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Communication

N-Acetylglutamic Acid Enhances Tolerance to Oxidative and Heat Stress in *Humulus lupulus*

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Abstract: *Humulus lupulus* (hop) is a necessary material in beer brewing because its female inflorescences (called hop cones) give a floral aroma, bitterness and foam stability to beer. Various aspects of growth conditions in the cultivation area, especially temperature, strongly affect the yield and quality of hop cones. Recent estimates suggest that climate change accompanied by global warming is negatively impacting hop production, with high temperatures reducing the expression of genes that regulate beneficial secondary metabolites in hops. This underscores the need for techniques to enhance hop tolerance to high temperatures. This study explores the potential of N-acectylglutamic acid (NAG), a non-proteinogenic amino acid, to confer hops with tolerance against oxidative and heat stress by suppressing ROS accumulation. Exogenous NAG treatment activated the expression of *HlZAT10/12* and *HlHSFA2*, which are putative homologues considered master regulators in response to oxidative and heat stress in *Arabidopsis thaliana* (Arabidopsis). Additionally, histone acetylation, a histone modification associated with transcriptional activation, was increased at these stress-responsive genes in the NAG-treated hops. These findings reveal NAG as a potential chemical compound to mitigate hop production reduction caused by high temperatures and suggest the conservation of epigenetic modification-mediated regulation of gene expression in response to environmental stresses in hops.

Keywords: *Humulus lupulus*; stress response; chemical priming; epigenetic modification; N-acetylglutamic acid

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1. Introduction

Humulus lupulus L. (hop), a diploid, perennial, dioecious and climbing plant belonging to the Cannabaceae family, and is cultivated in cold climate areas globally [1]. The female inflorescence of hops is recognised as the 'hop cone' and is formed by multiple bracts, with bracteoles surrounded by a strig. In the base of bracteoles, glandular trichomes (GTs), also known as lupulin glands, comprising basal cell, stalk and secretory cells, can biosynthesise and store various specialised secondary metabolites, such as prenylflavonoids (xanthohumol and demethylxanthohumol), essential oils (myrcene, α-humulene and β-caryophyllene) and bitter acids (α-acid [humulone] and β-acid [lupulone]) [2,3]. These secondary metabolites derived from hop cones give a floral aroma, bitterness and foam stability to beer, thus hops are used as an essential material in beer brewing.

Climate change, accompanied by global warming, has recently been estimated to impair the quality and yield of hops. This is because hops are cultivated in restricted areas, limited to small regions with suitable environmental conditions [4]. Temperature–yield correlation analysis over past decades reveals that high temperatures during the summer, coinciding with the hop growth period, have negatively affected the production of hop cones and their α-acid content in the Czech Republic, which, along with Germany, is one of the major hop production areas [5]. The combined analysis of meteorological measurements and projection models predicts that global warming, resulting in a 1.4 °C temperature

increase, will induce early ripening of hop cones. This is expected to trigger hop yield reduction, accompanied by decreased α-acid content, in both the Czech Republic and Germany [6]. Furthermore, high temperatures cause reduced expression of various genes regulating secondary metabolite biosynthesis, with a marked decrease observed in the valerophenone synthase gene, which plays a crucial role in bitter acid production [7]. These results highlight the need to develop strategies to counteract the detrimental effects of high temperatures on hop cultivation. However, fundamental research on hop plants remains insufficient, and the molecular mechanisms by which hops respond to stress remain incompletely elucidated.

Among techniques to enhance tolerance against environmental stresses, including high temperatures, chemical priming shows promise in plants. This method involves treating plants with functional chemical compounds to improve the stress response. It offers effective crop yield maintenance without genetic engineering amid climate changes [7]. For example, the amino acid γ-aminobutyric acid (GABA), whose content increases under high temperatures, modulates the expression of heat shock proteins (HSPs) and photosynthesis activity. This induces heat stress tolerance in *Actinidia chinensis* (kiwifruit) and *Agrostis stolonifera* (creeping bentgrass) [8–10]. Exogenous treatment with β-aminobutyric acid (BABA), an isomer of GABA, restores inhibited growth following heat stress in *Arabidopsis thaliana* (Arabidopsis) and *Brassica rapa* by upregulating abscisic acid-related genes and maintaining photosynthesis activity under high temperatures, respectively [11,12]. Furthermore, the activation of the response to misfolding proteins by ethanol enhances tolerance to heat stress in Arabidopsis and *Lactuca sativa* (lettuce) [13].

Under field conditions, high temperatures exacerbate negative effects on plants in conjunction with other environmental factors, particularly high light stress. This results in oxidative stress in plants, primarily reducing photosynthesis activity. High temperatures themselves induce oxidative stress by disrupting the ROS equilibrium in chloroplasts and mitochondria. This implies that chemical priming, activating the response to both oxidative and heat stress in hops, could effectively enhance growth under high temperatures. Previously, we found that exogenous treatment with N-acetylglutamic acid (NAG), a non-proteinogenic amino acid, confers heat and oxidative stress tolerance in Arabidopsis and *Oryza sativa* (rice), with improvements in heat- and oxidative-stress-responsive genes observed following the deposition of histone acetylation, which is an epigenetic modification closely related to gene activation [14,15]. Based on this finding, this study examined NAG effectiveness in alleviating heat and oxidative stress in hops.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

Humulus lupulus cv. 'Saaz' and 'Hersbrucker' were used as materials in this study. In vitro hop plants were established as previously described [16]. Stem explants were isolated from plants and sterilised with 70% (*v*/*v*) ethanol and 1% (*v*/*v*) sodium hypochlorite for 1 min and 5 min, respectively. After three washes with sterilised water, the explants were transferred to a 1/2 Murashige and Skoog (MS) medium containing 2% (*w*/*v*) glucose, 0.01 ppm benzyl adenine and 0.8% (*w*/*v*) agar at pH 5.8. The samples were moved into an incubator set at 20 °C with a 16 h light/8 h dark photoperiod. After 3 weeks, developed axillary buds with explants were transferred to a 1/2 MS medium containing 2% glucose and 0.8% agar to establish the in vitro plants.

2.2. Measurement of Chlorophyll Content

To measure the chlorophyll content of hop leaves, leaves of 1.5-month-old hop plants were treated with 0.5 mM NAG (Tokyo, Japan, Chemical Industry Co., Ltd.) in a 1/2 MS liquid medium containing 2% glucose (pH 5.8) for 24 h. After incubation, 5 µM methyl viologen (MV, Signa-Aldrich, St. Louis, MO, USA) was added to this medium, and the leaves of cv. 'Saaz' and 'Hersbrucker' were incubated in the medium for 48 h or 96 h, respectively. Three leaves were subjected to 1 mL *N*, *N*'-dimethylformamide (DMF) in

1.5 mL tubes at 4 °C for 24 h. The absorbance of the extraction liquid was measured at 646.8 nm and 663.8 nm on an NP80 spectrophotometer (Implen, Munich, Germany). Total chlorophyll amounts were calculated using the following formula: Chl a + b (μM) = 19.43 A646.8 + 8.05 A663.8. Each chlorophyll content (μM/mg) was determined relative to the chlorophyll content of leaves treated without NAG or MV. The values are presented as means and SEMs. Statistical analyses were conducted using Tukey's test. Sample sizes (*n*) and *p*-values are presented in the figure legends.

2.3. DAB Staining and Nitro Blue Tetrazolium Staining

For 3,3′-diaminobenzidine (DAB) and nitro blue tetrazolium (NBT) staining of hop leaves, leaves of 1.5-month-old hop plants (cv. 'Saaz') were treated with 0.5 mM NAG in a 1/2 MS liquid medium containing 2% glucose for 24 h. After incubation, 5 μM MV was added to this medium, and the leaves were incubated in the medium for 24 h. In heat stress assay, the leaves treated with 0.5 mM NAG for 24 h were incubated at 44 °C for 2 h. In DAB staining, three leaves were placed in a 1 mL DAB staining solution (peroxidase stain DAB kit, Nacalai, Kyoto, Japan) in 1.5 mL tubes, and then gently shaken in the dark for 2 h. The DAB-stained leaves were incubated in a DAB bleaching solution (60% ethanol, 20% acetic acid and 20% glycerol) and boiled at 95 °C for 15 min to remove the colour of chlorophyll. In NBT staining, three leaves were transferred to a 1 mL NBT staining solution (0.05% NBT, 50 mM potassium phosphate at pH 7.0) in 1.5 mL tubes for 30 min in the dark. The NBT-stained leaves were incubated in an NBT bleaching solution (96% ethanol, 4% acetic acid) to remove the colour of chlorophyll. Samples mounted with 50% glycerol were observed using an SZX16 (EVIDENT) equipped with a DP23 digital camera (EVIDENT). The experiment was conducted using three biological replicates.

2.4. Trypan Blue Staining

For trypan blue staining of hop leaves, leaves of 1.5-month-old hop plants (cv. 'Saaz') were treated with 0.5 mM NAG in a 1/2 MS liquid medium containing 2% glucose for 24 h. After incubation, 5 μM MV was added to this medium, and the leaves were incubated in the medium for 24 h. In trypan blue staining, three leaves were placed in a 1 mL trypan blue solution (Nacalai) in 1.5 mL tubes, followed by boiling at 95 °C for 1 h to stain dead cells. Trypan blue-stained leaves mounted with 50% glycerol were observed using an SZX16 equipped with a DP23 digital camera. The experiment was conducted using three biological replicates.

2.5. Measurement of Electrolyte Leakage

Electrolyte leakage was measured as previously described [17]. The leaves of 1.5-month-old hop plants (cv. 'Saaz') were treated with 0.5 mM NAG in a 1/2 MS liquid medium containing 2% glucose for 24 h. Then, the leaves were incubated at 44 °C for 2 h. After incubation, the leaves were infiltrated with 3 mL of deionized water for 10 min under vacuum and then shaken for 2 h. The conductivity (C1) of the leaves was measured using an F-74 conductivity meter with an electrical conductivity cell 3552-10D (HORIBA, Kyoto, Japan). After the deionized water containing the leaves was boiled at 95 °C for 15 min, the samples were cooled to room temperature. The conductivity (C2) of the cooled samples was then measured. The C1/C2 ratio divided by the fresh weight was calculated to determine electrolyte leakage. Electrolyte leakage was determined relative to that of leaves treated without NAG and heat stress. The values are presented as means and SEMs. Statistical analyses were conducted using Tukey's test. Sample sizes (*n*) and *p*-values are presented in the figure legends.

2.6. RNA Extraction and qPCR

Leaves of 1.5-month-old hop plants (cv. 'Saaz') were treated with 1 mM NAG in a 1/2 MS liquid medium containing 2% glucose for 2 h. Total RNA was isolated from the leaves using an RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). RNA extraction was

performed according to the manufacturer's instructions, and genomic DNA was removed using an RNase-free DNase set (QIAGEN). cDNA was synthesised from 1 µg total RNA with a Verso cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). qPCR analysis was performed using TB Green Premix Ex Taq II (TaKaRa Bio Inc., Shiga, Japan) and Light Cycler 480 (Roche, Basel, Switzerland).

For qPCR, gene-specific primers were constructed using Hopbase (https://hopbase.cgrb.oregonstate.edu/, accessed on 4 July 2022). Amino acid sequences of Arabidopsis *ZAT10*, *ZAT12*, *HSFA2*, *AOX1a*, *APX1* and *EF1α* were subjected to TBLASTN to search for nucleotide sequences with high homology to these genes, and genes with the highest similarity scores were regarded as putative homologues of these Arabidopsis genes in hops (*HlZAT10*, *HlZAT12*, *HlHSFA2*, *HlAOX1s*, *HlAPX1* and *HlEF1α*). Experiments were conducted using three biological replicates. The calculation of gene expression in qPCR was conducted by the $\Delta\Delta C_t$ method. *HlEF1α* was used as the internal control for qPCR. Expression in NAG-treated plants was determined relative to that of plants treated without NAG. The values are presented as means and SEMs. Statistical analyses were conducted using the two-tailed Student's *t*-test. Sample sizes (*n*) and *p*-values are presented in the figure legends. Primers used in qPCR and the annotation of the above putative homologues in hops are listed in Supplementary Table S1.

2.7. Chromatin Immunoprecipitation and qPCR

Chromatin immunoprecipitation (ChIP) was performed as previously described [18]. Five 1.5-month-old hop plants (cv. 'Saaz') were treated with 1 mM NAG in a 1/2 MS liquid medium containing 2% glucose for 2 h and fixed with 1% formaldehyde for 15 min, and then formaldehyde was quenched with glycine for 5 min. The fixed samples were ground in liquid nitrogen using a mortar and pestle. Chromatin fragmentation was performed using a Bioruptor® UCD-250 (Cosmo Bio., Ltd., Tokyo, Japan) in power mode H with an on/off cycle of 30 s/60 s, for a total duration of 12 min, on ice. Antibodies were added to the fraction of fragmented chromatins after preclearing, and then the samples were rotated overnight at 4 °C. An anti-H4ac antibody (Merck Millipore: 06-866) was used for immunoprecipitation. For qPCR, gene-specific primers were constructed using Hopbase. Nucleotide sequences of Arabidopsis *ZAT10*, *ZAT12*, *HSFA2* and *ACTIN2* were subjected to TBLASTN to search for nucleotide sequences with high homology to these genes, and contigs containing the sequence with the highest similarity score were regarded as putative homologues of these Arabidopsis genes in hop (*HlZAT10*, *HlZAT12*, *HlHSFA2* and *HlACT2*). qPCR analysis was performed using TB Green Premix Ex Taq II (TaKaRa) and Light Cycler 480 (Roche). The experiment was conducted using three biological replicates. The ratio of ChIP input was measured using the $\Delta\Delta C_t$ method and normalized to that of control loci to obtain enrichment levels. *HlACT2* was used as the control locus. Enrichment levels in NAG-treated plants were determined relative to those in plants treated without NAG. The values are presented as means and SEMs. Statistical analyses were conducted using the two-tailed Student's *t*-test. Sample sizes (*n*) and *p*-values are presented in the figure legends. The primers used in qPCR and the number of contigs containing the above putative homologues in hops are listed in Supplementary Table S1.

2.8. Prolonged Heat Stress Assay

For the heat stress assay, 1.5-month-old hop plants (cv. 'Saaz' and 'Hersbrucker') were used as a material. Stem explants with a node were transferred to a 1/2 MS medium containing 2% glucose and 0.8% agar with or without 0.5 mM NAG, and then incubated at 20 °C for 7 days. These samples were incubated at 30 °C and subjected to heat stress conditions. The fresh weight of the aerial parts and shoot lengths of cv. 'Saaz' and 'Hersbrucker' plants were measured after 14 and 28 days. As 'Hersbrucker' has a lower growth rate than 'Saaz', 'Hersbrucker' plants were subjected to a longer period of heat stress. The fresh weight and shoot length of NAG-treated plants were determined relative to those of plants that were not exposed to NAG. The values are presented as means and SEMs.

Statistical analyses were conducted using the two-tailed Student's *t*-test. Sample sizes (n) and p-values are presented in the figure legends.

3. Results

3.1. NAG Alleviates Oxidative-Stress-Triggered Chlorosis in Hops

To examine whether NAG could confer oxidative stress tolerance in hops, we observed the response to MV, which is an inducer of oxidative stress in plants, using leaves of tissue-cultured hop plants. We first confirmed that MV could induce the chlorosis and reduction of chlorophyll content in leaves 48 h after its treatment of cv. 'Saaz' (Figure 1A). MV-induced chlorosis and reduction of chlorophyll content were repressed in NAG-treated leaves compared to non-NAG-treated leaves (Figure 1B). Subsequently, to explore the potential of NAG in alleviating oxidative stress in another hop cultivar, we examined the leaf phenotype following treatment with both MV and NAG in the 'Hersbruker' cultivar. Chlorosis of leaves in the 'Hersbrucker' cultivar was observed 96 h after MV treatment (Figure 1C). The contrasting chlorosis induction times between the 'Saaz' and 'Hersbrucker' cultivars may indicate differing sensitivities to oxidative stress in these cultivars. Exogenous treatment with NAG reduced the frequency of MV-induced chlorosis and decreased chlorophyll content in leaves of hop cv. 'Hersbruker' as well as cv. 'Saaz', indicating that NAG could alleviate oxidative stress in several hop cultivars (Figure 1D).

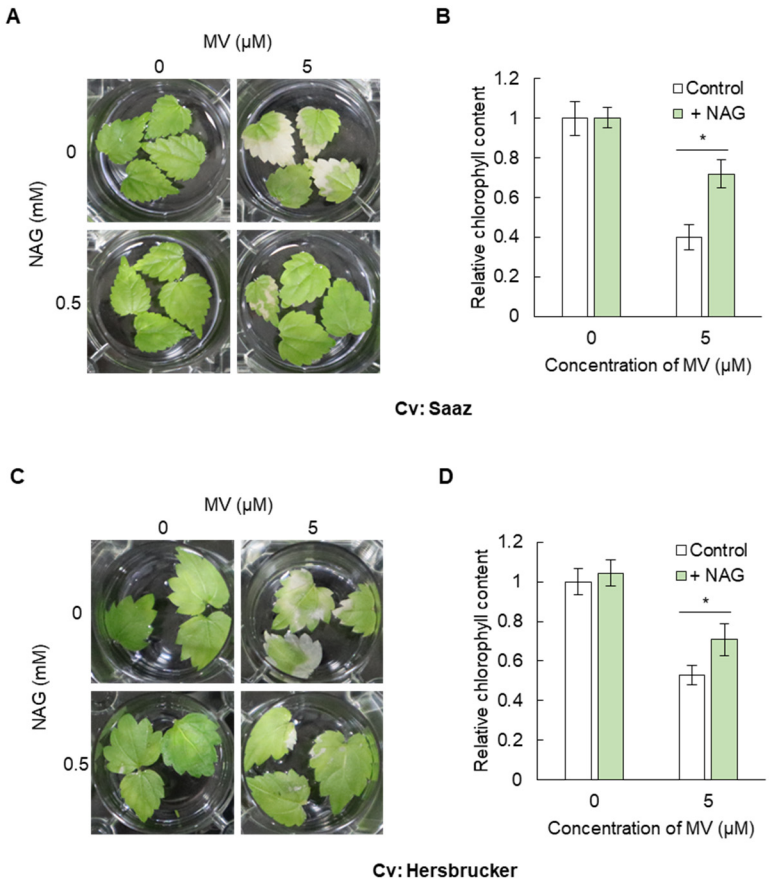

Figure 1. N-acetylglutamic acid (NAG) alleviates oxidative stress by repressing chlorosis in hops. (**A**) Leaves of hop cv. 'Saaz' treated with 0.5 mM NAG for 24 h, with or without 5 μM methyl viologen (MV) for 48 h. (**B**) Chlorophyll contents of leaves of hop cv. 'Saaz' treated with 0.5 mM NAG for 24 h, with and without 5 μM MV for 48 h. Error bars indicate standard error. $n = 12$. * $p < 0.05$ (Tukey test). (**C**) Leaves of hop cv. 'Hersbruker' treated with 0.5 mM NAG for 24 h, with or without 5 μM MV for 96 h. (**D**) Chlorophyll contents of leaves of hop cv. 'Hersbruker' treated with 0.5 mM NAG for 24 h, with and without 5 μM MV for 96 h. Error bars indicate standard error. $n = 6$. * $p < 0.05$ (Tukey's test).

3.2. NAG Enhances Expression Levels of Oxidative-Stress-Responsive Genes Associated with Histone Acetylation

Previously, we found that exogenous treatment with NAG enhanced basal tolerance to oxidative stress by increasing the expression levels of oxidative-stress-responsive genes with hyperacetylation under non-stress conditions in Arabidopsis, indicating that homologues of these genes are also activated in NAG-treated hops [14]. The qPCR experiment showed that the expression levels of *HlZAT10/12*, which had high homology to transcription factor Arabidopsis *ZAT10/12*, regarded as master regulators in response to oxidative stress in Arabidopsis, increased in NAG-treated plants relative to non-NAG-treated plants (Figure 2A). Additionally, chromatin immunoprecipitation (ChIP)–qPCR analysis confirmed that NAG enhanced the levels of histone H4 acetylation (H4ac) at *HlZAT10/12*, which indicates that the NAG-induced activation of *HlZAT10/12* associated with the upregulation of histone acetylation was conserved in hops as well as Arabidopsis (Figure 2B). Arabidopsis *ZAT10/12* increase the expression levels of ROS scavenging genes, alleviating oxidative stress by reducing ROS accumulation [19]. Thus, we examined whether exogenous treatment with NAG reduces ROS accumulation in hops with oxidative stress. The expression of *HlAOX1a* and *HlAPX1*, the nucleotide sequences of which were highly similar to those of the ROS-scavenging enzymes *Arabidopsis AOX1a* and *APX1*, respectively, functioning downstream of *ZAT12*, was enhanced in NAG-treated plants compared to that in non-NAG-treated plants (Figure 2C) [19]. DAB and NBT staining revealed the accumulation of H_2O_2 and $O_2^{\cdot-}$, respectively, in the leaves of MV-treated plants (Figure 2D,E). The frequency of MV-induced H_2O_2 and $O_2^{\cdot-}$ accumulation in the leaves of NAG-treated plants was lower than that in plants without NAG. Moreover, we examined the frequency of cell death in hops treated with MV using trypan blue staining, which enabled the detection of dead cells as blue-stained areas in plants. NAG-treated leaves showed a low frequency of MV-induced dead cells relative to non-NAG leaves (Supplementary Figure S1). Based on these results, NAG may mitigate ROS accumulation upon oxidative stress by basal activation of *HlZAT10/12*.

3.3. Exogenous Treatment with NAG Confers Heat Stress Tolerance in Hops

Heat stress induces ROS accumulation in plants [20]. Previously, we found that *Arabidopsis* and rice treated with NAG acquired heat and oxidative stress tolerance, suggesting that NAG can improve high-temperature-impaired growth in hops [14,15]. DAB and NBT staining revealed that NAG treatment reduced H_2O_2 and $O_2^{\cdot-}$ accumulation, respectively, induced by heat stress, compared with the findings in the absence of NAG treatment (Figure 3A,B). To further investigate the ability of NAG to alleviate cellular damage induced by ROS accumulation at high temperatures, we measured electrolyte leakage, which is related to the stability of the cellular membrane and is used as an index of cellular damage, in response to heat stress in hops. Consistent with the aforementioned findings, electrolyte leakage in heated leaves was reduced by NAG treatment compared with that in the absence of NAG exposure (Figure 3C). Next, to confirm whether NAG improved the growth and development of hop plants under high temperatures, we observed the phenotype of NAG-treated plants under prolonged heat stress. Under prolonged high-temperature conditions (30 °C, 14 days), NAG-treated plants exhibited greater fresh shoot weight and length than non-NAG-treated plants (Figure 3D–F). Prolonged heat-stress-induced reductions in fresh weight and shoot length were also repressed in cv. 'Hersbrucker' plants treated with NAG (Supplementary Figure S2). Finally, we confirmed that the expression of *HlHSFA2*, which was isolated as a putative homologue of *Arabidopsis HSFA2*, mediating transcriptional cascades and activating *APX1* in response to heat stress, was increased in NAG-treated hops as detected by qPCR (Figure 3G) [21,22]. NAG-treated hop plants also exhibited higher levels of histone H4ac in *HlHSFA2* than non-NAG-treated plants in the ChIP-qPCR experiment (Figure 3H), indicating that exogenous treatment with NAG could confer heat stress tolerance by activating *HlHSFA2* mediated by histone acetylation in hops.

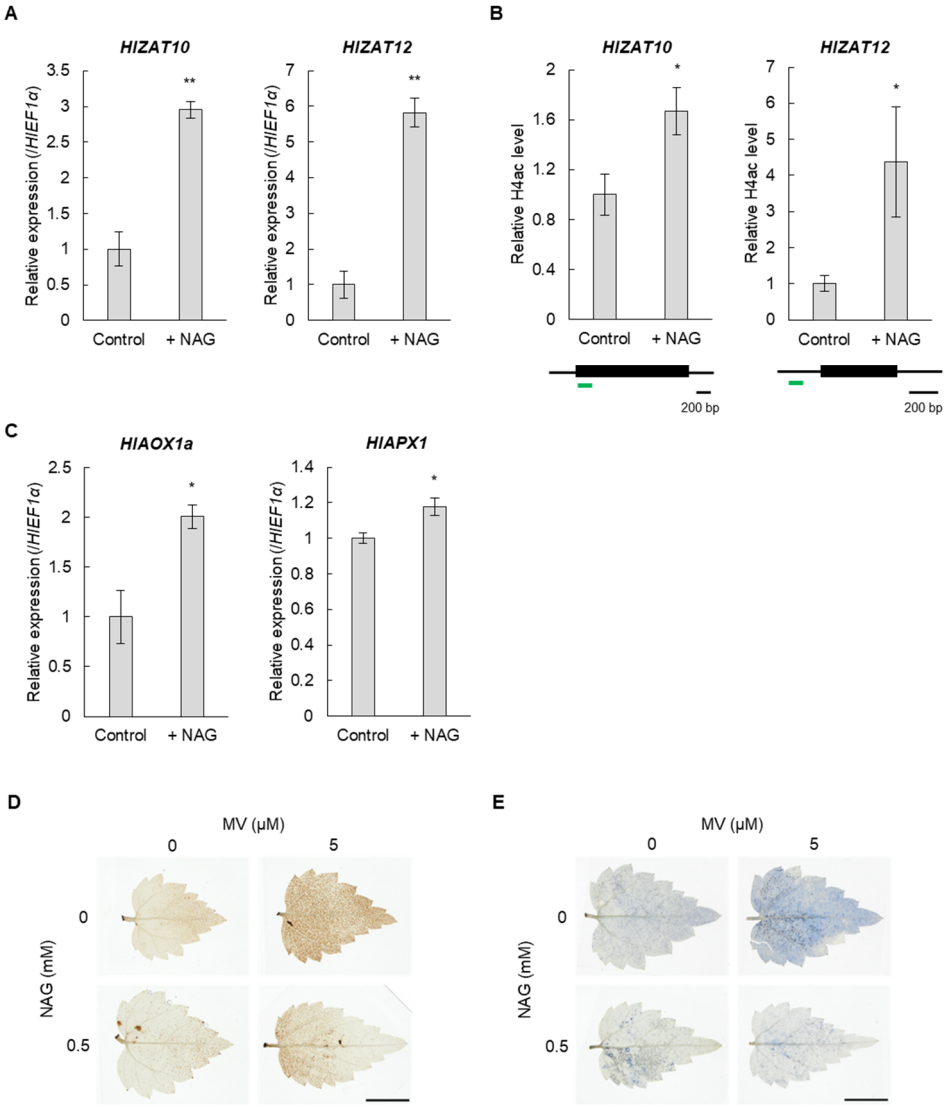

Figure 2. NAG activates the expression levels of oxidative-stress-responsive genes and reduces ROS accumulation in hops. (**A**) Expression levels of *HlZAT10/12* in leaves of hop cv. 'Saaz' treated with or without 1 mM NAG for 2 h. Error bars indicate the standard error. $n = 3$. ** $p < 0.01$ (Student's *t*-test). (**B**) Top: Levels of histone H4 acetylation at *HlZAT10/12* in leaves treated with or without 1 mM NAG for 2 h. Error bars indicate the standard error. $n = 3$. * $p < 0.05$ (Student's *t*-test). Bottom: Gene diagrams in ChIP-qPCR analysis. Bold bars and thin bars indicate exon and intron, respectively. Green bars indicate the amplified regions on *HlZAT10/12* in qPCR. (**C**) Expression levels of *HlAOX1a* and *HlAPX1* in leaves of hop cv. 'Saaz' treated with or without 1 mM NAG for 2 h. Error bars indicate the standard error. $n = 3$. * $p < 0.05$ (Student's *t*-test). (**D**) DAB staining for the detection of H_2O_2 in leaves of hop cv. 'Saaz' treated with 0.5 mM NAG for 24 h, with or without 5 μM MV for 24 h. Stained with brown area indicates the accumulation of H_2O_2. Three dependent experiments were conducted. Scale bar: 0.5 cm. (**E**) NBT staining for the detection of $O_2^{\cdot-}$ in leaves of hop treated with 0.5 mM NAG for 24 h, with or without 5 μM MV for 24 h. Stained with blue area indicates the accumulation of $O_2^{\cdot-}$. Three dependent experiments were conducted. Scale bar: 0.5 cm.

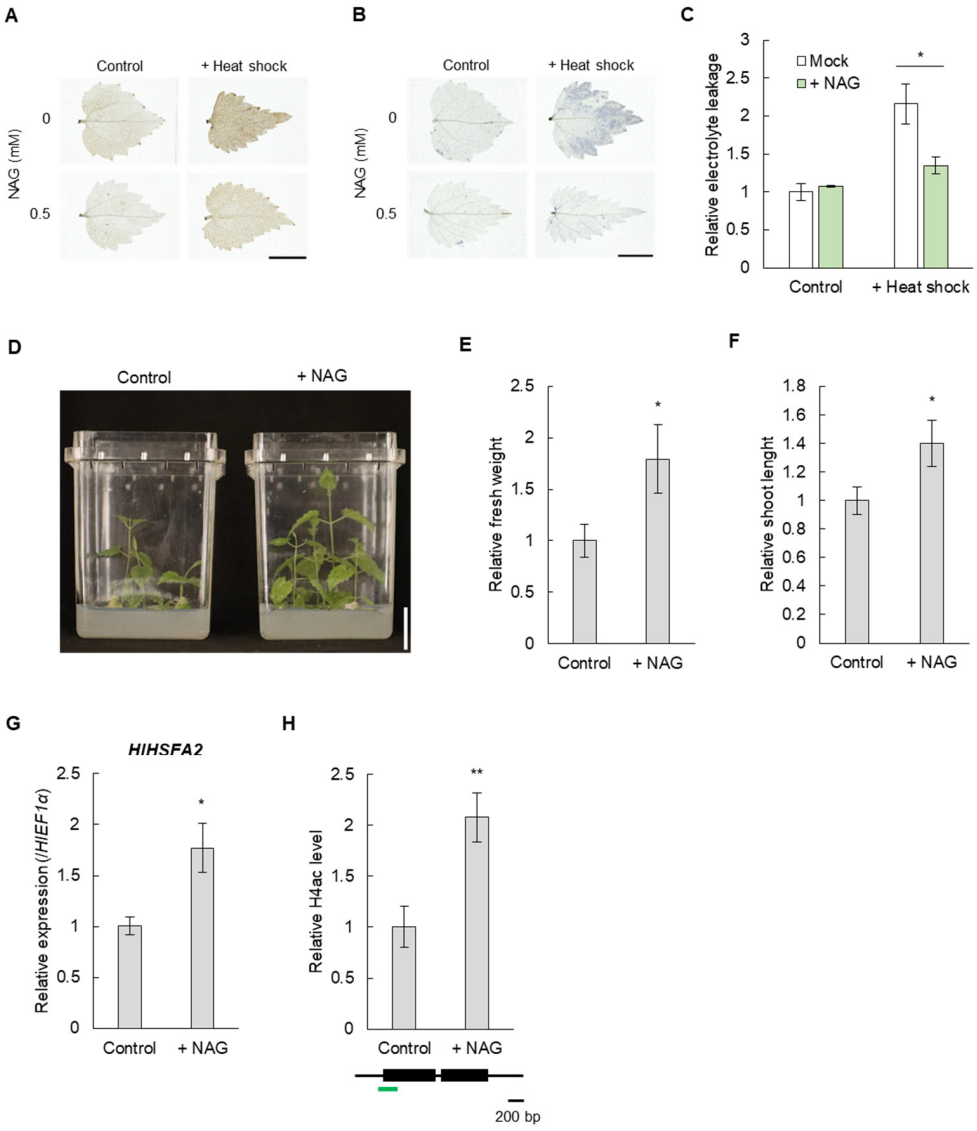

Figure 3. NAG-treated hops show heat stress tolerance. (**A**) DAB staining to detect H_2O_2 in the leaves of hop cv. 'Saaz' treated with 0.5 mM NAG for 24 h under heat stress (44 °C, 2 h). Brown staining indicates the accumulation of H_2O_2. Three independent experiments were conducted. Scale bar: 0.5 cm. (**B**) NBT staining to detect $O_2^{\cdot-}$ accumulation in the leaves of hops treated with 0.5 mM NAG for 24 h under heat stress. Blue staining indicates the accumulation of $O_2^{\cdot-}$. Three independent experiments were conducted. Scale bar: 0.5 cm. (**C**) Electrolyte leakage in hop leaves treated with 0.5 mM NAG for 24 h under heat stress. Error bars indicate the standard error. $n = 3$. * $p < 0.05$ (Tukey's test). (**D**) Plants of hop cv. 'Saaz' treated with 0.5 mM NAG for 7 days under prolonged heat stress conditions (30 °C, 14 days). Scale bar: 2 cm. (**E**) Fresh weight of aerial parts of plants treated with 0.5 mM NAG for 7 days under prolonged heat stress conditions. The fresh weight of NAG-treated plants was relative to that of the untreated plants. Error bars indicate standard error. $n = 17$. * $p < 0.05$ (Student's *t*-test). (**F**) Shoot length of plants treated with 0.5 mM NAG for 7 days under prolonged heat stress. The shoot length of NAG-treated plants is presented relative to that of non-NAG-treated plants. Error bars indicate the standard error. $n = 15$. * $p < 0.05$ (Student's *t*-test). (**G**) Expression levels of *HlHSFA2* in the leaves of plants treated with or without 1 mM NAG for 2 h. Error bars indicate the standard error. $n = 3$. * $p < 0.05$ (Student's *t*-test). (**H**) Top: Levels of histone H4 acetylation at *HlHSFA2* in leaves treated with or without 1 mM NAG for 2 h. Error bars indicate the standard error. $n = 3$. ** $p < 0.01$ (Student's *t*-test). Bottom: Gene diagrams in ChIP–qPCR analysis. Bold bars and thin bars indicate exon and intron, respectively. Green bar indicates the amplified regions of *HlHSFA2* in qPCR.

4. Discussion

In this study, we found that non-proteinogenic amino acid NAG alleviated damage resulting from oxidative or heat stress by activating stress-responsive genes in hops. NAG is an intermediate synthesised from glutamic acid (Glu) by *N*-acetylglutamate synthase (NAGS) in arginine biosynthesis in plants, and chemical priming experiments have revealed that Glu-related compounds can alleviate various abiotic stresses, including heat stress, in practical plants [23]. For example, exogenous treatment with Glu, Poly-γ-Glu and BABA ameliorated ROS signalling and improved photosynthesis efficiency under heat stress with enhanced carotenoid biosynthesis in *Brassica rapa* [11]. GABA application in *Helianthus annuus*, kiwifruit and creeping bentgrass increased the expression of HSP genes and the activity of antioxidant enzymes, such as superoxide dismutase, in response to heat stress [24,25]. GABA also protected *Vigna radiata* from heat stress by improving pollen viability and germination and activating antioxidant enzymes [26]. Although transgenic experiments have demonstrated that over-expression of *Solanum lycopersicum NAGS1* in *Arabidopsis* confers tolerance to drought and salinity stress, it had not been demonstrated whether NAG itself could enhance resistance to environmental stresses in plants [27]. Previously, we revealed the ROS scavenging-mediated ameliorating effect of NAG on heat and oxidative stress in *Arabidopsis* and rice as well as hops for the first time, suggesting that NAG has potential uses in alleviating these stresses in various plant species [14,15].

Recently, a draft genome assembly of the hop has permitted combination analysis of transcriptomics and metabolomics under high-temperature conditions [28,29]. These analyses demonstrated that ROS-scavenging genes are upregulated and Glu-related compounds accumulate in hop plants exposed to high temperatures, and an activated response might be required to maintain the biosynthesis of secondary metabolites, such as bitter acids [28]. We confirmed that NAG increased the expression of putative ROS-scavenging genes *HlAOX1a* and *HlAPX1* in hops. In *Arabidopsis*, *ZAT12* induces basal activation of *AOX1a* and *APX1*, and *HSFA2* upregulates *APX1*, conferring heat tolerance [19,22]. Thus, the removal of accumulated ROS is suggested to be necessary for the transcriptional cascade of oxidative and heat stress in plants. We observed using DAB and NBT staining that oxidative and heat-stress-induced H_2O_2 and $O_2^{\cdot -}$ accumulation was lower in NAG-treated leaves than in leaves treated without NAG, (Figures 2D,E and 3A,B). Thus, the repression of ROS accumulation might largely contribute to the effect of NAG in alleviating oxidative and heat stress, and NAG could prevent the inactivation of secondary metabolite biosynthesis by promoting ROS scavenging in hop plants.

We constructed the experimental system of ChIP–qPCR in hops and showed an increase in the levels of histone H4ac at *HlZAT10/12* and *HlHSFA2*, with upregulation of these genes in NAG-treated leaves (Figures 2B and 3G). Exogenous treatment with chemical compounds enhances tolerance to abiotic stresses by activating histone modifications in plants [7]. Among them, acetic acid has been suggested to be used as a substrate for histone H4 acetylation in *Arabidopsis*, resulting in enhanced drought tolerance with the activation of jasmonic acid-related genes [30]. Furthermore, the acetylation of tRNA by acetyltransferase *tmcAL* requires acetic acid as a substrate in *Bacillus subtilis*, indicating that NAG might be used as a donor substrate for acetyl groups in histone acetylation in oxidative- and heat-stress-responsive genes [31].

In Arabidopsis, histone acetylation at the promoter regions of *HSFA2* is deposited by histone acetyltransferase GCN5, and GCN5-binding sites are correlated to hyperacetylation and G-box motif (5′-CACGT, G-motif) regarded as a *cis* element recruiting two families of transcription factors, the basic helix-loop-helix (bHLH) and basic leucine zipper family (bZIP) family [32,33]. G-motifs in the *HSFA2* promoter are needed to recruit the transcription factor PIF4, which induces *HSFA2* and *HSPs* expression to enhance heat stress tolerance [34]. Hops have G-motifs in the putative regions of the promoter and first exon of *HlHSFA2*, and these motifs were conserved between draft genome-constructed cultivars ‘Saaz’ and ‘Cascade’, suggesting that G-motif-mediated change in histone acetylation and transcription state in *HSFA2* plays a crucial role in heat stress tolerance across species

of hops (Supplementary Figure S3). Under prolonged heat stress, NAG improved the growth of cv. 'Saaz' and 'Hersbrucker' plants (Figure 3D–F, Supplementary Figure S2). *Arabidopsis* carrying mutants of the RNA helicase *MAC7* and RNA-binding protein *MAC9/17*, which form the MOS4-associated complex suggested to interact with spliceosome and believed to be required for proper RNA splicing, exhibited low tolerance to prolonged heat stress [35,36]. Growth inhibition in *Arabidopsis* was also caused by deficiencies in *MIP3* and *ELM1*, which function in mitochondrial fission and ER homeostasis, respectively [37,38]. These findings suggest that proper regulation of RNA splicing and organelle dynamics is required for tolerance to prolonged heat stress in plants, and NAG might confer heat stress tolerance by controlling these phenomena in hops in addition to stress-responsive gene expression.

Beyond responses to environmental stress, epigenetic modification is involved in regulation during gene expression in hops and closely related species. For instance, analysis using methylation-sensitive amplified polymorphism in hops unveiled changes in the level of DNA methylation during development, from *in vitro* tissue-cultured plants to plants growing in the field, as well as during the regeneration process from callus to explants. DNA methylation plays a role in silencing gene expression and transposon elements [39,40]. *Artemisia annua* (*A. annua*), a medicinal plant similar to hops, has GTs and produces secondary metabolites, such as artemisinin, which is used in drugs against malaria. Transposase-Accessible Chromatin using sequencing (ATAC-seq) unveils chromatin accessibility, indicating the extent to which transcription factors can interact with chromatin and reflecting the potential for gene expression. The chromatin accessibility at GT-specific artemisinin biosynthesis genes differed between GT and leaf cells, highlighting the specificity of gene expression in artemisinin biosynthesis [41]. Furthermore, tissue-specific transcriptome analysis in hops revealed that the expression levels of secondary metabolite biosynthesis, including xanthohumol, bitter acid and terpenoids, were significantly elevated in lupulin glands compared to leaves. This implies that epigenetic modifications contribute to tissue-specific expression and the production of secondary metabolites, akin to artemisinin.

Heat and oxidative stress both impose negative impacts, such as high temperature and high light, respectively, on crop yield under field conditions. In hop cultivation, the yield and bitter acid (α-acid) content of hop cones decreased when the temperature in the growth period exceeded the annual norm in the Czech Republic [5]. Climate change accompanied by global warming is also predicted to cause reductions in hop yields and bitter acid content of 4–18% and 20–31%, respectively, by 2050 in Germany, the Czech Republic and Slovenia, whose microthermal climate represents suitable cultivation areas for high-quality aroma hops [6]. Interestingly, the flavour of some hop cultivars differs depending on the production area. The hop cultivar 'Centennial' has a tropical flavour in Belgium, but a citrusy and woodier flavour in the US [42]. Due to the location effect, also known as terroir, preserving the yield and quality of hop cones in suitable cultivation areas without relocating production sites due to climate change is crucial. A recent combination analysis of transcriptomics and metabolomics suggests that enhancing tolerance through the upregulation of stress-responsive genes is crucial for mitigating the reduction in secondary metabolite production under severe stress conditions in hops.

In this study, we revealed that exogenous treatment with NAG could enhance basal tolerance to oxidative and heat stress in hops through the activation of the putative transcriptional factors *HlZAT10/12* and *HlHSFA2,* without requiring specific techniques such as genetic engineering. Additionally, our research uncovered the involvement of histone acetylation in the response to oxidative and heat stress, suggesting that epigenetic modification underlies the environmental stress response of hop plants. While further studies are necessary to confirm its effect on hop cones, chemical priming with NAG holds promise as a means to enhance hop production amidst climate change, thereby meeting the demand for high-quality hops in beer brewing.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae10050484/s1. Figure S1: N-acetylglutamic acid represses the induction of cell death in hop; Figure S2: N-acetylglutamic (NAG) acid confers heat tolerances in the cultivar 'Hersbrucker'; Figure S3: *cis*-element G-motifs in promoter and exon regions of *HlHSFA2* in cv. 'Saaz' and 'Cascade'; Table S1: List of primers used in this study.

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