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Full Length Research Paper

Dynamic changes of main metabolic substances during anther-derived embryos development in loquat (*Eriobotrya japonica* Lindl. cv. 'Dawuxing')

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The main metabolic substances changes during the development process of anther-derived embryos in loquat (*Eriobotrya japonica* Lindl. cv. 'Dawuxing') were studied. These include water contents, dry mass contents, carbohydrates, soluble proteins and nucleic acids. In the developmental stages of anther-derived embryos, the fresh weight and the dry mass contents increased gradually with the anther-derived embryos development as a whole. Soluble sugars, soluble protein and nucleic acid were closely related to the development and maturation of embryos, changing significantly in metabolism at the important development turning points. Soluble sugar, fructose and starch contents had the same change trend. The two accumulation peaks appeared at globular and cotyledon stages. During the development of loquat anther embryos, the dynamics of protein synthesis were roughly in "S" shape. The two accumulation peaks appeared at globular and cotyledon stage, respectively, in accordance with the change of sugar and starch. Alkaline protein contents were higher than acidic protein contents. Alkaline protein contents and total soluble protein contents had the same change trend during the development process. DNA contents and total nucleic acid contents had the same change trend during the development of anther-derived embryos. The DNA synthesis peaks appeared at the embryogenic callus stage. RNA contents were very low at embryogenic callus stage and cotyledon stage, while DNA was actively synthesized at the two stages.

Key words: *Eriobotrya japonica*, anther culture, embryos, metabolic substances.

INTRODUCTION

Changes in metabolic activity seem to be a common process associated with embryogenic cell formation. A lot of researchers have found that accumulation of starch and other carbohydrates are closely related to somatic embryogenesis (Zhao et al., 2006). The accumulation peaks of starch and polysaccharide appear at the important turning point of embryogenesis and they provide energy for the new development phase (Zang et al., 2004; He and Qi, 2002). Changes in protein, nucleic acid and other biological macromolecules are the molecular basis for somatic embryogenesis. The metabolism of protein and nucleic acids as a dynamic process provides important information for somatic cell

differentiation (Sengupta and Raghvan, 1980). Therefore, it is essential to explore the influencing factors and molecular mechanism of somatic embryogenesis, by studying the physiological and biochemical changes during the process of plant somatic embryogenesis. Plant anther culture is an important way to obtain haploid plants. The physiological and biochemical changes in anther culture are more sensitive than the morphological changes during the process of somatic embryogenesis. Recently, the anther-derived embryos in loquat (*Eriobotrya japonica* Lindl. cv. 'Dawuxing') were obtained, and the embryogenic calluses subsequently developed into globular, heart, torpedo and cotyledonary stages of embryos (Li et al., 2008). However, few researches on physiological and biochemical changes in anther-derived embryos in loquat have been reported so far. In this research, the main metabolic substances during the

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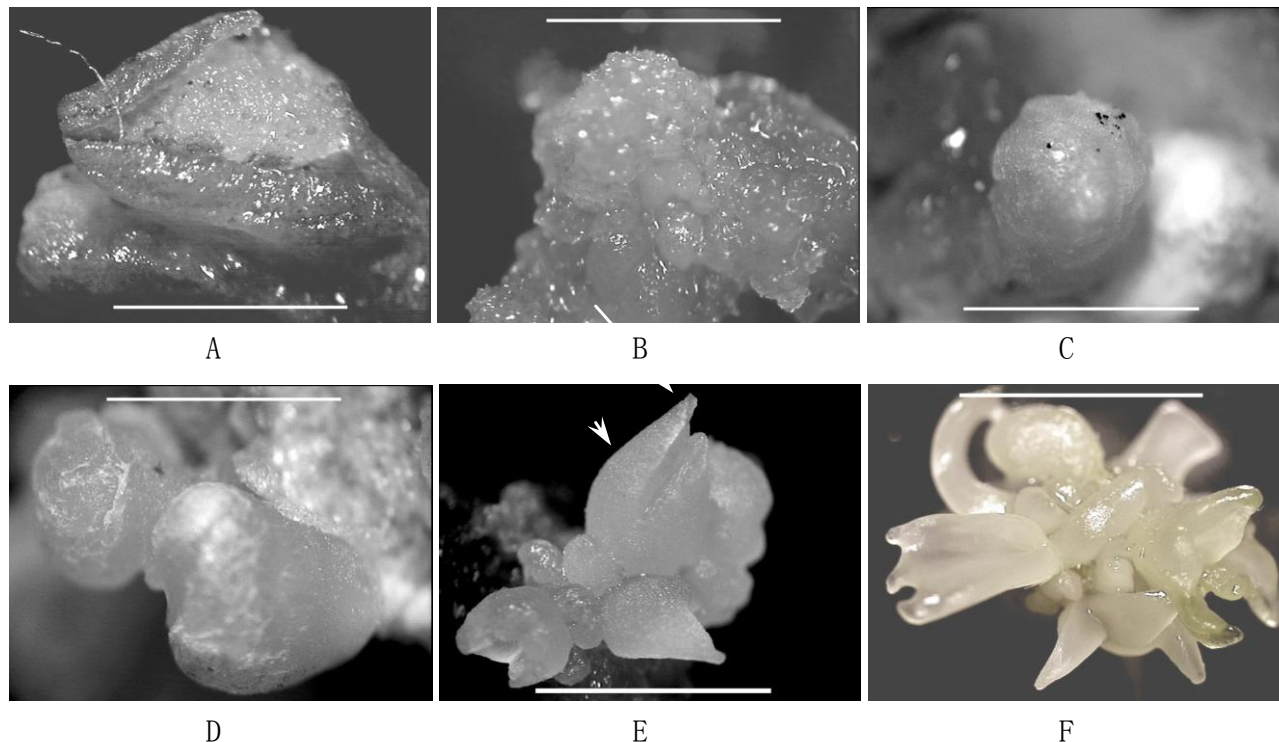


Figure 1. Callus induction and embryogenesis in cultured anthers of loquat (*E. japonica* L.). A: Anther-derived callus (bar = 1.0 mm); B: Embryogenic calluses (bar = 1.0 mm); C: Somatic embryos at the globular stage (bar = 0.5 mm); D: Somatic embryos at the heart-shape stage (bar = 0.5 mm); E: Somatic embryos at the torpedo stage (arrow head indicates the formation of cotyledonary embryos from torpedo embryos) (bar = 1.0 mm); F: Somatic embryos at the cotyledonary stage (bar = 2.0 mm).

development process of anther-derived embryos in loquat were studied, including flesh weight, dry mass contents, carbohydrates, soluble proteins and nucleic acids, in order to explore the influencing factors and molecular mechanism during the development process of anther-derived embryos and ultimately improve the plant regeneration system from anther culture in loquat.

MATERIALS AND METHODS

Anther-derived embryos of *Eriobotrya japonica* Lindl. cv. 'Dawuxing' were used for experimental materials (Figure 1) such as embryogenic calluses, globular embryos, heart-shape embryos, torpedo embryos and cotyledonary embryos. The embryos were frozen with liquid nitrogen and stored at -70°C.

Fresh weight and dry mass contents

The fresh weight of globular embryos, heart-shape embryos, torpedo embryos and cotyledonary embryos (mg) were measured. Number of embryos at each stage was 100 with 5 repeats.

The measurement of dry mass content was done using chemical seasoning. Embryogenic calluses and embryos at different stages were collected in conical flasks with a piece of solid sodium hydroxide, respectively, and kept at 25°C for 14 days. Then, their dry weight was measured.

Dry mass content (%) = dry weight (mg)/fresh weight (mg).

Soluble sugar and starch contents

Embryos (0.05 to 0.2 g) at different development stages were ground with 5 ml 80% ethanol followed by water bath (80°C, 20 min) and centrifugal treatment (4°C, 6000 r/min, 20 min). Then, the supernatant was poured into a pan. And the precipitation was extracted with 5 ml 80% ethanol, repeated twice. Then, all supernatant was merged with the pan and evaporated to dryness in 90°C water bath. Finally, the volume of precipitation was set to 25 ml. The contents of total soluble sugar and fructose were measured by anthrone colorimetry method (Wang and Gou, 2005).

Embryos (0.05 to 0.2 g) at different developmental stages were ground with 5 ml 80% ethanol followed by centrifugal treatment (4°C, 12000 r/min, 15 min). Then, sediment was suspended once with 5 ml distilled water and centrifuged under the same conditions. The sediment was suspended with 5 ml 80% $\text{Ca}(\text{NO}_3)_2$, and boiling water bath for 10 min. The supernatant after low speed centrifugation was poured into 25 ml volumetric flask. Meanwhile, the residue was extracted with 80% $\text{Ca}(\text{NO}_3)_2$, repeated twice. Finally, all supernatant was merged and volume was set to 100 ml. The measurement of starch contents was referred to the iodine staining method of Xu (1998).

Soluble protein and nucleic acid contents

The extraction of protein and nucleic acids was done following Zhu (1980). 4 ml nucleic acid sample was taken to measure the content of total nucleic acid in absorbance under 280 and 260 nm, respectively. Total nucleic acid concentration (mg/ml) = $0.0629 A_{260} - 0.0360 A_{280}$; DNA content was measured with the diphenylamine

Table 1. The fresh weight and dry mass content of embryos at different stages.

Developmental stage	Average fresh weight per embryo (mg)	Dry mass content (%)
Embryogenic callus	-	8.77 ^{bc}
Globular	0.758 ^c	9.51 ^b
Heart	0.921 ^c	7.42 ^c
Torpedo	1.438 ^b	9.64 ^b
Cotyledon	3.245 ^a	13.47 ^a

Means having the same letter in the columns are not significantly different according to Duncan's multiple range test (P = 0.05).

Table 2. The soluble sugar and starch contents of anther-derived embryos in loquat at different developmental stages.

Developmental stage	Total soluble sugar content (mg/g F. W.)	Fructoses content (mg/g F. W.)	Starch content (mg/g F.W.)
Embryogenic callus	20.700 ^c	9.476 ^c	5.906 ^e
Globular	26.657 ^b	11.101 ^b	24.478 ^b
Heart-shaped	22.722 ^c	9.979 ^{bc}	18.172 ^c
Torpedo	20.861 ^c	10.656 ^{bc}	15.627 ^d
Cotyledon	31.127 ^a	14.074 ^a	26.520 ^a

Means having the same letter in the columns are not significantly different according to Duncan's multiple range test (P = 0.05).

method (Wang and Gou, 2005); RNA content = total nucleic acid content - DNA content.

RESULTS

Fresh weight and dry mass contents

As a whole, the fresh weight increased with the anther-derived embryos development. Globular embryo's fresh weight was lowest, which is similar to that of heart-shape embryo. Cotyledonary embryo's fresh weight was highest and had significant difference with the other stages' embryos, about 4.3 times higher than globular embryo. In the whole developmental stages of anther-derived embryos, the water contents were high, and the dry mass contents were low (Table 1). The dry mass contents also increased gradually with the anther-derived embryos development, but there was a slight reduction in the heart-shaped stage, and it had the most important increase from torpedo stage to cotyledon stage with one peak at the cotyledon stage.

Soluble sugar and starch contents

In the entire developmental process, the soluble sugar and starch contents displayed significant differences at different developmental stages, showing obvious peak (Table 2). Their dynamic curves were significantly bimodal, the peaks of accumulation occurred at the

globular and cotyledon stages. Although, the change of fructose was not as significant as total soluble sugar and starch, their dynamic curves were still similar. Carbohydrates contents particularly starch were very low at embryogenic callus stage. The first peak appeared at the globular stage and the second one appeared at the cotyledon stage. The sharp increase in carbohydrates content at differentiation stage indicated the accumulation for gradual depletion of intracellular carbohydrates in the somatic embryogenesis.

Soluble protein contents

The soluble proteins contents of anther-derived embryos in loquat at different developmental stages are shown in Table 3. During the whole process, the early embryos, especially at globular stage, had the highest total soluble proteins content. The total soluble protein content of embryogenic callus was slightly lower than that at globular stage. The change of total soluble proteins was significant during developmental process and the dynamics of total proteins synthesis was roughly in "S" shape. The two accumulation peaks appeared at the globular and cotyledon stages, respectively. Alkaline proteins contents and total soluble proteins contents had the same trend during the developmental process of anther-derived embryos. Alkaline proteins content was higher than acidic proteins content during the process. The content of acidic proteins reached maximum at embryogenic callus, and it gradually reduced with the

Table 3. The soluble protein contents of anther-derived embryos in loquat at different developmental stages.

Developmental stage	Acidic protein content (mg/g F.W.)	Alkaline proteins content (mg/g F.W.)	Total soluble protein content (mg/g F.W.)
Embryogenic callus	2.093 ^a	2.702 ^b	4.695 ^a
Globular	1.811 ^{ab}	3.048 ^a	4.806 ^a
Heart-shaped	1.271 ^c	2.431 ^c	3.702 ^b
Torpedo	0.755 ^d	0.204 ^d	2.958 ^c
Cotyledon	0.878 ^d	2.488 ^{bc}	3.386 ^b

Means having the same letter in the columns are not significantly different according to Duncan's multiple range test ($P = 0.05$).

Table 4. The nucleic acid contents of anther-derived embryos in loquat at different developmental stages.

Developmental stage	Total nucleic acid content (mg/g F.W.)	DNA content (mg/g F.W.)	RNA content (mg/g F.W.)
Embryogenic callus	1.631 ^a	0.911 ^a	0.702 ^c
Globular	1.459 ^b	0.638 ^b	0.822 ^{ab}
Heart-shaped	1.375 ^{bc}	0.539 ^c	0.837 ^b
Torpedo	1.301 ^c	0.491 ^d	0.810 ^{ab}
Cotyledon	1.355 ^c	0.569 ^c	0.786 ^a

Means having the same letter in the columns are not significantly different according to Duncan's multiple range test ($P = 0.05$).

development of embryos. The most important decrease in acidic proteins content occurred from globular stage to heart-shaped stage. Generally, in the early embryo stages of anther-derived embryos, the contents of acidic proteins, alkaline proteins and total proteins all maintained a high level and with a peak of accumulation.

Nucleic acid contents

Changes in nucleic acid contents of loquat anther-derived embryos at different developmental stages are shown in Table 4. Nucleic acid content decreased from embryogenic callus to torpedo stage, and then it began to increase at the cotyledon stage. One peak of nucleic acid appeared at embryogenic callus stage. DNA contents and total nucleic acids content had the same trend. RNA content at embryogenic callus was the lowest one, and then it increased. And after edging down the torpedo and cotyledon stages, RNA content reached the highest level at differentiation stage. During the whole developmental process of loquat, anther-derived embryos DNA content was lower than RNA content, except for the embryogenic callus stage.

DISCUSSION

In the developmental stages of anther-derived embryos in loquat, the fresh weight and the dry mass contents increased gradually with the anther-derived embryos development as a whole. It indicated the substance

accumulation for somatic embryogenesis. But there was a slight reduction at heart-shaped stage, which might be induced by the huge depletion of intracellular substance at the early stages of somatic embryogenesis.

Soluble sugars, soluble protein and nucleic acid were closely related to the development and maturation of embryos, and at the important development turning points, they were changed significantly in metabolism.

In recent years, the studies have found that carbohydrates are the important regulators of plant growth and gene expression. They are not only the energy sources and structural substances, but also play a role in signal transduction, which is similar to hormones in the primary messenger. And in the process of somatic embryogenesis, carbohydrates mainly provide energy and play a role in morphogenesis (Zhao et al., 2006). Starch is one of the main sources of substances and energy, which is required for cell growth and development. And it is used to synthesize protein, cellulose, carbon of nucleic acid, substances of storage and structural frame (Guo et al., 2007). Researches on many plant embryos have shown that there is an accumulation process of carbohydrates at the embryogenic callus (Wang et al., 2008). However, the conclusion of the present study was different. At the embryogenic callus stage, the content of carbohydrates, especially starch, was very low. This might be because, with the continuing cell division, much starch and other carbohydrates would be consumed and may be related to the different experimental materials involved; this requires further studies. Moreover, the dynamic change of the soluble sugar and starch contents were similar during the

development process of anther-derived embryos in loquat. And two accumulation peaks, appeared at the globular and the cotyledon stages, respectively. The emergence of the two peaks was the reflections of preparation of energy for the development of embryos, maturation, germination and plant regeneration.

Many researches indicated that the change of most plant protein content was a double-peak curve and the first accumulation peak appeared at the stage of embryogenic callus (Qi et al., 2001; Guo et al., 2007). The protein content of asparagus and garlic only appear as an accumulation peak at the globular stage (Gao et al., 2001). The dynamics of protein metabolism of anther-derived embryos in loquat during the development process indicated that the two peaks appeared at the globular and cotyledon stages, and the content of the proteins at embryogenic callus was very close to that of globular stage. This might be due to the characteristics of anther-derived embryos in loquat themselves. The emergence of the two accumulation peaks of proteins proved the rapid synthesis and accumulation of proteins, which was the basis for proliferation and differentiation of cells, somatic embryogenesis and embryos development (Stamp, 1987; Zang et al., 2004). In general, at the different developmental stages of anther-derived embryos in loquat, the content of alkaline protein was higher than that of acidic protein and the change trend of alkaline protein contents was similar to that of the total soluble protein. It meant that the change in the total proteins was mainly expressed by the change of alkaline protein. Therefore, future research on the type and quantity of proteins should focus on alkaline protein. In the process of somatic embryogenesis and development, the change process of protein content was also a complex process of the emergence and disappearance of the specific proteins. And the expression and separation of the specific proteins, during the development process of anther-derived embryos in loquat, is worthy of further studies. DNA and other biological macromolecules are the molecular basis of embryogenesis (Zang et al., 2004). During the development process of anther-derived embryos in loquat, the dynamic change of DNA was similar to that of the total nucleic acid, which was in agreement with the results for wheat by Cui et al. (1997). During the development and differentiation process of somatic embryos, the variation of RNA and DNA ratios reflected the level of nucleic acid transcription to some extent. In the earlier development period of anther-derived embryos in loquat (from the embryogenic callus to the torpedo stage), the ratio significantly increased; which indicated that as the embryos developed, the transcription and translation function gradually increased, and proteins and ribosomes also gradually accumulate. However, the ratio gradually reduced in the later periods when DNA replication increased, which provided further conditions for the proliferation and differentiation of cell

(Cui et al., 2001). The peak of DNA synthesis in this experiment appeared at embryogenic callus, which was earlier than that of RNA, and was concordant with the results in other plants. The appearance of the synthesis peak of DNA and RNA indicated that the rapid synthesis of DNA replication was necessary for rapid division of cell in the earlier embryos and the accumulation of RNA was the basis for cell proliferation, differentiation and somatic embryogenesis. Due to the need for formation and differentiation of organs, the synthesis ratio of RNA and DNA was higher in the later embryos.

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