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전복 (*Haliotis discus hannai*) 에서 생체지표유전자를 이용한 건강도 평가 검증 모델 연구

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Development and Validation of a Biomarker Gene-Based Model for Health Assessment in Abalone (*Haliotis discus hannai*)

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ABSTRACT

The sustainable growth of abalone (*Haliotis discus hannai*) aquaculture requires reliable systems for seed quality verification and health assessment. Conventional morphological indicators are limited in sensitivity and speed, highlighting the need for molecular biomarkers. Differentially expressed genes (DEGs) identified through next generation sequencing (NGS) offer promising candidates, but their applicability as biomarker genes must be validated under controlled stress conditions. Juvenile abalones (2.2-2.3 cm shell length) were subjected to mechanical injury and oxidative stress (H_2O_2 exposure). Gill tissues were sampled at 3 h and 18 h post treatment, and gene expression was analyzed by quantitative real-time PCR (qPCR). Candidate biomarker genes included ferritin, Hsp70, IL-17, and NF-κB, representing oxidative defense, stress response, immune activation, and inflammatory regulation, respectively. Ferritin expression was significantly upregulated under both mechanical injury and oxidative stress, with the highest induction at 18 h, reflecting its role in iron storage and oxidative defense. Hsp70 showed rapid induction at 3 h, consistent with acute stress responses, and remained elevated at 18 h. IL-17 expression increased at both time points, indicating sustained immune activation in response to tissue damage and oxidative stimuli. NF-κB expression increased after 3 and 18 h, highlighting the role of NF-κB in initiating and maintaining the inflammatory response. These findings demonstrate that ferritin, Hsp70, IL-17, and NF-κB are responsive to mechanical and oxidative stress, supporting their utility as biomarker genes for assessing abalone health. The study establishes an experimental model to evaluate the applicability of NGS-derived DEGs as biomarkers, providing a foundation for the development of molecular based systems for seed quality verification and aquaculture health monitoring. Ultimately, this approach is expected to contribute to the sustainable growth of the abalone industry by improving disease resistance, enhancing stock quality, and advancing health management strategies.

Keywords: *Haliotis discus hannai*, NGS, DEGs, qPCR, ferritin, Hsp70, IL-17, NF-κB

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INTRODUCTION

The rapid expansion of aquaculture industries and the implementation of coastal resource enhancement policies have underscored the critical importance of seed quality in fisheries. Ensuring the production and dissemination of high quality seed stocks is one of the most effective strategies to improve

productivity, profitability, and sustainability in aquaculture. In parallel, globalization of the seed market and the strengthening of international agreements on variety protection have intensified competition among nations to secure intellectual property rights for superior indigenous genetic resources. Consequently, the preservation and development of high quality aquatic seed resources have emerged as both national and industrial priorities, driving active research worldwide.

Abalone (*Haliotis* spp.) represents one of the most economically valuable mollusks, with the Pacific abalone (*Haliotis discus hannai*) widely cultured along the Northeast Asian coasts and serving as a cornerstone of Korea aquaculture industry. However, rising seawater temperatures, increased stocking densities, pathogen infections, and other environmental stressors have led to reduced survival and productivity, thereby threatening the stability of abalone aquaculture. In artificial seed production, limited broodstock resources and repeated artificial breeding often result in diminished genetic diversity. This decline compromises immune competence and disease resistance, increasing susceptibility to pathogen transmission. The release of low quality seed into aquaculture systems or natural environments can further act as reservoirs of pathogens, perpetuating disease outbreaks and undermining both productivity and economic stability (Choi *et al.*, 2008; Li *et al.*, 2010; Zhang *et al.*, 2014; Wan *et al.*, 2017; Sutherland *et al.*, 2025). Therefore, establishing a reliable scientific system for seed quality verification and health assessment is urgently required.

Advances in molecular biology have provided new approaches for evaluating the health status of aquatic organisms (Swain *et al.*, 2022). Next-generation sequencing (NGS) has become a powerful tool for identifying differentially expressed genes (DEGs) under specific physiological or pathological conditions. Biomarker genes that respond sensitively to changes in health status offer distinct advantages over traditional morphological and physiological indicators, enabling faster and more quantitative assessments (Liu *et al.*, 2024; Oliva-Teles, 2024). However, rigorous validation is essential to determine whether DEGs identified through NGS can be effectively applied as biomarker genes. If the samples used for NGS analysis do not represent clear health contrasts, the applicability of the resulting DEGs as biomarkers may be limited.

In this context, four candidate genes ferritin, Hsp 70, IL 17, and NF-κB were selectively chosen for validation in the present study based on their established biological functions. Ferritin is a key iron storage protein that mitigates oxidative damage and has been reported as a stress responsive biomarker in aquatic animals (Kim *et al.*, 2015). Hsp70 is a highly conserved molecular chaperone that assists protein folding and is rapidly induced under acute stress conditions such as heat or mechanical injury Frontiers (Lyu *et al.*, 2020; Zhang and Qi 2025). IL-17 is a pro-inflammatory cytokine that mediates innate and adaptive immune responses, serving as an indicator of immune activation following tissue damage or pathogen exposure (Koh *et al.*, 2024). NF-κB is a central transcription factor regulating inflammation and immune signaling, known to be activated by oxidative stress and pathogen recognition (Lyu *et al.*, 2020). Together, these genes represent complementary aspects of oxidative defense, stress tolerance, immune activation, and inflammatory regulation, making them suitable candidates for biomarker validation in abalone health assessment (Oliveria *et al.*, 2024).

The present study aims to establish an experimental model for validating NGS-derived DEGs as biomarker genes applicable to health assessment in abalone. Juvenile abalones subjected to artificial physical injury and oxidative stress were employed as model organisms, and the expression of the four candidate biomarker genes was analyzed to assess their utility. Through this approach, the study seeks to provide foundational evidence for the development of molecular-based systems for seed quality verification and aquaculture health monitoring, ultimately contributing to the sustainable growth of the abalone industry.

Materials and methods

1. Preparation of Abalone Juveniles

A total of 50 juvenile abalones (*Haliotis discus hannai*) with shell lengths of 2.2–2.3 cm were used in this study. All individuals originated from the same hatchery and were spawned and stocked at the same time to ensure uniformity. The juveniles were divided into groups of 10 individuals each. The groups were assigned as follows: (i) control group, (ii) physical injury group sampled at 3 h post-treatment, (iii) physical injury group sampled at 18 h post-treatment, (iv) oxidative stress group sampled at 3 h post-treatment, and (v) oxidative stress group sampled at 18 h post-treatment.

2. Induction of Physical Injury and Oxidative Stress

To establish experimental models for validating biomarker genes, juvenile abalones (2.2–2.3 cm shell length) were subjected to physical injury and oxidative stress treatments. For the physical injury model, the epipodium was incised approximately 1 mm to induce tissue damage. For the oxidative stress model, individuals were exposed to 150 μM hydrogen peroxide (H_2O_2) (Alam *et al*, 2022). Gill tissues were collected from both physically injured and oxidative stress-induced abalones at 3 h and 18 h post-treatment. The collected tissues were immediately processed for total RNA extraction to be used in subsequent gene expression analyses.

3. Total RNA extraction and cDNA synthesis

Total RNA was extracted from gill tissues using RNA isoplus reagent (TaKaRa, Japan) according to the manufacturer's protocol. Briefly, 100 mg of tissue was homogenized in 1 mL of RNA isoplus, incubated at room temperature for 5 min, followed by the addition of 0.2 mL chloroform. After centrifugation at 4 °C for 15 min, the aqueous phase was transferred to a new tube, mixed with 0.5 mL isopropanol, and incubated for 10 min at room temperature. The RNA pellet was washed with 75% ethanol, air-dried, and dissolved in DEPC-treated water. RNA concentration and purity were determined using a spectrophotometer (NanoVue, GE Healthcare, USA), and samples with an A260/A280 ratio 1.8–2.0 were used for analysis. Residual genomic DNA was removed by DNase I treatment.

First-strand cDNA was synthesized from 1 μg of total RNA using the Cyclescript RT premix (dT) kit (Bioneer, Korea) following the manufacturer's instructions. The reaction was performed in a final volume of 20 μL under the following conditions: 20 °C for 30 s, 42 °C for 4 min, and 55 °C for 30 s, repeated for 12 cycles, followed by 95 °C for 5 min.

4. Quantitative real-time PCR (qPCR)

Gene expression analysis was performed using the SFCgreen Fast qPCR Master Mix (2 ×) kit (SFCprobes Co., Ochang, Korea) on a CFX Real-Time PCR Detection System (Bio-Rad, USA). Each 20 μL reaction contained 1 μL of cDNA, 1 μL of each primer (20 pmol), 10 μL of Master Mix, and nuclease-free water. The amplification protocol consisted of an initial denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 20 s, 58 °C for 20 s, and 72 °C for 20 s. Melting curve analysis was performed from 60 °C to 95 °C at 0.5 °C increments. Relative gene expression levels were calculated using the comparative Ct (2^-Δ ΔCt) method, with S9 ribosomal protein gene as the internal control. Primer sequences used for qPCR are listed in Table 1.

Table 1. Primer sequences used for qPCR analysis

Biomarker Gene	Forward primer	Reverse primer
Ferritin	5-TCCATAAGTATTTCCTGGCCGCGT-3	5-TCGTGAAGCTCTTCAGTCTCGTCA-3
Hsp70	5-GGATCGACCCGTATTCCTAA-3	5-TCCTGCTGTCTCAATGCCAA-3
IL-17	5-GCTGCTGCTACTGCTACTGT-3	5-CTGAGGTAGGTGGTGATGGT-3
NF-kB	5-AGACCTGCTGCTGATGACTT-3	5-TCTGGTGGTAGGTCTTGGTG-3
S9	5-CAGAATCCGAAAGTCAGCC-3	5-TCATCTTGCCCTCGTCCA-3

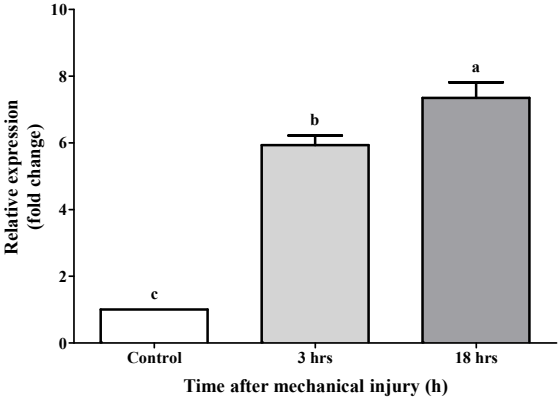

Fig. 1. Time-dependent expression of the ferritin gene in juvenile abalone (*Haliotis discus hannai*) following mechanical injury. Data are presented as mean ± SEM (n = 10). Different letters indicate statistically significant differences among groups as determined by one-way ANOVA followed by Tukey's multiple comparison test ($P < 0.05$).

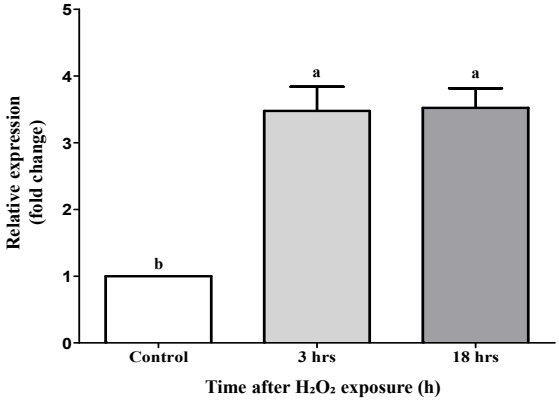

Fig. 2. Time-dependent expression of the ferritin gene in juvenile abalone (*Haliotis discus hannai*) following oxidative stress induction. Data are presented as mean ± SEM (n = 10). Different letters indicate statistically significant differences among groups as determined by one-way ANOVA followed by Tukey's multiple comparison test ($P < 0.05$).

5. Statistical analysis

All statistical analyses and graphical presentations were performed using GraphPad Prism software (version 5.0; GraphPad Software, San Diego, CA, USA). Data normality and homogeneity of variances were assessed prior to analysis. Differences among experimental groups were evaluated using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison post hoc test. Statistical significance was set at $P < 0.05$. Data are expressed as mean ± standard error of the mean (SEM).

RESULTS AND DISCUSSION

1. Ferritin gene expression

The expression of the ferritin gene in juvenile abalone (*Haliotis discus hannai*) was analyzed by qPCR using gill tissues collected at 3 h and 18 h after mechanical injury. Compared with the control group, ferritin expression was significantly upregulated in both treatment groups, with the highest level observed at 18 h (Fig. 1). Overall, ferritin displayed a time-dependent increase in response to mechanical injury.

Ferritin is a key iron-storage protein that stabilizes intracellular iron and mitigates oxidative stress caused by excess iron (Kim *et al.*, 2010). In aquatic organisms, ferritin has been reported as a critical biomarker gene involved in immune defense and stress responses (Qiu *et al.*, 2016; Nam *et al.*, 2013; Zhang *et al.*, 2022; Sutherland *et al.*, 2025). The observed time-dependent increase suggests activation of protective mechanisms against tissue damage and oxidative stress. The stronger induction at 18 h may reflect ferritin's role in recovery or sustained inflammatory responses. These findings support ferritin as a potential biomarker for assessing abalone health, offering rapid and quantitative evaluation compared with conventional morphological indicators.

Under oxidative stress induced by H_2O_2, ferritin expression was also significantly elevated at both 3 h and 18 h compared with the control (Fig. 2). Ferritin contributes to the detoxification of reactive oxygen species (ROS) and plays a central role in stress defense in mollusks (Nam *et al.*, 2013; Zhang *et al.*, 2022; Kim *et al.*, 2023). The consistent induction under oxidative stress highlights ferritin's utility as a molecular indicator of stress responses, while individual differences emphasize the need for population-level validation.

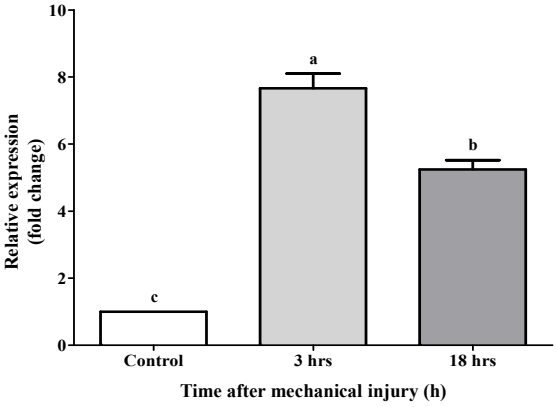

Fig. 3. Time-dependent expression of the Hsp70 gene in juvenile abalone (*Haliotis discus hannai*) following mechanical injury. Data are presented as mean ± SEM (n = 10). Different letters indicate statistically significant differences among groups as determined by one-way ANOVA followed by Tukey's multiple comparison test ($P < 0.05$).

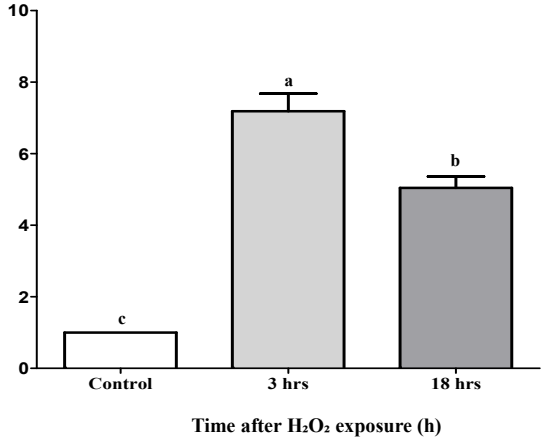

Fig. 4. Time-dependent expression of the Hsp70 gene in juvenile abalone (*Haliotis discus hannai*) following oxidative stress induction. Data are presented as mean ± SEM (n = 10). Different letters indicate statistically significant differences among groups as determined by one-way ANOVA followed by Tukey's multiple comparison test ($P < 0.05$).

2. Hsp70 gene expression

The expression of Hsp70 was significantly increased in juvenile abalone subjected to mechanical injury, with the highest induction at 3 h post-treatment (Fig. 3). At 18 h, expression declined compared with 3 h but remained higher than the control. This pattern indicates that Hsp70 responds rapidly to acute stress and gradually decreases as recovery progresses.

Hsp70 functions as a molecular chaperone, maintaining protein folding and stability, and repairing or removing damaged proteins under stress conditions. In aquatic species, Hsp70 is known to be induced by heat, mechanical injury, and oxidative stress, contributing to immune defense and cellular protection (De Zoysa *et al.*, 2009). The sharp increase at 3 h reflects activation of acute protective responses, while sustained elevation at 18 h suggests ongoing recovery or inflammation. These results highlight Hsp70 as a sensitive biomarker for acute stress in abalone, useful for early diagnosis and stock management.

Following oxidative stress, Hsp70 expression was also significantly elevated at both 3 h and 18 h compared with the control. Although individual variation was observed, most samples showed consistent induction. This confirms that Hsp70 is strongly responsive to oxidative stimuli, supporting its role as a biomarker for stress-related health assessment in abalone.

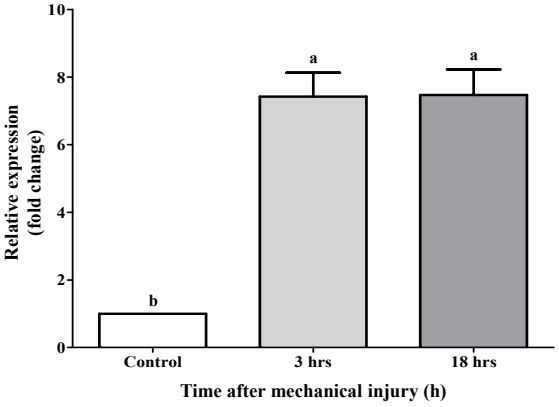

Fig. 5. Time-dependent expression of the IL-17 gene in juvenile abalone (*Haliotis discus hannai*) following mechanical injury. Data are presented as mean ± SEM (n = 10). Different letters indicate statistically significant differences among groups as determined by one-way ANOVA followed by Tukey's multiple comparison test ($P < 0.05$).

3. IL-17 gene expression

IL-17 expression was significantly upregulated at both 3 h and 18 h after mechanical injury compared

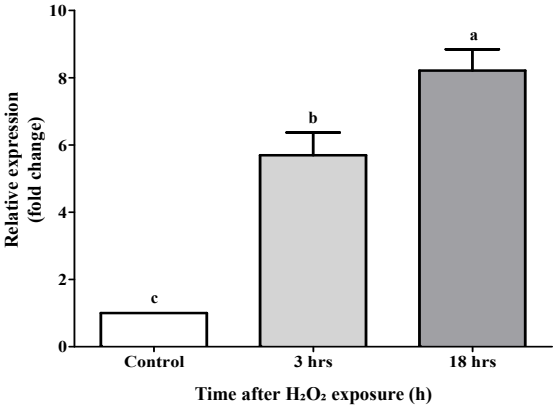

Fig. 6. Time-dependent expression of the IL-17 gene in juvenile abalone (*Haliotis discus hannai*) following oxidative stress induction. Data are presented as mean ± SEM (n = 10). Different letters indicate statistically significant differences among groups as determined by one-way ANOVA followed by Tukey's multiple comparison test ($P < 0.05$).

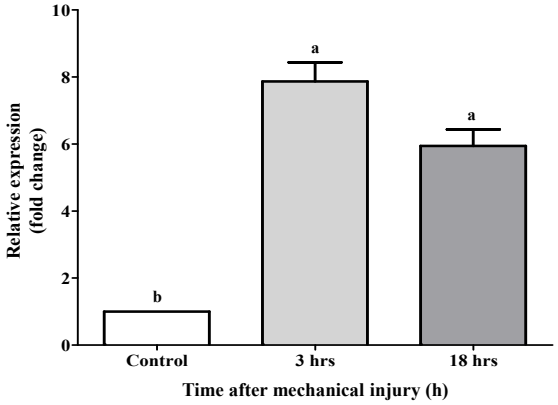

Fig. 7. Time-dependent expression of the NF-kB gene in juvenile abalone (*Haliotis discus hannai*) following mechanical injury. Data are presented as mean ± SEM (n = 10). Different letters indicate statistically significant differences among groups as determined by one-way ANOVA followed by Tukey's multiple comparison test ($P < 0.05$).

with the control (Fig. 5). Most individuals showed higher expression at 18 h, although some exhibited similar or greater induction at 3 h. This indicates that IL-17 responds rapidly to injury and remains elevated over time.

IL-17 is a pro-inflammatory cytokine that plays a crucial role in innate and adaptive immune responses. It is expressed mainly by T cells and other immune cells, and is induced by pathogen infection, tissue damage, and stress stimuli (Nam *et al.*, 2013; Dougan *et al.*, 2019: Li *et al.*, 2019; Zhang *et al.*, 2021; Ma *et al.*, 2024; Yogesha *et al.*, 2024). In mollusks, IL-17 has been reported to regulate immune responses and inflammation. The sustained induction observed in this study suggests that IL-17 participates in injury recognition and immune activation in abalone. These findings support IL-17 as a promising biomarker for evaluating immune competence and health status in juvenile abalone.

Under oxidative stress, IL-17 expression was also significantly increased at both 3 h and 18 h (Fig. 6), with higher levels generally observed at 18 h. This time-dependent induction further supports IL-17's role in mediating immune responses to oxidative stimuli.

4. NF-kB gene expression

NF-κB expression was significantly elevated at both 3 h and 18 h after mechanical injury compared with the control (Fig. 7). The rapid induction at 3 h indicates activation of acute inflammatory responses, while sustained elevation at 18 h suggests prolonged immune activity or recovery processes.

NF-κB is a central transcription factor regulating immune responses, inflammation, cell survival, and stress signaling. In mollusks, NF-κB is activated by pathogen recognition, tissue damage, and oxidative stress, functioning as a key regulator of innate immunity (Li *et al.*, 2020; Huang *et al.*, 2023; Zhang *et al.*, 2022). The consistent induction observed in this study highlights NF-κB as a reliable biomarker gene for immune activation in abalone.

Following oxidative stress, NF-κB expression was also significantly increased at both 3 h and 18 h (Fig. 8).

This study analyzed the expression of ferritin, Hsp70, IL-17, and NF-κB in juvenile abalone subjected to mechanical injury and oxidative stress, demonstrating their responsiveness as biomarker genes. Ferritin was strongly associated with oxidative defense, Hsp70 with acute stress responses, IL-17 with immune activation, and NF-κB with inflammation and immune regulation. Together, these genes provide a molecular framework for evaluating abalone health in terms of immunity,

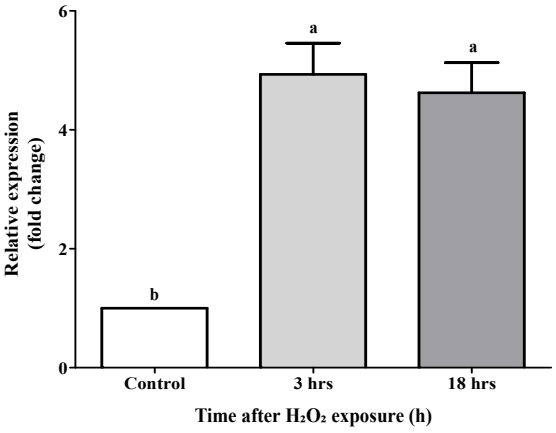

Fig. 8. Time-dependent expression of the NF-kB gene in juvenile abalone (*Haliotis discus hannai*) following oxidative stress induction. Data are presented as mean ± SEM (n = 10). Different letters indicate statistically significant differences among groups as determined by one-way ANOVA followed by Tukey's multiple comparison test ($P < 0.05$).

metabolism, and stress tolerance.

The ultimate goal of this research is to establish an experimental model to evaluate whether differentially expressed genes (DEGs) identified through next-generation sequencing (NGS) can be applied as biomarker genes for health assessment in abalone. By validating candidate DEGs under controlled stress conditions, this study provides foundational evidence for the development of new biomarker genes applicable to seed quality verification and aquaculture health monitoring. These findings are expected to contribute to the sustainable growth of the abalone aquaculture industry by improving disease resistance, enhancing stock quality, and advancing molecular-based management systems.

요 약

전복 (*Haliotis discus hannai*) 양식의 지속 가능한 성장을 위해서는 종묘 품질 검증 및 건강 평가를 위한 신뢰할 수 있는 시스템이 필수적입니다. 기존의 형태학적 지표는 민감도와 신속성이 제한적이어서 분자 바이오마커의 필요성이 대두되고 있습니다. 차세대 염기서열 분석 (NGS) 을 통해 확인된 차등 발현 유전자 (DEGs) 는 유용한 후보이지만, 객관적 검증된 스트레스 조건 하에서 생체지표유전자로서의 적용 가능성을 검증해야 할 필요성이 있다. 전복 치패 (패각 2.2-2.3 cm) 을 기계적 손상과 산화 스트레스 (과산화수소 노출) 에 노출시킨 후, 처리 후 3시간과 18시간에 아가미 조직을 수집하여 정량적 실시간 PCR (qPCR) 을 통해 유전자 발현을 분석했습니다. 검증 대상의 생체지표유전자로는 산화 방어, 스트레스 반응, 면역 활성화 및 염증 조절을 각각 나타내는 ferritin, Hsp70, IL-17 및 NF-κB입니다. Ferritin 발현은 기계적 손상과 산화 스트레스 모두에서 유의하게 상승 조절되었으며, 18시간 경과 후 가장 높은 발현을 보여 철 저장 및 산화 방어에서의 역할을 반영하였다. Hsp70은 급성 스트레스 반응과 일치하게 3시간 경과 후 빠르게 유도되었고 18시간 후에도 높은 수준을 유지하였다. IL-17 발현은 3시간 및 18시간 경과 후에서 모두 증가하여 조직 손상 및 산화 자극에 대한 지속적인 면역 활성화를 나타내었다. NF-κB 발현은 3시간 경과 후 증가했고 18시간 경과 후에는 더욱 증가하여 염증 반응의 시작 및 유지에 중요한 역할을 하는 것으로 나타났다. 이러한 결과는 ferritin, Hsp70, IL-17 및 NF-κB가 기계적 스트레스와 산화 스트레스에 반응함을 보여주며, 전복 건강 평가를 위한 생체지표유전자로서의 유용성을 보여주었다. 본 연구는 NGS 기반 차등 발현 유전자 (DEGs) 의 생체지표유전자로서의 적용 가능성을 평가하는 실험 모델을 제시하며, 종묘 품질 검증 및 양식 건강 모니터링을 위한 분자 기반 시스템 개발의 토대를 제공한다고 평가한다. 궁극적으로 이러한 접근 방식은 질병 저항성을 향상시키고, 종자 품질을 높이며, 건강 관리 전략을 발전시킴으로써 전복 산업의 지속 가능한 성장에 기여할 것으로 생각된다.

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