curcas | 9e–30  | 92         |
| 164 | XM_002519704.1   | RNA-binding protein, putative, mRNA | R. communis | 2e–29  | 99         |
| 175 | XM_002516598.1   | RNA-binding protein | R. communis | 4e–32  | 100        |
| 129 | XM_002512632.1   | Transaldolase, putative, mRNA | R. communis | 8e–29  | 100        |
| 41  | XM_007652067.1   | Lipid-transfer protein/seed storage 25 albumin superfamily protein | Theobroma cacao | 6e–14  | 91         |
| 44  | XM_002515342.1   | Aspartic proteinase nepenthesin-2 precursor, putative, mRNA | R. communis | 1e–56  | 99         |
| 45  | AAC37335.1       | UDP-glucuronic acid decarboxylase 2 | Populus tomentosa | 9e–16  | 97         |
| 47  | KF793885.1       | RNA-family small GTPase gene | Rhizophora apiculata | 3e–10  | 94         |
| 48  | XM_002515028.2   | Pyruvate dehydrogenase, putative, mRNA | R. communis | 1e–13  | 100        |
| 54  | XM_002527361.6   | dTDP-glucose 4-6-dehydratase, putative, mRNA | R. communis | 2e–17  | 91         |
| 78  | XM_002527361.6   | ATP binding protein, putative, mRNA | R. communis | 2e–06  | 91         |
| 83  | XM_002528374.1   | Hypothetical protein, mRNA | R. communis | 4e–21  | 100        |
| 87  | XM_002525459.1   | Histidyl-tRNA synthetase, putative, mRNA | R. communis | 8e–23  | 100        |
| 107 | XM_002533865.1   | UDP-glucosyl transferase, putative, mRNA | R. communis | 3e–10  | 100        |
| 108 | XM_002521787.1   | ATP binding protein, putative, mRNA | R. communis | 3e–04  | 100        |
| 177 | XM_00253835.2    | Acetyl-CoA carboxylase 1 | R. communis | 2e–52  | 100        |
| 139 | XM_002510134.1   | Cysteine protease, putative, mRNA | R. communis | 2e–17  | 100        |
| 196 | XM_002510241     | Cysteine protease, putative, mRNA | R. communis | 3e–28  | 95         |
| 148 | XM_002513096.1   | Conserved hypothetrical protein, mRNA | R. communis | 2e–12  | 96         |
protein, transaldolase, lipid-transfer protein, aspartic proteinase, UDP-glucuronic acid decarboxylase, pyruvate dehydrogenase and histidyl-tRNA synthetase. Additionally, we statistically analyzed the number of TDFs in each seed development phase (Table 3). One TDF was detected in the initial three stages, whereas six, five and twelve fragments were found in the 40, 50 and 60-day-old developing seeds, respectively. This indicated that 40–60 days was the vital phase of fatty acid synthesis and accumulation in castor seeds, displaying various temporal patterns; therefore, those TDFs could be involved in the process of fatty acid synthesis. cDNA-AFLP analysis as a differential display technique could tremendously reduce the number of false positives using restriction enzymes to generate specific tags; thereby, it is a reliable, suitable and economical system to detect differentially expressed transcripts without requiring prior sequence knowledge [8,14]. Rantong et al. [19] identified differentially expressed transcripts in lace plant leaf shape during development using cDNA-AFLP, and annotated 79 TDFs related to photosynthesis, biosynthesis pathways, gene regulation and stress responses. Leymarie and Corbineau [20] reported differentially expressed transcripts involved in barley seed germination and dormancy using cDNA-AFLP, and sequenced 25 TDFs. In our study, 143 TDFs were isolated and sequenced, and almost half of these TDFs were homologous to unknown-function genes, suggesting that those TDFs could be directly or indirectly implicated in novel fatty acid synthesis or storage pathways.

Fatty acid biosynthesis in the cell uses acetyl-CoA produced by glycolysis, as a raw material for initial synthesis, along with a series of other biochemical reactions and enzymes. Acetyl-CoA carboxylase (ACC) is a rate-limiting biotinidase involved in the first committed step of fatty acid synthetic pathway. Davis et al. [21] firstly tested the role of ACC in vivo and identified that its overproduction would result in a high increase in the synthesis of fatty acids. In addition, ACC catalyzes the conversion of acetyl-CoA to malonyl-CoA, which is a key metabolite in regulating fatty acid synthesis. Turnham and Northcote [22] investigated the role of ACC in regulating fatty acid oxidation and showed that ACC was activated with an increase in malonyl-CoA inhibition of fatty acid oxidation. Intriguingly, in our study, TDFs No. 177 was differentially expressed in different seed development stages, which was consistent with other findings [22–24]. The accumulation of lipids along with a marked rise in ACC activity started from 16 days after pollination during the formation of rape-seeds [22]. Also, researchers detected and purified ACC from developing soybean seeds using chromatography analysis. In the developing castor oil seed, Simcox et al. [24] found that the rate of fatty acid synthesis decreased during seed maturation, and demonstrated a corresponding diminution in the activity of ACC.

In most organisms, the pyruvate dehydrogenase complex (PDC) catalyzes the pivotal irreversible reaction that oxidizes pyruvate into acetyl-CoA [25]. Also, Sutendra et al. [26] found that knockdown of PDC would decrease the de novo synthesis of acetyl-CoA. The properties and subcellular localization of the pea chloroplast PDC demonstrated its role in providing acetyl-CoA and NADH for fatty acid synthesis [27]. Smith et al. [28] suggested that malate and pyruvate were used as carbon sources for fatty acid synthesis, and that the activity of ACC might limit the fatty acid synthesis. Although PDC was required in fatty acid biosynthesis in castor bean, it was not necessary in the plastid to provide acetyl-CoA during seed germination [29,30]. In addition, researchers have also studied the relationship among pyruvate dehydrogenase, acetyl-CoA and fatty acid synthesis in developing seeds, suggesting a predominant role for plastidic pyruvate dehydrogenase in acetyl-CoA formation during lipid synthesis of seeds [31,32]. Here, TDF No. 48 was identified as PDC and was isolated 60 days after pollination, which was consistent with the measurements of the fatty acid content (Table 1), suggesting that fatty acid synthesis in castor bean was mainly a process in the later seed developmental stages.

The results obtained in this study could facilitate the efforts to efficiently improve the content of castor oil in breeding programmes. Given the amount of attention that castor oil with its numerous industrial applications has attracted around the world, there are hopes that molecular methods could greatly shorten

| Development stage (d) | No. (band size, bp) | Number of bands |
|-----------------------|---------------------|-----------------|
| 10                    | 148 (67)            | 1               |
| 20                    | 21 (119)            | 1               |
| 30                    | 44 (260)            | 1               |
| 40                    | 45 (73)/47 (73)/129 (103)/139 (226)/196 (102)/177 (65) | 6 |
| 50                    | 41 (230)/54 (60)/87 (85)/185 (213)/178 (132) | 5 |
| 60                    | 7 (96)/24 (190)/30 (104)/34 (58)/38 (76)/48 (69)/78 (65)/83 (82)/107 (63)/108 (52)/164 (101)/175 (104) | 12 |

Table 3. Statistical results of transcript derived fragments at different development stages.
the selective breeding time as compared to traditional breeding, which need several years.

Conclusions

In this study, we identified and isolated TDFs in the seed development of *R. communis* using cDNA-AFLP analysis, which detected many genes involved in different seed developmental phases. One TDF, identified as PDC, was isolated 60 days after pollination, and the fatty acid content significantly increased at later developmental stages. These results provided evidence that fatty acid synthesis in castor bean mainly occurred in the later seed developmental stages.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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