GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Association between polymorphisms in exons 4 and 10 of the *BPI* gene and immune indices in Sutai pigs

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Genet. Mol. Res. 14 (2): 6048-6058 (2015)
Received January 12, 2014
Accepted January 19, 2015
Published June 8, 2015
DOI http://dx.doi.org/10.4238/2015.June.8.2

ABSTRACT. The bactericidal/permeability-increasing protein (*BPI*) gene has been identified as a candidate gene for disease-resistance breeding. We evaluated whether polymorphisms in exons 4 and 10 of the *BPI* gene are associated with immune indices [interleukin-2 (IL-2), IL-4, IL-6, interferon-β (IFN-β), IL-10, and IL-12]. In this study, we identified one mutation (C522T) in the *BPI* exon 4 site and two mutations (A1060G and T1151G) in the *BPI* exon 10 site. Correlation analysis revealed that in the Sutai pig population, the effect of genotypes at the *BPI* exon 4 site on the level of IL-6 was significant ($P < 0.05$), with an effective genotype of CD; moreover, the effect of genotypes at the *BPI* exon 10 site on the level of IL-12 was significant ($P < 0.05$), and the effective genotype was AB. The optimal combined genotype was CD-AB, which was more effective regarding the IL-6 and IL-12

levels compared to the other combined genotypes ($P < 0.05$). These results indicate that single nucleotide polymorphisms and the combined genotypes of *BPI* exons 4 and 10 affect immune indices in Sutai pigs. Therefore, these genotypes should be further examined as effective markers for disease-resistant breeding of pigs.

Key words: *BP1* gene; Combined genotype; Immune indices; Pig; Single nucleotide polymorphisms

INTRODUCTION

Bactericidal/permeability-increasing protein (BPI) belongs to the lipopolysaccharide (LPS)-binding protein family. It is an endogenous cationic protein present in humans and other mammals and is primarily present in the aniline blue-staining particles of polymorphonuclear leukocytes. BPI not only kills Gram-negative bacteria and neutralizes endotoxins and LPS (Akın et al., 2011), but also promotes complement activation and opsonization for increased phagocytosis, inhibits angiogenesis, inhibits inflammatory mediator release, and inhibits infection by fungi and protozoa. Therefore, BPI plays a key role in the natural defense system of the animal body (Weiss et al., 1978; Elsbach, 1998). The function of BPI is related to its structure, which includes a cationic, lysine-rich N-terminus with antibacterial and LPS (endotoxin)-neutralizing activities (Iovine et al., 1997) and a C-terminus that improves stability and opsonic activity (Ooi et al., 1991). Binding of the BPI protein to the negatively charged LPS envelope activates the mononuclear phagocyte system, leading to the release of cytokines such as tumor necrosis factor-α, interleukin (IL)-6, and IL-2, which contribute to the subsequent inflammatory reaction (Mannion et al., 1989). Under normal physiological conditions, specific immune indices reflect the overall immune reaction activity involved in disease resistance (Ma et al., 2011).

The porcine *BPI* gene is located at 7q21-23 and consists of 15 exons and 14 introns (Shi et al., 2003). Recent studies have shown that polymorphisms are present in the porcine *BPI* gene and have demonstrated their impact on disease resistance/susceptibility. Shi et al. (2003) reported that polymorphisms in exon 4 of the *BPI* gene in York, Pig Improvement Company (PIC), and Meishan pigs were related to resistance against *Salmonella* infection. Moreover, Christopher et al. (2004) reported that the *Ava*II and *Hpa*II restriction fragment length polymorphism (RFLP) sites in exons 4 and 10 of *BPI* were related to susceptibility to *Salmonella* infection. Some single nucleotide polymorphism (SNP) sites in the *BPI* gene may be important molecular markers of porcine disease resistance, specifically resistance against Gram-negative bacteria (Cao, 2008). Based on these and other findings, *BPI* was identified as a candidate gene for disease-resistance breeding.

Enterotoxigenic *Escherichia coli* and *Salmonella* spp are the main Gram-negative bacteria found in the swine intestine. Previous research has shown that the *BPI* gene is related to the susceptibility to *Salmonella* infection; we found that genetic variation in exon 10 of the *BPI* gene is associated with *E. coli* F18 (Liu et al., 2013). To determine whether the *BPI* gene is an effective marker for anti-disease breeding, we detected polymorphisms in exons 4 and 10 in the *BPI* gene. Important immune indices were assessed using an enzyme-linked immunosorbent assay, and the effect of single genotypes and combined genotypes of the *BPI* gene on immune indices were analyzed. In this study, we examined the effect of polymorphisms in the

BPI gene and provided a theoretical basis for genetic marker selection for resistance against Gram-negative bacteria.

MATERIAL AND METHODS

Experimental materials

Sutai pigs (N = 196) from 10 lineages were provided by the Jiangsu Engineering Research Centre for Molecular Breeding of Pigs located in Suzhou city. Approximately 1.0 g ear tissue was collected from each individual and stored in a 1.5-mL microcentrifuge tube in an icebox. DNA was extracted using the conventional phenol-chloroform method and stored at -20°C for later use.

Polymerase chain reaction (PCR)-single-strand conformation polymorphism (SSCP)/RFLP analysis

The *BPI* mRNA sequence (accession No. EF436278) and genomic sequence (accession No. FP339579.2) were obtained from the Spidey database (http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/). The 15 exon sequences for the *BPI* gene were determined using the genomic sequence obtained. Using the Primer Premier 5.0 software, primers were designed based on the acquired sequences (Table 1). The primers were synthesized by Shanghai Biological Engineering Technology Co. Ltd. (Shanghai, China). The amplification reaction system consisted of 2.5 μL 10X PCR buffer, 1.5 μL 2.5 x 10^3 μM dNTPs, 1 μL 10 μM primers (upstream), 1 μL 10 μM primers (downstream), 0.2 μL *Taq* enzyme (5 U/μL) (TaKaRa Biotechnology Dalian Co. Ltd., Shiga, Japan), 1 μL 100 ng/μL DNA template, and 17.8 μL ddH_2O; the total volume was 25 μL. The PCR amplification program consisted of denaturation at 94°C for 4 min, 32 cycles of denaturation at 94°C for 30 s, annealing (specific temperatures are shown in Table 1) for 30 s, and extension at 72°C for 30 s, followed by a final extension at 72°C for 7 min; the reaction was held at 4°C.

Table 1. Primer sequences for *BPI* exons 4 and 10.

Primer	Primer sequence	Annealing temperature (°C)	Expected length (bp)
Exon 4	F: 5'-TCAGGTTGGTTACCGCAGAG-3' R: 5'-ACCCTGTTGATGTGGCTTCT-3'	62.4	200
Exon 10	F: 5'-CCCAACATGGAGATGCAGTTC-3' R: 5'-CAATGAATCAATGAGCACACC-3'	57.0	445

For PCR-SSCP analysis of the *BPI* exon 4, 4 μL of each PCR product was mixed with 7 μL denaturing loading buffer (0.05% xylene-cyanide, 0.05% bromophenol blue, and 5.5 mM EDTA, pH 8.0, in formamide), denatured at 98°C for 15 min, and snap-chilled on ice for at least 5 min. Samples were then loaded onto 12% polyacrylamide gels (acrylamide:bis-acrylamide = 29:1) and electrophoresed at a constant voltage of 110 V; the gel was stained with silver and visualized under ultraviolet light.

For PCR-RFLP analysis of the *BPI* exon 10, 10 μL of each PCR product was digested overnight at 37°C using the *Hpa*II (5 U/μL) restriction enzyme. Digested fragments were

electrophoresed on 10% polyacrylamide gels in 1X tetrabromoethane at a constant voltage of 120 V, stained with silver, and visualized under ultraviolet light. For sequencing, SNPs in *BPI* exons 4 and 10 were detected by sequence alignment using DNAMAN version 5.0 (Lynnon Biosoft, Quebec, Canada).

PCR products were extracted using a gel extraction kit (Bio Basic, Inc., Quebec, Canada) and sequenced using the ABI PRISM 377 DNA auto sequencer (Shanghai Biotechnology Co. Ltd., Shanghai, China).

Determination of immune indexes

Jugular venous blood was collected from 35-day-old weaning piglets. EDTA was used as an anticoagulant. Blood serum was separated using conventional methods, including centrifugal separation at 2000-3000 rpm. Sample absorbance was determined using an enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN, USA). The absorbance values were used to draw the standard curve, which was used to measure the following 6 indices: IL-2, IL-4, IL-6, interferon-β (INF-β), IL-10, and IL-12.

Statistical analyses

The frequencies of the gene and genotypes were calculated according to Hardy-Weinberg equilibrium principle: $p = P + H/2$, $q = Q + H/2$, $\chi^2 = \sum d^2/e$. In this equation, $d = e - o$, which is the difference between the predicted value and the detected value; p and q represent the allele frequencies at specific positions; P and Q represent the homozygote frequency at certain positions; H represents the heterozygote frequency at different positions; χ^2 represents chi-square value. The general linear model was established using the SPSS version 18.0 software (SPSS, Inc., Chicago, IL, USA) to analyze the effects of the genotypes at *BPI* exons 4 and 10 on measured traits. The following linear models were used:

Model 1: $Y_{ij} = \mu + \text{Genotype}_i + e$

Model 2: $Y = \mu + \text{Genotype}_{BPI\,\text{exon 4}} + \text{Genotype}_{BPI\,\text{exon 10}} + \text{Genotype}_{BPI\,\text{exon 4 - }BPI\,\text{exon 10}} + e$

Y_{ij} represents the measured traits; μ represents the overall mean; Genotype_i is the genotypic effect at *BPI* exon 4 or *BPI* exon 10; and e is the residual error.

RESULTS

PCR-SSCP analysis of the *BPI* exon 4

A clear DNA band was observed at 200 bp; this was in agreement with the predicted amplified fragment size. SSCP analysis indicated that there were 3 genotypes: CC, CD, and DD. The electrophoresis map is shown in Figure 1.

PCR-RFLP analysis of the *BPI* exon 10

A clear DNA band was observed at 445 bp; this was in agreement with the predicted amplified fragment size. A restriction enzyme recognition site for *Hpa*II was present in the *BPI-10* gene, which was completely digested by *Hpa*II to produce the BB phenotype (304/142

bp). If this position contained a B → A mutation, the *Hpa*II enzyme was unable to digest the fragment, resulting in the AA phenotype (445 bp). When both alleles existed together, they produced the AB type (445/304/142 bp). The restriction map is shown in Figure 2.

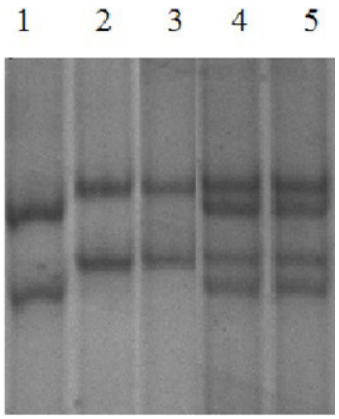

Figure 1. SSCP electrophoresis patterns of PCR products at exon 4 of the *BPI* gene. *Lane 1* = DD type; *lanes 2* and *3* = CC types; *lanes 4* and *5* = CD types.

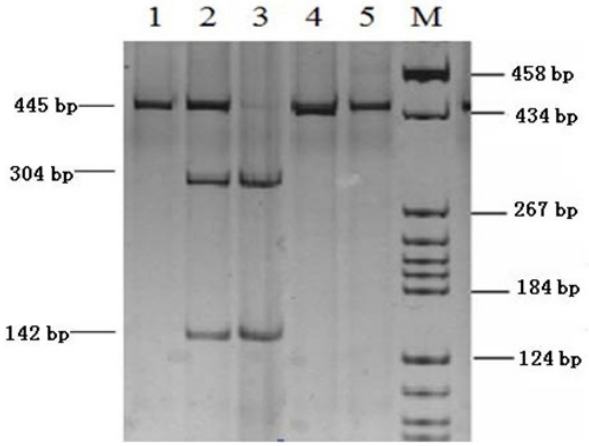

Figure 2. RFLP electrophoresis patterns of PCR products at exon 10 of the *BPI* gene. *Lanes 1*, *4*, and *5* = AA types; *lane 2* = BB type; *lane 3* = AB type; *lane M* = pUC19 DNA/*Msp*I (*Hap*II) marker.

Sequence analysis

Sequencing of the BPI exon 4

Sequence analysis of the PCR products of genotypes CC and DD revealed that the sequence of genotype CC corresponded to the sequence provided in GenBank (EF436278); however, genotype DD contained a C → T mutation at the 522-bp position, which did not result in an amino acid change. The sequencing map is shown in Figure 3.

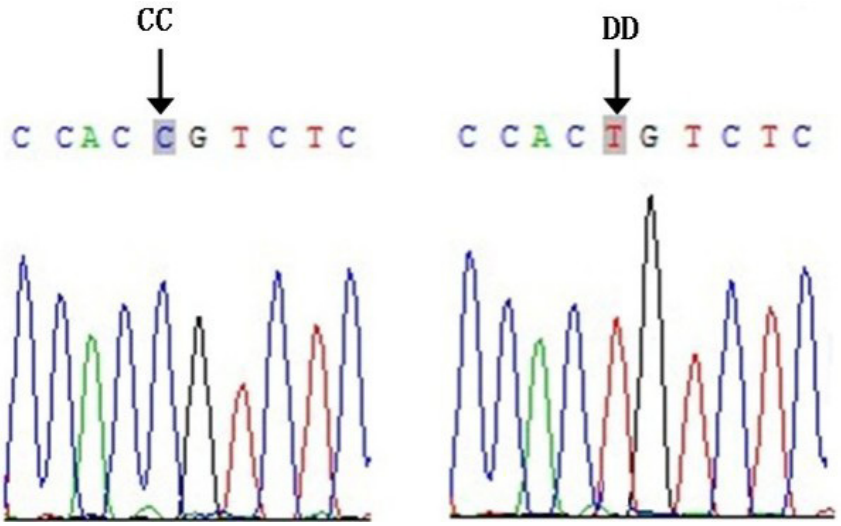

Figure 3. Sequencing results at exon 4 of *BPI* c.522 C > T locus.

Sequencing of the BPI exon 10

Sequence analysis of the PCR products for genotypes AA and BB showed that the sequence of genotype AA corresponded with the sequence provided in GenBank (EF436278). However, genotype BB contained an A → G mutation at the 1060-bp position, resulting in an amino acid substitution (Thr353Ala), as well as a T → G mutation at the 1151-bp position, which also resulted in an amino acid substitution (Leu383Arg). The sequencing map is shown in Figures 4 and 5.

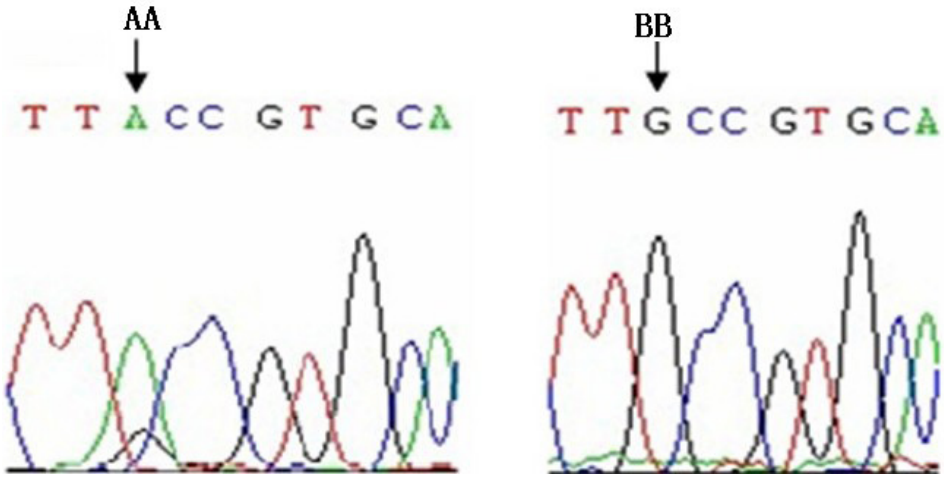

Figure 4. Sequencing results at exon 10 of *BPI* c.1060 A > G locus.

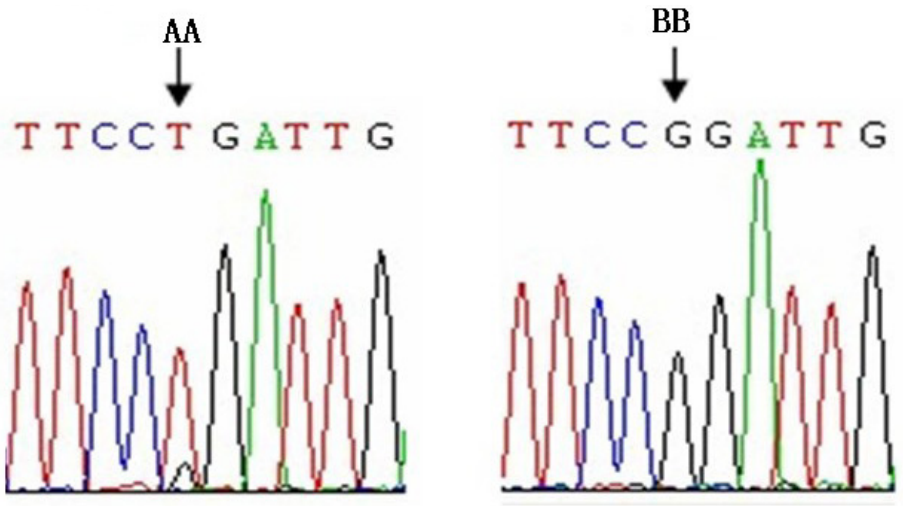

Figure 5. Sequencing results at exon 10 of *BPI* c.1151 T > G locus.

Polymorphisms in *BPI* exons 4 and 10 in Sutai pigs

Among the 196 Sutai pigs, 148 showed the CC genotype, 36 showed the CD genotype, and 12 showed the DD genotype at the *BPI* exon 4 site. The frequencies of the CC, CD, and DD genotypes were 0.755, 0.184, and 0.061, respectively, and allele C was the dominant allele. The χ^2 goodness-of-fit test showed that the sample was in genetic equilibrium ($P > 0.05$, Table 2).

The genotype distribution at the *BPI* exon 10 site was as follows: 50, AA type; 106, AB type; 40, BB type. The frequencies of the AA, AB, and BB genotypes were 0.255, 0.541, and 0.204, respectively. The proportion of allele A was similar to that of allele B. According to the χ^2 goodness-of-fit test, the sample showed significant deviation from Hardy-Weinberg equilibrium ($P < 0.05$, Table 2).

Table 2. Genotype and allele frequencies at *BPI* exon 4 and 10 sites in Sutai pigs.

Gene	Genotype frequency			Allele frequency		χ^2 value
BPI exon 4	CC	CD	DD	C	D	8.331
	0.755 (148)	0.184 (36)	0.061 (12)	0.847	0.153	
BPI exon 10	AA	AB	BB	A	B	0.699
	0.255 (50)	0.541 (106)	0.204 (40)	0.526	0.474	
Combined genotype	CCAA	CCAB	CCBB	CDAA	CDAB	DDAA
	0.082 (16)	0.388 (76)	0.214 (42)	0.163 (32)	0.133 (26)	0.020 (4)

The genotype quantity is shown in the parentheses, $\chi^2_{0.05}(1) = 3.84$, $\chi^2_{0.01}(1) = 6.64$.

Relationship between the *BPI* exon 4 and 10 polymorphisms and immune indices

There were no significant differences in the performance of 196 Sutai pigs from 10 lineages; thus, these pigs were analyzed without partition. With regard to *BPI* exon 4, the IL-6 level in individuals with genotype CD was significantly higher than that in individuals with

genotypes CC and DD ($P < 0.05$; Table 3). The IL-2, IL-4, IFN-β, and IL-10 levels showed no significant differences between the 3 genotypes. With regard to *BPI* exon 10, the IL-12 level was significantly higher when pigs showed the AB phenotype ($P < 0.05$, Table 4).

Table 3. Relationship between different genotypes at the *BPI* exon 4 site and immune indices (mean ± SD).

Genotype	Immune indexes (pg/mL)					
	IL-2	IL-4	IL-6	IFN-β	IL-10	IL-12
CC	38.78 ± 16.84	6.53 ± 1.96	125.11 ± 34.75^{a}	9.48 ± 3.39	21.55 ± 6.06	31.86 ± 8.78
CD	48.02 ± 27.79	7.89 ± 3.57	157.88 ± 59.75^{b}	9.24 ± 5.07	23.55 ± 8.53	31.73 ± 9.18
DD	33.79 ± 13.23	6.03 ± 1.03	99.97 ± 19.29^{a}	6.61 ± 3.36	19.83 ± 2.44	22.73 ± 6.97

Different letters in a column indicate significant difference ($P < 0.05$).

Table 4. Relationship between different genotypes at the *BPI* exon 10 site and immune indexes (mean ± SD).

Genotype	Immune indexes (pg/mL)					
	IL-2	IL-4	IL-6	IFN-β	IL-10	IL-12
AA	47.02 ± 24.25	7.50 ± 3.92	135.43 ± 56.63	8.46 ± 4.96	22.37 ± 8.73	27.79 ± 7.28^{a}
AB	37.61 ± 20.23	6.69 ± 1.93	134.53 ± 44.34	9.66 ± 3.82	22.08 ± 6.46	33.39 ± 9.42^{b}
BB	48.44 ± 21.45	7.32 ± 2.76	140.73 ± 52.65	8.72 ± 3.72	22.09 ± 6.16	29.01 ± 8.69^{a}

Different letters in a column indicate significant difference ($P < 0.05$).

Based on the effect of a single genotype at the *BPI* exon 4 or 10 site, we attempted to combine genotypes at the *BPI* exon 4 and 10 sites to examine the effect of the combined genotypes on the immune indices (Table 5). The combined genotypes showed a significant effect on IL-2, IL-6, and IL-12 levels ($P < 0.05$). In individuals with the CDAB genotype, IL-6 level was significantly higher than that in individuals with the CCAB genotype ($P < 0.05$), and the IL-12 level was significantly higher than that in individuals with the DDAA genotype ($P < 0.05$). Overall, the IL-6 and IL-12 levels for the CDAB genotype were the highest, and thus this combined genotype can be considered superior in terms of disease resistance.

Table 5. Relationship between combined genotypes at *BPI* exon 4 and 10 sites and immune indices (mean ± SD).

Genotype	Immune indexes (pg/mL)					
	IL-2	IL-4	IL-6	IFN-β	IL-10	IL-12
CCAA	43.71 ± 3.12^{ab}	6.03 ± 1.48	107.25 ± 1.95^{ab}	7.35 ± 1.25	18.19 ± 6.37	27.60 ± 2.76^{ab}
CCAB	33.68 ± 13.23^{a}	6.20 ± 1.49	119.59 ± 22.87^{a}	10.06 ± 3.33	21.65 ± 6.22	33.66 ± 8.98^{a}
CCBB	48.44 ± 21.45^{ab}	7.32 ± 2.77	140.73 ± 52.65^{ab}	8.72 ± 3.72	22.09 ± 6.16	29.01 ± 8.69^{ab}
CDAA	52.04 ± 27.52^{b}	8.17 ± 4.68	150.81 ± 63.93^{ab}	9.11 ± 5.76	23.97 ± 10.17	29.68 ± 7.09^{ab}
CDAB	43.27 ± 28.85^{ab}	7.35 ± 2.43	160.52 ± 61.55^{b}	8.64 ± 4.60	22.95 ± 7.35	34.17 ± 10.06^{a}
DDAA	32.50 ± 15.89^{ab}	6.25 ± 1.14	102.99 ± 22.44^{ab}	7.04 ± 3.98	19.80 ± 2.81	21.63 ± 8.10^{b}

Different letters in a column indicate significant difference ($P < 0.05$).

DISCUSSION

In our study, one mutation, C522T, at the *BPI* exon 4 site, and two mutations, A1060G and T1151G, at the *BPI* exon 10 site, were detected in Sutai breeds. These mutations differ from those reported in other studies. Cao (2008) reported that 6 SNPs were present in exon 4

of the *BPI* gene, including C512T, T551G, C563T, T573C, G599A, and T607C, among which T573C and T607C caused amino acid mutations. Tuggle et al. (2006) reported that one mutation, G599A, in exon 4 of the *BPI* gene was related to resistance in swine. The difference in results between this study and previous studies may be because of differences in breed or individuals used; therefore, our results need to require further investigation.

With regard to the immune indices, IL-6 levels were found to be significantly higher in pigs with the CD genotype than in pigs with the other 2 genotypes at the *BPI* exon 4 site, and IL-12 levels in pigs with the AB genotype were significantly higher than in pigs with the other 2 genotypes at the *BPI* exon 10 site. Because cytokines play a significant role in immune responses in the body, including humoral and cellular immunity, cytokine levels can be used as an accurate physiological index for stress response and resistance. IL-6 is a cytokine with multiple biological activities, a number of which are involved in various aspects of immune and inflammatory responses (Kishimoto and Hirano, 1988). IL-6 is produced by various cell types, such as fibroblasts, macrophages, T and B lymphocytes, endothelial cells, glia cells, and keratinocytes, upon exposure to a variety of extracellular stimuli (Ray et al. 2008). IL-6 is not produced under normal conditions. However, once bacterial and viral infections or tissue injuries occur in the host, IL-6 is expressed rapidly and transiently and participates in host defense. A decrease in the expression of IL-6 in swine lymphocytes leads to continuous immunosuppression (Chen et al., 2010). IL-12 was independently discovered by Kobayashi et al. in 1989 and Stern et al. in 1990 as "natural killer-stimulating factor" and "cytotoxic lymphocyte maturation factor", respectively. IL-12 has the extensive capacity to activate cytotoxic lymphocytes, including Th1-mediated CD4+ cell differentiation, and stimulate the activation of natural killer cells and the production of interferon-γ (Shrayer et al., 2002; Yoon et al., 2003), and thus it was considered to be a target gene for bioprevention. Therefore, higher expression of IL-6 or IL-12 contributes to improved general disease resistance in the body. Therefore, pigs with the CD and AB genotypes had higher levels of IL-6 and IL-12, respectively, and showed strong stress resistance.

Most economic traits of livestock and poultry are regulated by polygenic sites at different levels. The effect of a single genetic marker is relatively weak, and therefore cannot be directly applied to actual production; thus, it is necessary to consider complex interactions between different sites. The effect of polygenic sites is the additive effect of 1 or more single sites. The effect of combined genotypes is not only likely to be stronger than that of single genotypes (Liu et al., 2009, 2010), but also has no corresponding advantages compared to single genotypes (Chang et al., 2009). Currently, gene polymerization is mainly used to analyze animal growth traits and reproductive performance, for which genetic improvement of livestock and poultry by marker-assisted selection is more useful (Zeng et al., 2011). In our study, the optimal combined genotype at the *BPI* exon 4 and 10 sites was CDAB, as it resulted in higher IL-6 and IL-12 levels compared to the other combined genotypes. The combined genotype CDAB (the value of IL-6 and IL-12 was 160.52 and 34.17 pg/mL, respectively) was significantly more effective than the CD genotype (the value of IL-6 was 157.88 pg/mL), and the AB genotype (the value of IL-12 was 33.39 pg/mL), further indicating that combined genotypes were more effective than single genotypes. The effect of combined genotypes should be further examined and validated in a larger population of Sutai pigs, particularly with regard to their role as genetic markers of resistance. Moreover, the immune ability of Sutai pigs resistant to disease should be analyzed by using cytokine and lymphocyte subtype detection to provide a basis for breeding disease-resistant strains of Sutai pigs.

ACKNOWLEDGMENTS

Research supported by grants from the National Natural Science Funds (#31372285, #31172183), the Science and Technology Supporting Project of Jiangsu Province (#BE2014357, #BE2012330, #BE2013345), and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

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