Artificial Gynogenesis and Sex Reversal in *Hypophthalmichthys molitrix*

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ABSTRACT For gynogenesis experiments, spermatozoa from *carp* was exposed to UV irradiation to destroy genetic materials. Eggs from silver carp (*Hypophthalmichthys molitrix*) were 'fertilized' by irradiated spermatozoa which only play the role of stimulating egg. Heat shock was applied for 3 min after fertilization in 41℃ to double the genome ploidy of the resulting haploid 'fertilized egg'. When the fish had reached 10 cm in length, Silastic tube containing methyltestosterone was implanted into the fish body. Although the gonad could develop to reach Phase II, the regression of gonad was observed in test groups compared with controls, suggesting the hormone released from Silastic tube can affect gonad development.

Keywords: *Hypophthalmichthys molitrix*, artificial gynogenesis, sex reversal

1. INTRODUCTION

Silver carp (*Hypophthalmichthys molitrix*) is the major economic fish species cultured in China, whose annual production in 1998 amounts to 4.7×10^6 tons or about 36.15 percent of total annual production in freshwater. During the long-term cultures, it has suffered from inbreeding and other artificial behavior which give rise to early-maturation, body decrease and slow growth rate. Therefore, it is of significance to purify and improve its species quality, which has been listed to the key project in China. In theory, artificial gynogenesis, combined with sex reversal technique, could be used to obtain the inbreeding line in silver carp in a short time and could overcome the limitation of long cycle in routine inbreeding. However, so far, no functional male fish from female gynogenetic fish by sex reversal was obtained. In this study, by implanting the Salilastic tube (Silastic Corp, USA), which could release hormone slowly to affect gonad development, into the gynogenetic fish body, we observed the sex reversal in *Hypophthalmichthys molitrix*.

2. MATERIALS AND METHOD

2.1. Artificial gynogenetic fish

Experiments were performed in spawning seasons with silver carp (*Hypophthalmichthys molitrix*) and carp from Freshwater Fisheries Research Center, Wuxi, China. Eggs from silver carp and spermatozoa from carp were obtained according to routine method[1]. Three experiments below have been done:

(1) Fertilization between eggs from silver carp and spermatozoa from carp;

(2) Fertilization between egg from silver carp and UV-irradiated spermatozoa from carp;
(3) The 'fertilized egg' obtained above was subjected to heat shock for 3 min at 41℃.

2.2. Sex reversal experiment

When the gynogenetic fish reached 10 cm in length (the fist half of October), 20 of them were implanted into Silastic tube containing 17α-methyltestosterone. After one year, survival rate and external morphology were inspected to compare the difference among gynogenetic fishes, sex reversal ones and controls. Also, 3 of each group were killed to subject to gonad section observation.

2.3. Electrophoresis assay

Blood samples from caudal vein were collected from each 4 of control carp (300g), control silver carp (295g) and gynogenetic silver carp (305g) to isolate serum. 5% polyacrylamide slab gels electrophoresis was carried out by LKB2117 isoelectrofocusing apparatus in a ladder of pH4~6 at the voltage, current and power of 1500V, 25mA and 20W, respectively for 2h. After electrophoresis, the serum esterase staining was as described[3].

3. RESULTS

3.1. Artificial-induced gynogenesis

Experiment 1

After fertilization between eggs from silver carp and spermatozoa from carp, the early cleavage of fertilized egg shows no apparent abnormality followed by the increase of abnormality and then death at the stage of fry[1]. This result is in agreement with that of Stlanley's with the crossing of grass carp and carp[4] .

Experiment 2

After fertilization between eggs from silver carp and UV-irridated spermatozoa from carp, the resulting embryos are usually haploid and can't survive[2].

Experiment 3

After the 'fertilized egg' obtained above was subjected to heat shock, the elasticity of its membrane became lower than that of controls. The early cleavage appears normal and abnormality increased after gastrulation. 100 normal fishes were obtained.

3.2. External morphology and sex reversal

Examination in the next year found that 15 of 20 tubed fishes survived with the survival rate of 75%. No significant difference of individual weight (500g) was detected between the tubed

fishes and controls, suggesting Silastic tube did not affect the survival and growth. There was no significant difference in external morphology among the three groups, which demonstrated no parental traits appear in test fishes.

The thick and thin gonad in control group corresponds to the female and male fishes, respectively. All 3 of gynogenetic fishes and tubed ones show thick gonad, suggesting they are all female ones.

As shown in **Fig.1**, section observation found the oocyte in gonad of all three groups could develop to Phase II, but the regression was observed in tubed fishes. This result is explained by the fact that hormone released from Silastic tube can affect the development of gonad.

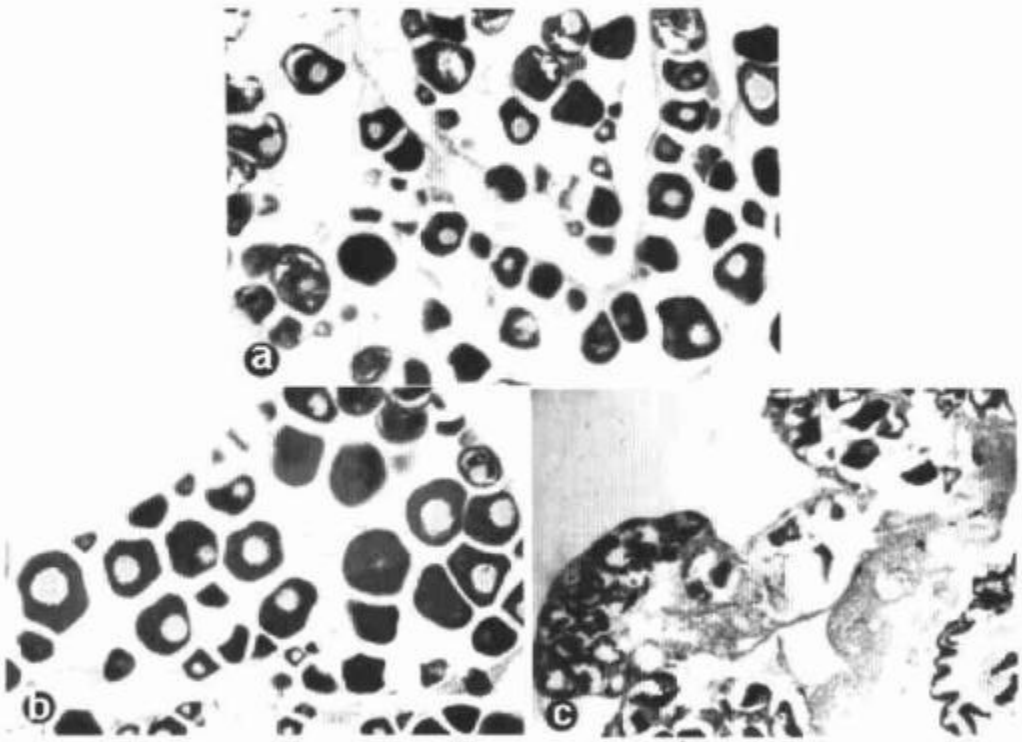

Fig.1 Gonad section of control (**A**), gynogentic (**B**) and sex reversal (**C**) of *Hypophthalmichthys molitrix*.

3.3. Serum esterase

The electrophoretic patterns (**Fig.2**) of 4 gynogenetic fish (4~7) were identical to those of control ones (1~3), which were basically distinguished from that of carp. In particular, a band at pH4.32 in carp was not detected in gynogenetic fish. These results fully prove that the gynogentic fish was developed from maternal egg and the irridated spermatozoa functioned only as a stimulating factor.

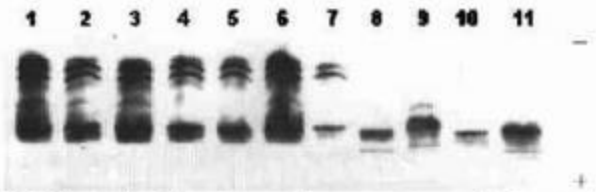

Fig.2 Electrophoretic patterns of serum esterase. **1~3**, controls; **4~7**, gynogenetic fishes; **8~11**, carp. The arrow show carp-specific band at pH 4.32.

4. DISCUSSION

The evidences below demonstrated that gynogenetic fish was obtained in this study.

(1) No viable fish could be obtained through the crossing between silver carp. When eggs were 'fertilized' by spermatozoa which had received UV irradiation followed by post-fertilization heat shock, we obtained viable fishes. This result can be explained by the mechanism that the genetic material of the spermatozoa was destroyed and it only play a role in stimulating egg. The resulting 'fertilized eggs' are usually haploid and produce diploid individuals following heat shock.

(2) No significant parental traits of carp were observed in gynogenetic fish compared with control ones.

There was no significant difference between the serum esterase patterns of gynogenetic fishes and controls, suggesting no genetic materials of carp appear in gynogenetic fishes.

Artificial gynogenetsis technique was first established to produce inbred lines, from which hybrid vigor[5] could be gained by interline cross. In addition, this technique also play an important role in selection breeding and genetics in fish[6].

Artificial gynogenesis had been studied for a long time and gynogenetic fish has been reported by foreign authors[7~12]. In China, this technique was first established in 60s, gynogenetic fish has been reported in grass carp (Pan Guang-Bi, unpublished data), carp[13] and rainbow trout[14]. Although sex reversal was successful in carp, no report was found in silver carp due to the unsuitability of oral administration to silver carp. Our results demonstrated the implantation of Silastic tube containing hormone into the fish body could achieve this goal. By using the same method, after treating 460~656 d, Jensen *et al*[16] had obtained 27 sex reversal gynogenetic fishes. Among them, 5 have testis, 9 are dissect, 8 have gonad but no germ cell appears and 5 have abnormal ovary. Our experiments showed gonad changes could be detected after 13 months treatment, which lays a foundation on establishing inbred line in silver carp.

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Isolation and Expression of *HAP2*, A Homolog of *AP2* in *Hyacinthus orientalis* L.[1]

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ABSTRACT By precisely controlling exogenous hormones applied in the cultural process, particular type of floral organs, such as tapels, stamens, or ovules, can be regenerated from perianth explant of *Hyacinthus orientalis* L. To further study the molecular mechanism of the specific organgenesis in the system, an *AP2* homolog, *HAP2* was isolated from regenerated tepals by using RT-PCR. Results from RT-PCR combined with Southern hybridization showed that *HAP2* was expressed in leaves, perianth, regenerated tepals and regenerated stamens. The possible functions of *HAP2* on hormone-regulated floral organ regeneration are discussed.

Keywords: *Hyacinthus orientalis*, regenerated floral organ, *HAP2*

1. INTRODUCTION

In recent years, detailed genetic analysis on floral organ development of *Arabidopsis* and *Antirrhinum* led to the proposal of the "ABC" model[1,2], which describes how three classes of homeotic genes act combinatorially in discrete domains to specify the identity of floral organ types. Homeotic genes that affect the floral organ identities have been isolated from many plant species[3,4]. In *Arabidopsis*, floral organ identities were determined by the activity of genes *APETALA1* (*AP1*), *APETALA2* (*AP2*), *APETALA3*(*AP3*), *PISTILLA* (*PI*) and *AGAMOUS* (*AG*)[4]. Among them, *AP2* plays an important role in the regulation of flower development. In *Hyacinthus orientalis,* the floral organ differentiation of regenerated floral buds could be regulated by the age of explants and exogenous hormones *in vitro*[5]. At a high concentration of hormones in the medium, tapel differentiation occurs from the regenerated floral buds. At a lower concentration of hormones, stamens and ovules could be induced respectively[6]. It is obvious that the hormones are involved in the differentiation of floral organs[5]. This phenomenon addresses an interesting question that whether the hormonal regulation of the particular organ differentiation is mediated, directly or indirectly of the expression of "ABC" genes. To test this possibility, we isolated several homologs of "ABC" genes from *Hyacinthus orientalis* and showed that *HAG1*, a homolog of *AGAMOUS*, expressed differently in regenerated stamen and regenerated tepals[7]. In this study, we isolated a homeotic gene *HAP2* from the regenerated tepals and determined its expression.

2. MATERIALS AND METHODS

2.1. Plant materials

Bulbs (three-year-old) of *Hyacinthus orientalis* (cv. Delft blue) were used as experimental materials. In early November, bulbs were planted in the soil, and floral buds were collected at right stages.

2.2. Induction and culture of regenerated tapels and stamens

Suitable perianth were selected as the explants. Isolation and sterilization of the explants were performed as described previously[8]. The induction and culture of regenerated tapels and stamens were conducted as described previously[5,8].

2.3. Reverse transcription and PCR

Total RNA isolation was conducted according to the method of Cathala *et al*.[9] 2 μg total RNA was reversely transcripted into cDNA. Based on the conserved domains of AP2 homologs, two degenerated primers were designed for PCR amplification. 5' and 3' oligonucleotides were 5'-TGGGA(A/G)TC(G/C/T)CA(C/T)AT(C/T)TG GGA-3' and 5'-TCCCA(A/G/C)(C/T)(G/T)(A/G) CC(A/G)CA(C/T)TT (A/G)TG-3', respectively. PCR amplification was conducted in a total volume of 50 μL with 5 μL 10× reaction buffer, 4 μL of 2.5 mM dNTP, 4 μL of 5 μM of the two primers, respectively, and 0.5 μL Taq polymerase. Thermocycling was performed at 94℃ for 60 sec, 56℃ for 60 sec and 72℃ for 120 sec for 35 cycles. 5' and 3' RACE were conducted by using GIBCO BRL Kit.

2.4. Gene cloning and sequencing

Target gene cloning was performed according to the protocol of pGEM-T Easy Vector Systems (Promega). Sequencing was carried out by using ABI PRISM™ 377 DNA Sequencer with BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elemer).

2.5. RT-PCR and Southern hybridization

Total RNA isolated from leave, perianth, regenerated tepals and regenerated stamens were digested with DNase respectively, then 2μg total RNA was reversely transcripted into cDNA. According to the sequence of *HAP2*, two specific primers were designed. RT-PCR was conducted with control lacking reverse transcriptase, to determine whether there is contamination of genomic DNA. After amplification, 8 μl PCR product was electrophoresed in 1.0 % agarose gel for 6 h, and DNA was blotted onto a nylon membrane. The probe for hybridization was labeled by using random priming method (Promega). Blots were hybridized at 65℃ overnight in 5×SSPE, 10% dextran sulfate, 1% SDS, and 0.2 mg/mL denatured salmon sperm DNA. Blots were washed once at room temperature for 30 minin 0.2×SSC, 0.5% SDS and twice at 55℃ for

60 minin 0.1× SSC, 0.5% SDS. Autoradiography was performed at -70℃ for 1 day.

3. RESULTS

3.1. Induction and regeneration of tapels and stamens

Floral buds could be regenerated from the perianth explants in the medium containing high concentration of hormones. Maintaining this level of exogenous hormones in the medium, the tepals can be continuously induced from the regenerated floral buds. However, when the level of the exogeneous hormones was decreased, tepal differentiation is inhibited, and stamen differentiation occurs. Detailed descriptions can be found in our previous studies[5~7].

3.2. *HAP2* gene cloning and sequence analysis

According to previous work in *Arabidopsis*, AP2 is involved in determining sepal and petal identities[10], so we tried to isolate the homologs of *AP2* from the floral organs of *Hyacinthus orientalis.* RT-PCR amplification was conducted by using total RNA of regenerated tepal primodia as templates. Using degenerated primers, a fragment of 280 bp was amplified from the total RNA of regenerated tepals. The sequencing and blast search results showed that the fragment has 89% to AP2 at amino acid sequence level, which demonstrated that a fragment homologous to *AP2* had been isolated from *Hyacinthus orientalis*. Further, the 5'- and 3'- end sequences were isolated by using 5' and 3' RACE and a full length cDNA was obtained. This gene was designated *HAP2* (for *Hyacinthus* AP2), and its accession number in GenBank is AF134116. **Fig.1** shows both nucleotide and amino acid sequences of *HAP2*. The *HAP2* gene is 1597 bp in full length, and encodes a putative polypeptide of 368 amino acids.

AP2 homologs contain two copies of 68-amino acid repeat sequences that are referred to as the AP2 domain, both of which contain about 18 amino acid residues capable of forming amphipathic α-helic structures that may participate in protein-protein interactions[11]. In *Arabidopsis*, these two domains are designated as AP2-R1 and AP2-R2, respectively[11]. As **Fig.2** shows, HAP2 also contains the two repeats, HAP2-R1 and HAP2-R2. The residues capable of forming amphipathic α-helice structure between HAP2-R1 and AP2-R1 is 100% identical, but the residues capable of forming amphipathic α-helice structure in HAP2-R2 lack 9 amino acids compared with those in AP2-R2. Besides, AP2 homologs have a highly basic 10-amino acid domain that includes a putative nuclear located sequence KKSR[12,13], suggesting that AP2 may function in the nucleus[11], and this structure was also found in HAP2. The 5' end of coding region of AP2 contains a serine-rich acidic domain analogous to regions that function as activation domains in a number of RNA polymerase II transcription factors[14], but this structure is not evident in HAP2. Taken together, these findings suggest that, samilar to *AP2*, *HAP2* also encodes a putative nuclear protein.

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   TGAAGAGAAGCAAGTGACTGTGATGCCCAAACTCAGAGCAGAGAGCACAGAGAAAGAC          58
AGTGTGAAGACTGAATAGAGAGCGAGAGAGCAAAAATAACAGCAAGTTTTCTTCTTTTCT         118
TCTTTCTCTCTCCGTCTTCAAGAAGCAAATCTTCTGCTGATGTTTTTCTCTGGAGATGGA         178
AAATGACAAAAAGGTGGGATCTTGGTGATCAGAAGCTCCTCAGTTGGAGAAAAGCAGCGG         238
CAGGGTAAATAGAGAGATTGGGAGCGAGAGAGAAGAAAAGGGCATCGGTTTCTCTATCTG         298
GGGAGTTGGAAATTGATAAAAAGGAGGGATCTTGGTGAGAGGTTCCGGTGGTTGCGGGAT         358
TTCAACGGCTTGCCCTAGAGAATGGGGGATCCCGTCACCTTCCAGTTCTTTCCTGTCGGT         418
                        M  G  D  P  V  T  F  Q  F  F  P  V  G
GAGCCGGAGGAGGAGGAGAGCCCTGGTGGTGAGTCTACCGTCGCCGCCGGAGGAGCGCCG         478
 E  P  E  E  E  E  S  P  G  G  E  S  T  V  A  A  G  G  A  P
CGGGGCCACTGGGCGGAGGTGAAGTTTGTTGAGAAGGAGGCGGACGTGTCGCCGGCGATC         538
 R  G  H  W  A  E  V  K  F  V  E  K  E  A  D  V  S  P  A  I
AAGAAGAGCCGGCGGGGGCCGAGGTCTAGGAGCTCGCAGTACAGGGGAGTGACTTTCTAC         598
 K  K  S  R  R  G  P  R  S  K  S  S  Q  Y  R  G  V  T  F  Y
AGGAGGACTGGGAGATGGGAGTCGCATATTTGGGATTGCGGCAAACAGGTCTATTTGGGA         658
 R  R  T  G  R  W  E  S  H  I  W  D  C  G  K  Q  V  Y  L  G
GGATTTGACACTGCTCATGCTGCTGCAAGAGCTTACGATCGAGCTGCGATTAAGTTTCGA         718
 G  F  D  T  A  H  A  A  A  R  A  Y  D  R  A  A  I  K  F  R
GGGGTTGATGCTGATATCAATTTCAATCTCAGTGATTATAATGAGGATCTGAAGCAGATG         778
 G  V  D  A  D  I  N  F  N  L  S  D  Y  N  E  D  L  K  Q  M
ATGAACTTAGCCAAAGAGGAGTTTGTTCACATCCTTCGAAGGCAGAGCACTGGGTTTTCG         838
 M  N  L  A  K  E  E  F  V  H  I  L  R  R  Q  S  T  G  F  S
AGGGGCAGCTCGAAATATCGAGGGGTGACTCTCCACAAGTGCGGCCGCTGGGAAGCTAGG         898
 R  G  S  S  K  Y  R  G  V  T  L  H  K  C  G  H  W  E  A  R
ATGGGCCAATTCCTTGGCAAAAAGGCTTATGACAAAGCGGCTATCAAGAGCAGTGGAAGG         958
 M  G  Q  F  L  G  K  K  A  Y  D  K  A  A  I  K  S  S  G  R
GAAGCTGTAACCAATTTTGAGCCAAGTTCTTATGAACGGGAAGTGCTCACTGAGGCAGAT        1018
 E  A  V  T  N  F  E  P  S  S  Y  E  R  E  V  L  T  E  A  D
AGTGATGCCATTGGCCATGACATCGATCTGAACTTGAGGATATCTCAACCGAATGTTAGC        1078
 S  D  A  I  G  H  D  I  D  L  N  L  R  I  S  Q  P  N  V  S
AGTCCAAAGAGGCGTGATAATCCAGTTGGCGTCCAGTTCCATTTGGGCTCCTTCGAATCG        1138
 S  P  K  R  R  D  N  P  V  G  V  Q  F  H  L  G  S  F  E  S
TCTGACGCCAAGAAAGCTATGATTGACACCCATTCATCAATTTTGGTTGGTCAGCCCCAT        1198
 S  D  A  K  K  A  M  I  D  T  H  S  S  I  L  V  G  Q  P  H
ACGGCGGCAATGACATCCGAGGCTTCTCGAGTATGGTCTGCCCTTTATCCTGGATTCTAT        1258
 T  A  A  M  T  S  E  A  S  R  V  W  S  A  L  Y  P  G  F  Y
CCCCCTATTGAGCTACGAGCCAAAGACAAGATGTCTATGGTCGGTTCAGCAGCCCTACCA        1318
 P  P  I  E  L  R  A  K  D  K  M  S  M  V  G  S  A  A  L  P
AATTGGACATGGCAATTGCACGGGCCAATGCCATCGCCGATGTTCACTTCTGCAGCATCA        1378
 N  W  T  W  Q  L  H  G  P  M  P  S  P  M  F  T  S  A  A  S
TCAGGATTCGCCACCACCCATGCCACATCCTCCATTACCCCCCCTGCCAGCCATCATCTT        1438
 S  G  F  A  T  T  H  A  T  S  S  I  T  P  P  A  S  H  H  L
CAGTTCCCTCCACCACCCAACACCAACTACTATTCCAGGAGCTGAAGCCGATGGAAATGC        1498
 Q  F  P  P  P  P  N  T  N  Y  Y  S  R  S  *
ATTCTAATGGTACATACATGTGTCATTGTATGTAAAGCACAAATTCGGGAGTAAAGCAGG        1558
ATGGTGCATGTTTTTACTGACAAAAAAAAAAAAAAAAAA                              1597
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Fig.1 Nucleotides and predicted amino acid sequences of *HAP2*.

Fig.2 Alignment of the deduced amino acid sequences for HAP2 cDNA with AP2 homologs from Arabidopsis (AP2) and Petunia (PHAP2B).

The asterisks designate the same amino acid. The underlined amino acids indicated HAP2 domain repeated motif HAP2-R1 and HAP2-R2, in which the residues capable of forming amphipathic **α**– helices are boxed. A 10-amino acid basic region that contains a putative nuclear located signal sequence (KKSR) is also underlined.

3.3. *HAP2* expression analysis

Since the expression of *HAP2* was hard to be detected by Northern hybridization, we combined RT-PCR and Southern hybridization to determine its expression. Based on the sequence of *HAP2*, two specific primers were designed, and RT-PCR results is shown in **Fig.3**. A specific 900 bp size DNA fragment can be amplified from leaves, perianth, regenerated tepals and

regenerated stamens. Further, when the 3'end cDNA was used as a probe, the PCR Southern hybridization result is consistent with that of RT-PCR. *HAP2* is expressed in both non-floral and floral organs *in vivo*, which is the same as the pattern of *AP2*[10,11].

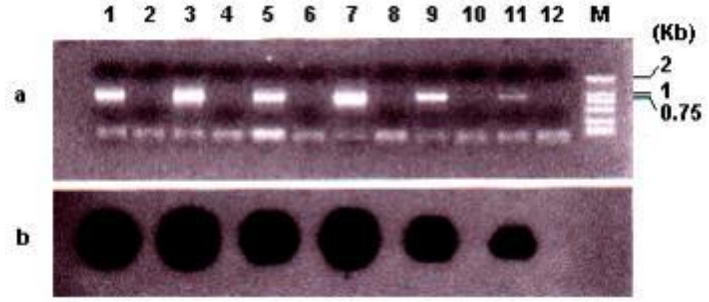

Fig.3 *HAP2* expression analysis by using RT-PCR combined with Southern hybridization. (**a**) RT-PCR result. (**b**) Southern hybridization. **Lane 1** and **2**, mature regenerated stamens; **3** and **4**, young regenerated stamens; **5** and **6**, mature regenerated tepals; **7** and **8**, young regenerated tepals; **9** and **10**, perianth; **11** and 1**2**, leaves; **2**, **4**, **6**, **8**, **10** and **12**, controls without reverse transcriptase.

4. DISCUSSION

In *Arabidopsis*, *AP2* plays a critical role in the regulation of flower development. According to the "ABC" model, *AP2* and *AG* are mutually antagonistic genes[1,2]. *AP2* negatively regulates *AG* expression in sepal and petal, and conversely *AG* negatively regulated *AP2* gene expression in stamens and carpels. Our result revealed that *HAP2* is also expressed in stamens, suggesting *HAP2* expression in stamen is not repressed at RNA level by *AG*-like genes. Similar data were also reported of organogenesis on a single organ type without interference of other organs or tissues. Recently we have isolated several homeotic genes, such as *HAG1*, *HPI1* and *HPI2*[7,16]. Further using molecular methods such as *in situ* hybridization to analyze their expression in regenerated flower primordia and floral organs may provide important information on understanding their function and relationship and especially the mechanism of the regulation of floral development by hormones.

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