Identification of a novel DNA-binding protein to osmotin promoter

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Received April 14, 1998

Abstract One novel osmotin promoter-binding-protein (OPBP1) gene, was isolated from salt-adapted tobacco suspension cells using yeast one-hybrid system. The OPBP1 interacted specifically *in vivo* with FA, a DNA sequence from the 5′ upstream region of osmotin gene, which was essential for osmotin responsiveness. The deduced amino acid sequence of OPBP1 contained a conserved motif of a new gene family, AP2 family. This protein did not contain the typical motif found in the most known DNA-binding proteins and transcription factors.

Keywords: osmotin, DNA-binding protein, one-hybrid screen.

Osmotin is a 24 kD basic protein that was originally identified as the most prominent polypeptide present in salt-adapted tobacco cells. The expression of osmotin is induced by many stresses and environmental factors including pathogen infection, ethylene, ABA, Methyl jasmonate, salicylic acid, wounding, and salt stress[1]. The messages of those environmental factors are transferred into nucleus through signal transduction pathway to induce the osmotin promoter to overexpress osmotin. The accumulation of osmotin in salt-adapted tobacco cells is up to 12% of total protein[2]. It was shown that the osmotin was closely related with many important physiological phenomena such as osmotic stress, drought tolerance, salt tolerance and pathogen resistance[1,2].

The upstream of 1 052 base pairs of osmotin gene contributed to osmotin expression. The minimal essential responsiveness *cis*-acting element to environment was identified between −248 and −108, termed fragment A (FA). It contained a G box, an AT box and two PR (pathogenesis related) boxes in different orientations[2]. It will facilitate to understand the transcription regulation of osmotin to study DNA binding proteins to FA and isolate transcription factors involved in osmotin expression. It will also help to reveal the induction process of osmotin expression by environmental factors through signal transduction pathway.

Yeast one-hybrid system is a basic tool developed currently to isolate novel genes encoding proteins that bind to *cis*-acting regulatory elements[3]. In this system, a target *cis*-acting element is normally inserted upstream of a report gene in a plasmid. The plasmid is integrated into yeast genome by homologous recombination. Then the yeast cell containing the integrated fragment is isolated according to its nutrition deficiency. The mRNA is isolated from target animal or plant

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and a cDNA library in which the cDNA fragments are fused with GAL4 active domain is constructed. The cDNA library is transformed into the yeast cells containing the *cis*-element and the report gene in their genome. When a cDNA coding protein interacts with *cis*-element the fused GAL4 active domain will express the report gene downstream of the *cis*-element and the yeast cell containing the cDNA will be isolated. Several *trans*-factors were identified in this way[3,4]. In the present study, we screened a cDNA library of the salt-adapted tobacco suspension cells for proteins interacting with osmotin FA using yeast one-hybrid system. More than 100 yeast colonies were obtained. One of them was further studied and sequenced. It was demonstrated that the clone codes a novel DNA binding protein related to a new family of DNA binding proteins in plants.

1 Materials and methods

1.1 Materials

Fifteen days-cultured salt-adapted suspension cells were harvested for RNA isolation[1]. The one-hybrid system was purchased from Clontech Co. The system of cDNA library fused with GAL4 active domain was purchased from Strategene Co. All of restriction enzymes and other enzymes were purchased from Promega Co. The reagents and chemicals were purchased from Sigma Co. Isotopic was purchased from Amersham Co. The primers were synthesized by BRL Co.

1.2 Methods

1.2.1 PCR amplification. The primers 5′-AAG GAT CCT TAC AAG TGT CAC GTT AC-3′ and 5′-CCA GAT CTT CTC AAG GAT GCT TTG G-3′ and template plasmid pGUS1B-10[1] were used in PCR to amplify *cis*-element of osmotin gene. To decrease the mistake during the PCR, high fidelity DNA polymerase pfu (Strategene Co.) was used. PCR was performed under the condition: 94℃ pre-denature for 5 min, then 30 cycles of 94℃ for 45 s, 45℃ for 1 min, 72℃ for 1 min. The PCR product was ligated with pBluescript SK- (Stratagene Co.) digested with EcoR V. The ligated DNA was transformed into *E. coli* DH5α. The plasmid was isolated from transformant cell and sequenced to confirm.

1.2.2 DNA manipulation and RNA purification. Common DNA manipulation methods, such as electrophoresis, recovery of DNA fragment from gel, ligation, transformation and Sanger's DNA sequence method, were performed as described[5]. Total RNA was isolated using acidic phenol method as described[1]. The mRNA was purified after precipitation with 8 mol/L LiCl and column chromatography (Clontech Co.).

1.2.3 Yeast transformation and plasmid isolation. Yeast transformation was performed using LiAC/PEG method. Plasmid isolation from yeast cells was performed using acidic glass beads[5].

1.2.4 cDNA library construction and screening. cDNA library construction, amplification and mass excision were performed as described in the manual of manufacturer. The cDNA was inserted into pADGAL4 and fused downstream of GAL4 protein active domain. A large scale of mass excised and amplified cDNA library was purified using CsCl centrifugation method and transformed into yeast cells. The screening for positive yeast colonies was performed as the manual of manufacturer.

2 Results

2.1 Construction of yeast one-hybrid system

The target DNA containing tandem copies of the *cis*-acting element must be constructed upstream of report genes in yeast one-hybrid system. To facilitate construction of tandem FA in the same direction, PCR was used and BamH I and Bgl II sites were introduced into the ends of FA (fig. 1). The DNA fragment digested with BamH I and Bgl II was recovered from gel and ligated using T4 DNA ligase. Small amounts of BamH I and Bgl II were added during ligation. Because BamH I and Bgl II produce cohesive ends that can ligate each other, the ligated fragments could not be digested again since both sites were destroyed. However, the fragments from self-ligation should be digested with BamH I or Bgl II. Polymers of tandem FA in the same direction were formed in this way. The polymers were then separated by gel electrophoresis. The pentamer was

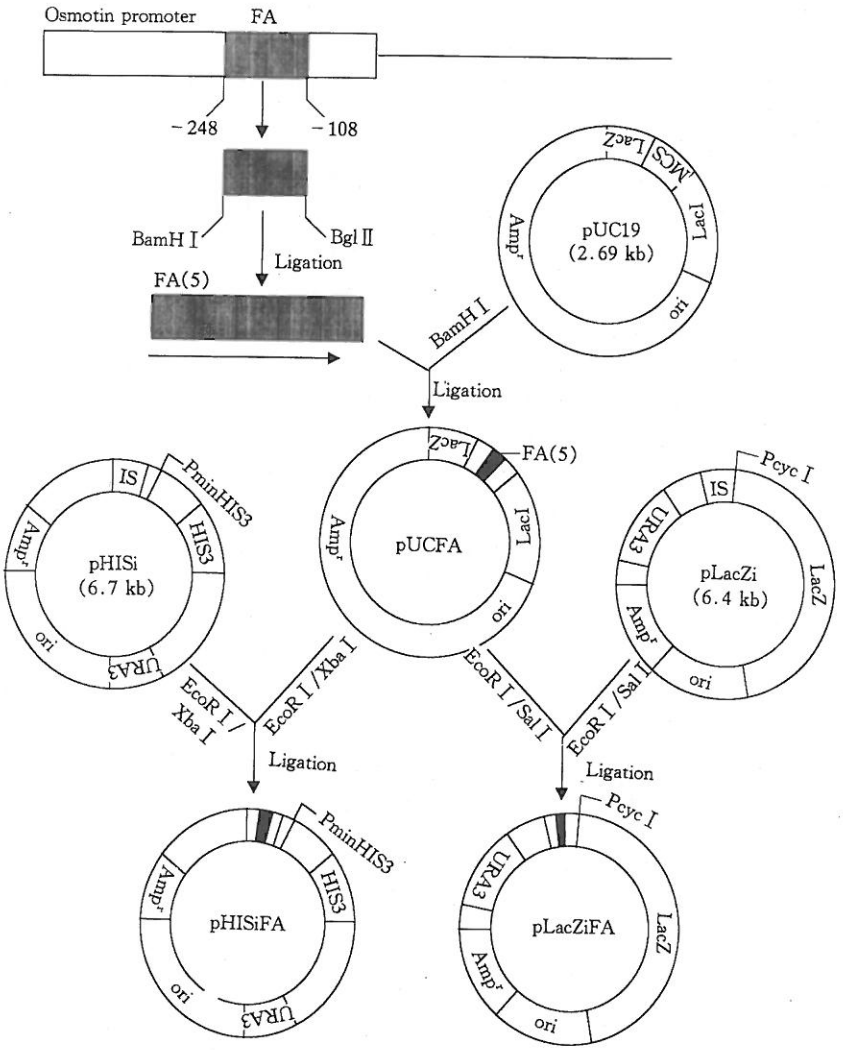

Fig. 1. Cloning strategy of fragment A of osmotin promoter. FA, Fragment A of osmotin promoter; FA(5), pentamer of FA.

recovered from gel and inserted into pUC19 digested with BamH I to form pUCFA. pUCFA was analyzed with restriction enzymes to determine the direction of the insertion. The pUCFA upstream of LacZ report gene with correct FA direction was digested with EcoR I/Xba I and EcoR I/Sal I. The FA(5) fragments were recovered and inserted into pHISi (digested with EcoRI/Xba I) and pLacZi (digested with EcoRI/Sal I) respectively. The formed plasmids, pHISiFA and pLacZFA, were linearized and transformed into yeast YM4271 cells. The transformed cells were spread on SD/-Ura plates. YM4271, a *ura*$^-$ strain, could not grow on the selective plate without Ura by itself. The pHISiFA or pLacZFA contains URA3 and could be integrated into yeast genome to make yeast *ura*$^+$. The yeast cells harboring integrated pHISiFA growing on SD/-Ura plate were isolated and used as recipients for transforming cDNA library. Because of HIS3 leaky the yeast cells might grow on SD/-His plate without target element activation. The yeast cells harboring pHISiFA were suspended in H_2O and spread on SD/-His selective plates with different concentrations of 3-aminotriazole (3-AT). The 3-AT concentrations, 0, 7.5, 15, 30, 45 and 60 mmol/L, were titered to identify the concentration of 3-AT that completely inhibited the background growth from leaky HIS3 expression. It was shown that 7.5 mmol/L 3-AT inhibited background growth completely and the concentration was used in cDNA library screening.

2.2 Identification of cDNA clones encoding DNA binding proteins

The yeast cells harboring the pHISiFA were transformed with the cDNA library amplified and purified with CsCl. The transformants were spread on SD/-His-Leu plates containing 7.5 mmol/L 3-AT. The positive colonies were picked up after 4—7 d incubation. 102 positive colonies were obtained from 106 transformants. These colonies were streaked on fresh selective plate and grew well. Sixty colonies were inoculated into liquid SD/-His-Leu containing 7.5 mmol/L 3-AT with shaking and the growth of the cells was observed. Nine colonies growing well were chosen for large scale culture. The plasmids from the large scale culture yeast cells were isolated and transformed into competent DH5α cells. DNA was isolated from the transformant and sequenced with T7 primer to analyze the 3′ end of cDNA. It was shown that 3 clones from 9 clones were identity. No homologue sequences were found in GenBank with about 250 nt of the three clones.

One of the three clones, OPBP1, was used for further study. The plasmid DNA from OPBP1 clone was retransformed into yeast cells harboring pHISiFA and spread on SD/-His-Leu plate containing 7.5 mmol/L 3-AT. Many transformants grew on the selective plate. The DNA was also transformed into yeast cells harboring pLacZiFA and spread on SD/-Leu plate. The β-galactosidase activities of the growth colonies were determined. All the growth colonies appeared in blue color in the test. It suggested that OPBP1 plasmid encoded a DNA binding protein gene to FA.

2.3 Sequence analysis

The restriction enzyme map of OPBP1 plasmid was made. It contained one Hind III and two EcoR I sites without BamH I and Pst I sites. Two fragments from the plasmid digested with Hind III and Sal I were subcloned into pBluescript SK-. Full length DNA sequence of the insert was obtained (fig. 2). It was 952 bp in length and encoded an open reading frame of 277amino acids. As expected, the OPBP1 open reading frame was fused in-frame to the upstream transcription activation domain in pADGAL4. It produced a 45 ku fused protein.

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GAATTCGGCACGAGGAAAAACAAAATGGATTCTTCTTCTTGTTCTTCTCATTTTTTCTACCCTATGAATT      70
                          M  D  S  S  S  C  S  S  H  F  F  Y  P  M  N  S      16
CTGATCTTTCTTCTGATTCTTCTTGGGAATGGTCCAATTTAAACTCAACTTCTCTTCCTTTAACGTTAA     140
  D  L  S  S  D  S  S  W  E  W  S  N  L  N  S  T  S  L  P  F  N  V  N       39
CGATTCCGAAGAGATGCTTCTTTTTGGTGTTCTTACTAACACAGCTCAAGAAACAACGTCGGAAACAGTT     210
  D  S  E  E  M  L  L  F  G  V  L  T  N  T  A  Q  E  T  T  S  E  T  V       62
ACCTCGTACCATGTTAAGGAAGAGGAAGTTAGTTCAAAATCTAAGGTTATAAAAGAAATTGAAGAAAAAC     280
T  S  Y  H  V  K  E  E  E  V  S  S  K  S  K  V  I  K  E  I  E  E  K  P      86
CGGCCAAGGAGAAGTCGTTTCGAGGCGTTAGAAGGCGGCCGTGGGGGAAATTCGCGGCGGAGATAAGGGA     350
  A  K  E  K  S  F  R  G  V  R  R  R  P  W  G  K  F  A  A  E  I  R  D      109
TTCTACTAGGAATGGTGTAAGAGTATGGTTAGGGACATTTGATAGTCCTGAGGCAGCTGCTTTAGCTTAT     420
  S  T  R  N  G  V  R  V  W  L  G  T  F  D  S  P  E  A  A  A  L  A  Y      132
GATCAAGCCGCTTTTTTAATGCGGGGTACATCAGCAATCTTGAATTTTCCTGTGGAAACAGTCCAAGAGT     490
D  Q  A  A  F  L  M  R  G  T  S  A  I  L  N  F  P  V  E  T  V  Q  E  S     156
CGTTACGTGACATGAAATGTCACGTAGACGAGGAATGTTCCCCTGTGGTGGCGCTAAAAAAGCGCCACTC     560
  L  R  D  M  K  C  H  V  D  E  E  C  S  P  V  V  A  L  K  K  R  H  S      179
ATTGAGGAAGAAGAGCTTGAGTTCCAAGAAAAGCAATAGTAGTAGTAATAGTAAAGTTGTGAGGGAAGTA     630
  L  R  K  K  S  L  S  S  K  K  S  N  S  S  S  N  S  K  V  V  R  E  V      202
AAAATGGAGAATGTAAATGTTGTAGTTTTTGAAGATTTGGGTCCTGATTACTTGGAACAACTTTTGAGTA     700
K  M  E  N  V  N  V  V  V  F  E  D  L  G  P  D  Y  L  E  Q  L  L  S  S     226
GTAGTTCAAGTGATCATAGTAGTTGTAATGAAGCTTTCTACCATGTAAATCAAAGATTTACCCAACTTTG     770
  S  S  S  D  H  S  S  C  N  E  A  F  Y  H  V  N  Q  R  F  T  Q  L  C      249
TATTTATTTATTTATTTATTTATATTATTTTTGTTTATTTTCTTTTAGGGGCAGGGAAATAGGGGAATTC     840
  I  Y  L  F  I  Y  L  Y  Y  F  C  L  F  S  F  R  G  R  E  I  G  E  F      272
CATCTCTTTGTTCTTTAGTTGGAGATCACTTTCTAGTGTCTCTATTGTATAAATTTTGGTGATGGTGGTA     910
H  L  F  V  L  <                                                           277
TTTAACTTAAAAGGATTTTTAAATGTGAAAAAAAAAAAAAAA                                 952
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Fig. 2. The nucleotide sequence of OPBP1 and its deduced amino acid sequence.

No identical sequence was found from GenBank for OPBP1 full-length sequence. It was found that OPBP1 contained a conserved motif homologue to a new family of proteins found in plants currently during comparing protein conserved domains (fig. 3). The sequence comparison showed that there are a conserved domain in APETALA2 (AP2) and AINTEGUMENTA (ANT) from *Arabidopsis*, ethylene responsive element binding proteins (EREBPs) from tobacco, and several other unknown function proteins . AP2 is a central player in the gene network controlling *Arabidopsis* flower homeotic gene expression and flower development. It is also required during seed development[6]. ANT is another gene from *Arabidopsis* which is critical for the development of ovule and female gametophyte. ANT maps to close location with AP2 on chromosome 4 in *Arabidopsis*. It suggests that a gene duplication occurred to form ANT and AP2 in evolution[7]. EREBPs from tobacco are involved in the induction of β-1, 3-glucanase. Ohme-Takogi and Shinshi show that EREBPs contain a conserved domain of 59 amino acids[8]. The conserved domain is essential to binding GCC box from glucanase gene promoter because the deletion of the conserved domain results in losing binding ability. The GCC box is found in a number of ethylene inducible pathogenesis-related (PR) protein genes. There are two repeat copies of PR box that has a consensus sequence of GCC box in osmotin promoter. It was shown, in our experiment, that OPBP1 protein had the same conserved domain with EREBPs and bound specifically to FA that contained two PR boxes. It suggested that the OPBP1 bound to PR box specifically and did not bind to G box and AT box.

AP2, ANT, EREBPs and OPBP1 contain the same motif (fig. 3). None of them has been found to contain typical protein structures found in known DNA binding proteins and transcription factors, such as bZIP and zinc finger. It is classified as a new family of DNA binding proteins[7,9]. Sequence analysis indicates that the AP2 family can be divided into two groups: one group containing a single domain and the second group containing two similar domains and a

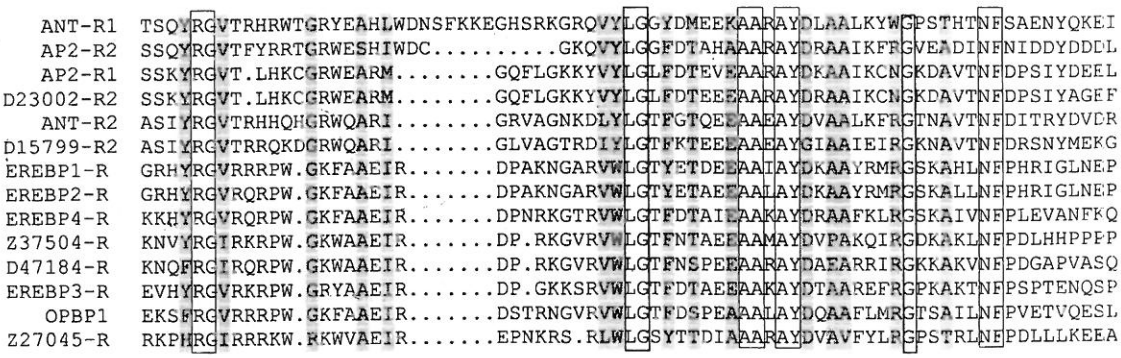

Fig. 3. The sequence analysis of the conserved domain in OPBP1. The conserved domain of OPBP1 is compared with other proteins[6]. The consensus amino acids in all members are indicated in open box. ANT (GenBank accession number U40256), AP2 (GenBank accession number U12546), Z37504 and Z27045 are from *Arabidopsis*. D23002, D15799 and D47184 are from rice. EREBP1—4 (GenBank accession number D38123, D38126, D38124 and D38125) and OPBP1 (GenBank accession number U81157) are from tobacco. R1 and R2 indicate two conserved domains respectively of ANT and AP2.

conserved linker region. Both of the domains are crucial for full activity because point mutations in the conserved domain result in the gene products' losing activity. OPBP1, which contains a single domain, belongs to the first group. OPBP1 conserved domain, compared with EREBPs, is close to EREBP3. Because the conserved sequence region is also very similar to an unknown function rice gene, Z47184, it is suggested that the conserved domain is distantly related in plants, such as dicotyledons and monocotyledons.

3 Discussion

Osmotin plays an important role in plant stress physiology. Its expression is related to many stresses and environmental factors. One novel protein, OPBP1 that interacted with osmotin gene *cis*-acting element, was isolated using yeast one-hybrid system from tobacco salt-adapted suspension cells. The protein bound to the osmotin FA specifically in the yeast system.

The expression of tobacco osmotin is induced by a member of environmental signals. Different signals cause different levels of osmotin expression. For example, wounding and low temperatures cause the increasing of osmotin mRNA without significant accumulation of osmotin in tobacco. However, salt stress, fugal infecting and ethylene and JA treatment induce the accumulation of osmotin. It suggests that there is a posttranscription regulation in expression of osmotin[10]. Our experiment showed that OPBP1 bound to osmotin promoter specifically in yeast. The role of the binding protein in the signal transduction pathway needs further exploration.

References

1 Xu, Y., Chang, P., Liu, D. et al., Plant defense genes are synergistically induced by ethylene and methyl jasmonate, *The Plant Cell*, 1994, 6:1077.

2 Liu, D., Raghothama, K. G., Hasegawa, P. M. et al., Osmotin overexpression in potato delays development of disease symptoms, *Proc. Natl. Acad. Sci. USA*, 1994, 91:1888.

3 Wang, M. M., Reed, R. R., Molecular cloning of the olfactory neuronal transcription factor Olf-1 by genetic selection in

yeast, *Nature*, 1993, 364(8):121.

4 Sieweke, M. H., Tekotte, H., Frampton, J. et al., MafB is an interaction partner and repressor of Ets-1 that inhibits Erythroid differentiation, *Cell*, 1996, 85:49.

5 Ausubel, F. M., Brent, R., Kingston, R. E. et al., *Current Protocol of Molecular Biology*, New York: John Wiley and Sons, Inc., 1995.

6 Jofuku, K. D., den Boer, B. G. W., Van Montagu, M. et al., Control of *Arabidopsis* flower and seed development by the homeotic gene APETALA2, *The Plant Cell*, 1994, 6:1211.

7 Klucher, K. M., Chow, H., Reiser, L. et al., The AINTEGUMENTA gene of *Arabidopsis* required for ovule and female gametophyte development is related to the floral homeotic gene APETALA2, *The Plant Cell*, 1996, 8:137.

8 Ohme-Takagi, M., Shinshi, H., Ethylene-inducible DNA binding proteins that interact with an ethylene-responsive element, *The Plant Cell*, 1994, 7:173.

9 Ecker, J. R., The ethylene signal transduction pathway in plants, *Sciences*, 1995, 268:667.

10 LoRosa, P. C., Chen, Z., Nelson, D. E. et al., Osmotin gene expression is posttranscriptionally regulated, *Plant Physiol.*, 1992, 100:409.

FSHβ subunit gene is associated with major gene controlling litter size in commercial pig breeds

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Received April 14, 1998

Abstract An insertion fragment in porcine FSHβ subunit gene was cloned by PCR. Sequencing data show that the insertion is a retroposon of 292 bp siting in intron I at the site between +809 and +810 base. Based on these results, a PCR programme was created to genotype animal individuals in different pig breeds at FSHβ locus and polymorphism of FSHβ gene was analyzed. With the combination of genotype and litter size of sows, it was demonstrated that FSHβ locus is closely associated with major gene controlling litter size in commercial pig breeds, such as Yorkshire, Landrace, Durco. Averagely the AA sows give more 1.5 piglets than BB sows do per litter.

Keywords: **FSHβ subunit gene, litter size, major gene, candidate gene approach.**

Litter size is a very important quantitative trait to pig production and difficult to improve by traditional breeding technology due to its low heritability. Marker-assisted selection (MAS) is thought as a new approach, which can be used to improve production traits of low heritability effectively. In order to make full use of MAS, the gene controlling these traits has to be localized precisely. Generally two ways are being employed to search genes responsible for quantitative traits. One of them is the so-called "genome scanning", where genomic region influencing the trait could be concluded by linkage analysis of phenotype and the selected DNA markers in resource family, then all of gene loci would be identified in these possible regions. Not as some successful cases in human or animal, the genes controlling pig litter size are still hard to study by this method due to lack of appropriate resource population[1]. The alternative way, candidate gene approach, is based on the knowledge of physiology and biochemistry in formation of phenotype. Polymorphisms of the related genes would be well studied in animal breeds with largely varied phenotype. In final, whether mutation of the related gene can result in variation in phenotype or not must be demonstrated at molecular level. By this approach, chicken sex-linked dwraf gene and porcine malignant hyperthermia syndrome (MHS) gene were successfully mapped.

Porcine follicle-stimulating hormone (FSH) is a kind of glycoprotein secreted from anterior pituitary. Through its receptor on gonad cell, it stimulates proliferation of granular cell, differentiation of endomembrane cell, production of E_2 and induces the production of receptors of LH, PRL. Mammalian FSH consists of α and β subunits, α subunit is common to LH, TSH. Generally, biological function of FSH depends on specificity of FSHβ subunit. In this paper FSHβ gene is chosen as candidate gene controlling the pig litter size.