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Physiological Response of the Target Stemborer *Chilo suppressalis* to Elevated CO_2 as Reared with Transgenic *Bt* Rice during Different Plant Growth Stages

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Abstract: Transgenic *Bt* rice (abbr. *Bt* rice) has provided a powerful tactics to control the striped stemborer *Chilo suppressalis* as one key lepidopteran pest in the paddyfields of China. Globally rising carbon dioxide (i.e., CO_2) concentration has been predicted to affect the Cry protein contents in plant tissues of *Bt* rice and thus might affect its control efficiency to target insect pests. To reveal the resistance ability and the corresponding mechanism of *C. suppressalis* to *Bt* rice during different growth stage under elevated CO_2 (eCO_2), we carried out this experiment to measure the Bt toxin contents in *Bt* rice stems grown under ambient CO_2 (aCO_2) (400 ppm) and eCO_2 (800 ppm) at seedling, tillering and heading stages, and to observe the larval mortality and bioassay the activity of midgut protease and the expression levels of Bt-toxin-receptor genes, aminopeptidases (APNs) in *C. suppressalis* larvae. Compared with aCO_2, eCO_2 increased the Bt-toxin content of *Bt* rice at seedling stage (+6.66%), and decreased that at heading stages (−13.99%), and significantly reduced the Bt-toxin content at tillering stage (−15.21%). And the larval mortality of *C. suppressalis* was lower as reared with *Bt* rice stems during tillering stage grown under eCO_2 in contrast to aCO_2. In addition, eCO_2 significantly increased the activity of total protease, tryptase-like enzyme and aminopeptidase of *C. suppressalis* larvae fed on *Bt* rice during seedling stage, and significantly reduced the activity of tryptase-like enzyme and aminopeptidase of *C. suppressails* larvae fed on *Bt* rice during tillering and heading stages respectively. Moreover, eCO_2 significantly increased the expression level of APN1 and APN5 of *C. suppressails* larvae fed on *Bt* rice during seedling stage, and significantly reduced the expression level of APN5 of *C. suppressalis* larvae fed on *Bt* rice during tillering and heading stages respectively. In summary, the control efficiency of *Bt* rice to target insect pests under eCO_2 showed a downward trend during tillering and heading stages, and especially during tillering stage.

Keywords: elevated CO_2; transgenic *Bt* rice; *Chilo suppressalis*; mortality; midgut protease; Bt protein receptor genes; control efficiency

Citation: Liu, Y.; Wang, Y.; Li, S.; Xie, B.; Chen, G.; Chen, F. Physiological Response of the Target Stemborer *Chilo suppressalis* to Elevated CO_2 as Reared with Transgenic *Bt* Rice during Different Plant Growth Stages. *Agronomy* **2023**, *13*, 3000. https://doi.org/10.3390/agronomy13123000

Academic Editor: Luca Ruiu

Received: 20 October 2023
Revised: 27 November 2023
Accepted: 30 November 2023
Published: 6 December 2023

1. Introduction

The striped stemborer, *Chilo suppressalis* Walker (Lepidoptera: Pyralidae) is one of the key insect pests to rice production in Asia [1]. To control this insect pest, the genetically engineered rice plants producing Cry proteins derived from *Bacillus thuringiensis* (*Bt*) have been developed to control these target pests [2–4]. The transgenic *Bt* rice (abbr. *Bt* rice) expressing *Cry1C*, *Cry1Ab*, *Cry1Ac*, *Cry2A* genes and the fusion gene *Cry1Ab/Ac* have been proven remarkably effective in the control of target lepidopteran pests though previous laboratory and field experiments [5–7].

The action mode of Cry protoxins in the susceptible lepidopteran larvae is that the Bt toxins are dissolved and released in the midgut, and then the released protoxins are cleaved by midgut proteinases (e.g., trypsins, chymotrypsins) into a stable toxin core, and the toxin core is capable of binding to specific receptors on the midgut epithelium, which is recognized as an essential step for toxicity, and these events promote the toxin oligomerization and lead to the formation of toxin pores causing lysis of osmotic cells [8–10]. In this series of events, any physiological modification can decrease insect susceptibility to Cry toxins. For example, the significantly decreased activity of chymotrypsin-like enzyme in *Bt*-resistant *Plodia interpunctella* larvae were resulted in the decreased activation of Cry protoxins [11].

To date, several specific receptors to Bt toxins in target insect pests have been identified, including cadherin (Cad) proteins [12], alkaline phosphatases [13], glycolipids [14] and aminopeptidases (APNs) [15]. Toxin receptors mutations, such as deletion mutation and down-regulated of gene expression level, were associated with toxin resistance in target insect pests [16,17]. For instance, the lacking of *APN1* expression influenced the resistance of *Spodoptera exigua* colony to Cry1Ca [18]. Some documents showed that the silencing of APNs expression in *C. suppressalis* larvae by RNA interference (RNAi) reduced the larval susceptibility to Cry1Ab, Cry1Ac and Cry1C [19,20]. In addition, the silencing of expression of CADs in *C. suppressalis* larvae by RNAi decreased the larval susceptibility to Cry2Aa and Cry1Ca [21].

Globally atmospheric CO_2 level has risen steadily and is expected doubling (about 800 ppm) by the end of this century [22]. Generally, those plants grown under elevated CO_2 (eCO_2) were found to have higher photosynthetic rate, biomass and carbon: nitrogen (C: N) ratio [23–25]. Under eCO_2, the assimilation and re-assignment of C and N resources in plant tissues of transgenic *Bt* crops will alter the Bt-toxin content, and further alter the control efficiency to target insect pests [26–28]. In previous studies, we found that the foliar Cry1Ab/1Ac content in *Bt* rice (cv. HH1 with *Cry1Ab* + *Cry1Ac*) during the tillering stage was significantly lower as grown under eCO_2 in contrast to ambient CO_2 (aCO_2) [29]. However, during different growth stage (e.g., seedling, tillering or heading stage) for *Bt* rice grown under eCO_2, how the changes of Bt-toxin content in plant tissues and how will the target insect, *C. suppressalis* larvae respond to the change of Bt protein content and the responsive mechanism are still unclear.

In this study, we carried out a series of experiments to measure the Bt-toxin content of *Bt* rice grown under aCO_2 (400 ppm) and eCO_2 (800 ppm) at seedling, tillering and heading stages, and observe the mortality of the target insect pest, *C. suppressalis* and bioassay its midgut protease activity and the expression level of Bt-toxin-receptor genes (APNs) in order to understand the physiological responses and the responsive mechanism of *C. suppressalis* larvae to *Bt* rice grown under eCO_2.

2. Materials and Methods

2.1. Plants Growth Conditions

This research was performed in the artificial climate chambers (GDN-400D-4/CO_2, Ningbo Southeast Instrument CO., Ningbo, China) connected with CO_2 tanks for maintaining the desired CO_2 level. The temperature in the chambers was maintained at 28 °C (day) and 25 °C (night) under a 16L: 8D photoperiod. Two CO_2 levels were set at aCO_2 (400 ppm) and eCO_2 (800 ppm) with three artificial climate chambers for each CO_2 treatment.

2.2. Plants and Insects

Bt rice (cv. HH1 with a fused *Cry1Ab/CryAc* gene driven by the *actin-I* promoter) and its corresponding non-*Bt* rice (cv. MH63) were tested in this study. The seeds of these two rice cultivars were kindly provided by Prof. Yongjun Lin and Hongxia Hua (Huazhong Agricultural University of China). The seeds were soaked in water for 1 d, and sprouted on a board covered with wet cotton gauze for 1 d, and then the rice seeds were sown into plastic foam covering (0.6 cm thick) on plastic boxes (22 cm length: 15 cm width: 12 cm

height) and placed in the artificial climate chambers (GDN-400D-4/CO_2, Ningbo, China) of aCO_2 and eCO_2 treatments. Eight plastic boxes with 8 plants per box during seedling and tillering stages were placed in each artificial climate chambers, and 4 plastic boxes with 8 plants per box were maintained in each artificial climate chambers during the heading stage. The boxes were filled with modified culture solution and the solution was replaced with fresh solution every day [29].

The larvae of *C. suppressalis* larvae were obtained from a laboratory colony that collected from paddy fields in Nanjing (China) in 2019. The larvae were kept at 27 ± 2 °C and 70 ± 10% RH under a 16: 8 h light/dark photoperiod in the artificial climate chambers, and maintained on an artificial diet as previously described [30].

2.3. Measurement of Bt-Toxin Content in Bt Rice Plants

At the seedling, tillering and heading stages, 5 stems of *Bt* rice (cv. HH1) and non-*Bt* rice (cv. MH63) grown under aCO_2 and eCO_2 were randomly selected from each artificial climate chamber to measure the Bt-toxin content by using the ELISA kit (EnviroLogix, Portland, ME, USA; catalog number AP003), respectively. The samples were respectively put into 2 mL tube with two steel balls, and homogenized in a Tissue Lyser II (Qiagen, Haan, Germany) by shaking for 2 min at 30 Hz, and then mixed with extraction buffer PBST. The Bt protein content were measured as dictated in the kit instruction.

2.4. Bioassay of Tested Insect Larvae

The 4th instar larvae of *C. suppressalis* fed on the stems of *Bt* rice (cv. HH1) and non-*Bt* rice (cv. MH63) grown under aCO_2 and eCO_2, were used for the following bioassay of *C. suppressalis* larvae. The stems of *Bt* rice and non-*Bt* rice were randomly selected at the seeding, tillering and heading stages, which were cut into 6 to 7-cm fragments. A thin layer of moist cotton wool was put on the bottom of a 9 cm-diameter Petri dish, and a moistened 8 cm-diameter filter paper was placed on the cotton layer. The rice stems and one 4th instar larvae of *C. suppressalis* were placed in a Petri dish, which was subsequently sealed with Parafilm. The Petri dishes were covered with black cloth and placed in the artificial climate chambers.

Each combination of CO_2 levels (ambient vs. elevated), rice cultivars (HH1 vs. MH63) and growth stages (seedling, tillering and heading) were carried out four replicates, and each replicate contained 30 larvae of *C. suppressalis*. The mortality and body weight of *C. suppressalis* larvae were recorded every day and the rice stem fragments were changed every day. The bioassay tests were terminated until the pupation of *C. suppressalis* larvae.

2.5. Analysis of Midgut Enzyme Activity of C. suppressalis Larvae

Forty 4th instar larvae of *C. suppressalis* were individually put into each Petri dish (9 cm diameter: 2.5 cm height) and fed on the stem fragments of *Bt* rice (cv. HH1) and non-*Bt* rice (cv. MH63) grown under aCO_2 and eCO_2, respectively. Each combination of CO_2 levels (ambient vs. elevated), rice cultivars (HH1 vs. MH63) and growth stages (seedling, tillering and heading) had 40 Petri dishes. After 1, 2, 3, 4 days of feeding exposure, the midgut of 10 larvae of *C. suppressalis* was daily dissected and washed with ice-cold 0.7% NaCl solution, and immediately stored at −80 °C for the following RNA extraction.

Prior to analysis, the midgut samples of *C. suppressalis* larvae were put in 1 mL 0.15 mol/L NaCl, and then homogenized at 4 °C and centrifuge at 15,000× *g* for 10 min. The supernatant was collected and the protein concentration was quantified using bovine serum albumen (BSA) as the standard method introduced by Bradford [31]. The activity of midgut protease was measured similar to that described by Zhou et al. [32] and Wang et al. [33].

The substrate of azocasein (2 mg/mL in 0.15 mol/L NaCl) was used to measure the total protease activity in the larval midgut of *C. suppressalis*. Ten μL enzyme sample, 100 μL azocasein solution (2 mg/mL in 0.15 mol/L NaCl) and 40 μL 0.1 mol/ L Glycine-NaOH Buffer (pH 10.0) were added into the centrifuge tube and reacted at 30 °C for 1 h. and 150 μL pre-cold 10% trichloroacetic acid was added into the tube to terminate the reaction.

The solution was centrifuge at 12,000 rpm for 15 min, and the supernatant was mixed with equal volume 0.1 mol/L glycine-NaOH Buffer. Then the absorbance was measured at 415 nm.

N-*α*-benzoyl-DL-arginine-*p*-nitroanilide (BApNA) and *N*-succinyl-$(Ala)_2$-Pro-Phe-*p*-nitroanilide (SAAPFpNA) were used as substrate to measure the activity of typsin and chymotrypsin, respectively. BApNA (50 mg/mL in DMSO) and (SAAPFpNA) (50 mg/mL in DMF) was respectively diluted to 1.0 mg/mL with universal buffer. BApNA (100 µL) and SAAPFpNA (90 µL) solution was respectively added into individual wells containing enzyme sample and Glycine-NaOH Buffer, and the reaction product absorbance was measured at 405 nm in 10 s intervals for 15 min.

Aminopeptidase (APN) activity was assayed using 10 µL enzyme sample with 100 µL 1 mM L-leucine p-nitroanilide (L-pNA) and 140 µL 50 mM Tris–HCl (pH 8.0) buffer. Then the the reaction product absorbance was measured using a UV-Vis spectrophotometer at 410 nm in 30 s intervals for 10 min.

2.6. Quantitative RT-PCR of APNs in C. suppressalis Larvae

Total RNA was extracted from the larval midgut of *C. suppressalis* using TRIzol reagent (Invitrogen, CA, USA) according to the kit instruction and treated with DNase I (Takara, Kyoto, Japan) to remove the DNA. Then the first-strand cDNA was immediately synthesized using the PrimeScriptTM RT Reagent Kit with gDNA Eraser (Takara, Kyoto, Japan). The sequences encoding aminopeptidase genes (i.e., APN) of *C. suppressalis* larvae were obtained from GenBank, including APN1 (GenBank No. DQ342305.3), APN3a (GenBank No. JF519739.1), APN3b (GenBank No. JQ088280.1), APN4 (GenBank No. HQ901596.1) and APN5 (JQ088281.1). The specific primers for the APNs in *C. suppressalis* larvae were designed by the Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA), and the reference gene of elongation factor (EF) and β-Actin1 were used as the internal standard to analyze the expression level of the target genes (Table 1). The expression level of APNs was quantified following the $2^{-\Delta\Delta Ct}$ normalization method, respectively [34]. Three technical replicates were also performed for each cDNA sample.

Table 1. Primers used to measure the transcript expression of APNs in the larvae of the rice stemborer *Chilo suppressalis* in the quantitative real time PCR.

Primer	Sequence (5′→3′)	Primer	Sequence (5′→3′)
EF-F	CGCTGGCGACTCCAAAA	APN3a-R	TGTCTCATTAGCAGGAACATCG
EF-R	CACAATGACTTGAGCCGTGAA	APN3b-F	AGAAGTTGACGAATGCCGTGAAGC
β-actin-F	TCTTGGGTATGGAAGCTAACGGCA	APN3b-R	AAATGTACGACAGAGCGGTGGTTG
β-actin-R	CATCGTTGATGGCGCTAAAGCAGT	APN4-F	TGTTAATATGTCAACTGGTCTC
APN1-F	CTGCTGGCGTTTATAGTATCTC	APN4-R	GTTCTGAATTGTATGTGAATCG
APN1-R	GTGTAATCTTCCATCGCTCTAA	APN5-F	CCTTGAACAGCAATCATAATG
APN3a-F	GATCAACAACAGGGTGATAGGA	APN5-R	CCAGGAATAGTAACTTGTATCTT

2.7. Data Analysis

The statistical analysis was performed using SPSS 20.0 (Chicago, IL, USA). Two-way analysis of variances (ANOVAs) were used to analyze the effects of CO_2 level (ambient vs. elevated), rice growth stage (seedling, tillering and heading) and their interaction on Bt content of *Bt* rice. And three-way repeated-measures ANOVAs were used to analyze the effects of CO_2 levels, rice cultivars (*Bt* rice vs. non-*Bt* rice), growth stages and their interactions on the mortality, enzyme activity, and the transcript expression level of *APNs* in the midguts of *C. suppressalis* larvae with feeding time as the repeated measures, and the univariate-ANOVA was also used to analyze the effects of CO_2 levels, rice cultivars, growth stages and their interactions on the mortality enzyme activity, and the transcript expression level of APNs in the midguts of *C. suppressalis* larvae with feeding time as the repeated measures. The Student's *t*-test was performed to analyze significantly difference

between aCO_2 and eCO_2, and between *Bt* and non-*Bt* rice at $p < 0.05$, and the Turkey test was performed to analyze significantly different among three rice growth stages at $p < 0.05$.

3. Results

3.1. Bt Content in Bt Rice Stems Grown under aCO_2 and eCO_2 during Seedling, Tillering and Heading Stages

CO_2 level ($F = 5.59$, $p = 0.027$) and its interaction with rice growth stage ($F = 6.26$, $p = 0.006$) both significantly impacted the Bt content in *Bt* rice stems, and there were significant differences among the seedling, tillering and heading stages ($F = 50.81$, $p < 0.001$; Table 2). Compared with aCO_2, eCO_2 significantly reduced the Bt content in *Bt* rice stems during tillering stage (−15.21%, $p < 0.05$), and also decreased the Bt content during heading stages (−13.99%, $p > 0.05$), while increased the Bt content during seedling stages (+6.66%, $p > 0.05$; Figure 1). The Bt content in *Bt* rice stems continuously decreased with the plant growth from seedling stage to heading stage under aCO_2 and eCO_2 (Figure 1). Under aCO_2, the Bt content during heading stage was significantly lower than that during seedling stage (−23.08%) and tillering stage (−17.45%), respectively ($p < 0.05$; Figure 1). Under eCO_2, the Bt content during tillering stage (−25.93%) and heading stage (−37.97%) was significantly lower than that during seedling stage respectively ($p < 0.05$; Figure 1), and the Bt content during heading stage was significantly lower than that during tillering stage (−16.26%; $p < 0.05$, Figure 1).

Table 2. Two-way ANOVAs of CO_2 levels (ambient vs. elevated), rice growth stages (seedling, tillering and heading) and their interaction on Bt-toxin content in stems of transgenic *Bt* rice stems (abbr. *Bt* rice); and three-way repeated-measures ANOVAs of CO_2 levels, rice cultivars (*Bt* rice vs. non-*Bt* rice), growth stages and their interactions with feeding time as the repeated measures on the measured indexes of enzyme activity and the transcript expression level of APNs in the midguts of rice stemborer, *C. suppressalis* larvae (*F*/*p* values).

Measured Indexes		CO_2 Level (CO_2)	Rice Cultivar (Cultivar)	Rice Growth Stage (Stage)	CO_2 × Cultivar	CO_2 × Stage	Cultivar × Stage	CO_2 × Cultivar × Stage
Bt-toxin content (μg/g FW)		5.59/0.027 *		50.81/<0.001 ***		6.26/0.006 **		
Larval mortality (%)		0.49/0.48	567.64/<0.001 *	11.81/<0.001 *	0.26/0.61	0.88/0.41	3.25/0.039 *	0.98/0.37
Enzyme activity (mU/mg)	Total protease	0.01/0.92	461.22/<0.001 ***	444.92/<0.001 ***	27.62/<0.001 ***	1.05/0.37	49.27/<0.001 ***	6.48/0.006 **
	Trypsin-like enzyme	0.43/0.52	22.65/<0.001 ***	307.82/<0.001 ***	4.74/0.040 *	1.26/0.30	7.52/0.003 **	7.19/0.004 **
	Chymotrypsin-like enzyme	0.74/0.39	39.84/<0.001 ***	788.71/<0.001 ***	0.81/0.38	2.90/0.07	2.43/0.11	0.75/0.48
	Aminopeptidase	0.004/0.95	100.92/<0.001 ***	443.35/<0.001 ***	0.54/0.47	2.78/0.08	13.89/<0.001 ***	4.49/0.022 *
Expression level of APNs	APN1	0.58/0.46	24.27/<0.001 ***	24.35/<0.001 ***	0.01/0.91	3.12/0.06	14.66/<0.001 **	4.66/0.020 *
	APN3a	2.65/0.12	4.74/0.040 *	6.79/0.005 **	0.05/0.83	1.03/0.37	3.44/0.049 *	3.35/0.052
	APN3b	0.38/0.55	16.21/<0.001 ***	2.84/0.078	3.56/0.07	0.09/0.91	1.34/0.28	1.32/0.29
	APN4	0.70/0.41	5.89/0.023 *	25.78/<0.001 ***	0.40/0.53	1.83/0.18	2.38/0.11	3.80/0.037 *
	APN5	11.04/0.003 **	1164.93/<0.001 ***	5.10/0.014 *	9.98/0.004 **	13.51/<0.001 ***	37.17/<0.001 ***	7.54/0.0029 **

Note: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

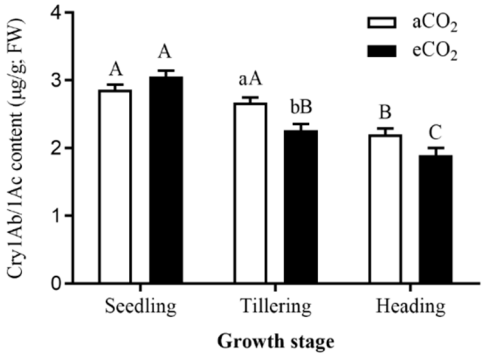

Figure 1. Bt-toxin content in the stems of transgenic *Bt* rice (cv. HH1) grown under ambient CO_2 (aCO_2) and elevated CO_2 (eCO_2) during seedling, tillering and heading stages. Note: Different lowercase letters indicated significantly different between aCO_2 and eCO_2, at same rice growth stage by the Student's *t* test at $p < 0.05$, and different uppercase letters indicated significantly different among three growth stages under same CO_2 level by the Tukey-test at $p < 0.05$.

3.2. Mortality of C. suppressalis Larvae Fed on Bt and Non-Bt Rice Plants Grown under aCO_2 and eCO_2

The larval mortality of *C. suppressalis* was significantly affected by rice cultivar ($F = 567.64$, $p < 0.001$), growth stage ($F = 11.81$, $p < 0.001$) and their interactions ($F = 3.25$, $p = 0.039$) (Table 2). There was no *C. suppressalis* larvae survived to pupal stage as they fed on *Bt* rice stems grown under aCO_2 and eCO_2 during seedling, tillering and heading stages (Figure 2). The mortality of *C. suppressalis* larvae fed on *Bt* rice stems was significantly higher than that of *C. suppressalis* larvae fed on non-*Bt* rice stems grown under aCO_2 and eCO_2 during seedling, tillering and heading stages respectively ($p < 0.05$; Figure 2). And the mortality of *C. suppressalis* larvae under aCO_2 was significantly higher as fed on *Bt* rice stems during seedling stage in contrast to tillering and heading stages, respectively ($p < 0.05$; Figure 2).

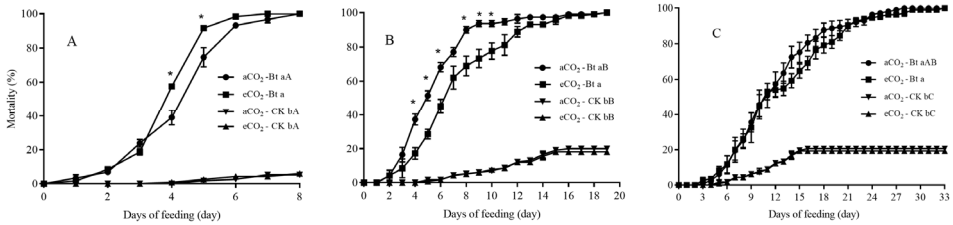

Figure 2. Larval mortality of the rice stemborer, *Chilo suppresalis* fed on the stems of *Bt* rice (cv. HH1) and non-*Bt* rice (cv. MH63) grown under aCO_2 and eCO_2 during seedling (**A**), tillering (**B**) and heading (**C**) stages. Note: * and different lowercase letters indicated significantly difference between aCO_2 and eCO_2 for same rice cultivar and at same rice growth stage, and between *Bt* rice and non-*Bt* rice grown under same CO_2 level and at same rice growth stage by the Student's test at $p < 0.05$, respectively; Different uppercase letters indicated significantly difference among different rice growth stages for same rice and under same CO_2 level by the Tukey test at $p < 0.05$.

The mortality of *C. suppressalis* larvae fed on *Bt* rice grown under eCO_2 was significantly higher than that under aCO_2 during seedling stage for 4 and 5 days respectively ($p < 0.05$; Figure 2A). After fed on *Bt* rice grown under eCO_2 for 4, 5, 6, 8, 9 and 10 days during tillering stage, the larval mortality of *C. suppressalis* was significantly lower than that under aCO_2 respectively ($p < 0.05$; Figure 2B). After fed on *Bt* rice grown under aCO_2 for 28 days during heading stage, the mortality of *C. suppressalis* larvae reached 100%, and the larval mortality of *C. suppressalis* reached 100% after fed on *Bt* rice grown under eCO_2 for 33 days (Figure 2C).

3.3. Enzyme Activity in C. suppressalis Larvae Fed on the Stems of Bt and Non-Bt Rice Plants Grown under aCO_2 and eCO_2

3.3.1. Total Protease

The total protease activity in *C. suppressalis* larvae was significantly affected by rice cultivar ($p < 0.001$), growth stage ($p < 0.001$) and their interaction ($p < 0.001$), and the interactions between CO_2 level and rice cultivar ($p < 0.001$), and among CO_2 level, rice cultivar and growth stage ($p = 0.006$) (Table 2). Compared with aCO_2, eCO_2 significantly increased the activity of total protease in *C. suppressalis* larvae during seedling stage ($p < 0.05$, Figure 3A) and significantly decreased that during tillering stage ($p < 0.05$, Figure 3C) when they fed on *Bt* rice for 4 days.

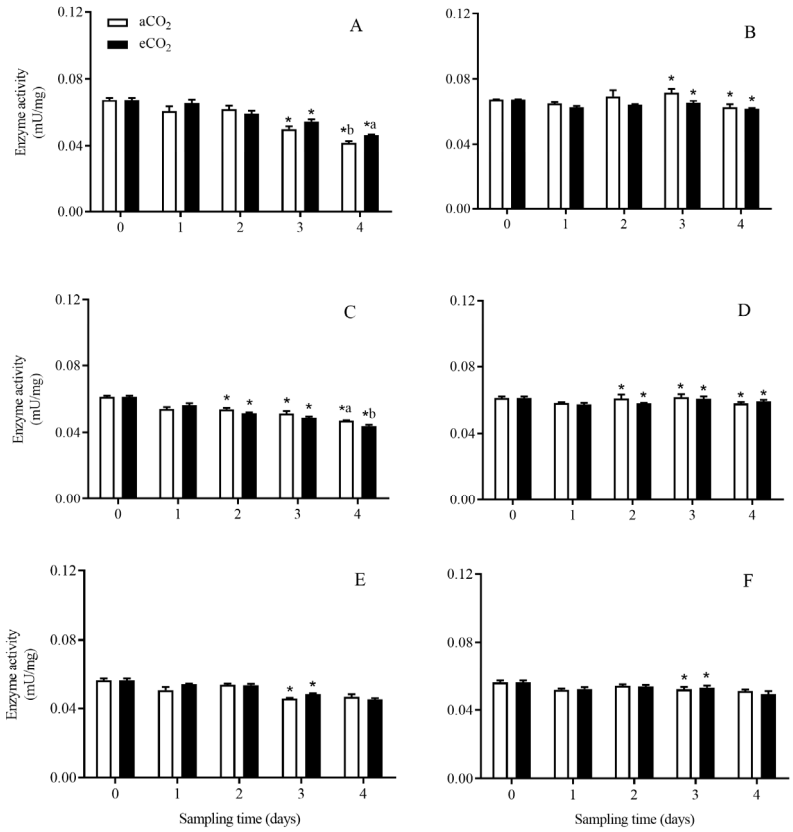

Figure 3. The activity of total protease in *C. suppressalis* larvae fed on *Bt* rice and non-*Bt* rice grown under aCO_2 and eCO_2 for 0, 1, 2, 3 and 4 days, during seedling, tillering and heading stages. Note: (**A**,**B**)-seedling stage; (**C**,**D**)-tillering stage; (**E**,**F**)-heading stage; (**A**,**C**,**E**)-*Bt* rice, (**B**,**D**,**F**)-non-*Bt* rice. Each value represents means ± SE. *, different lowercase letters indicate significant difference between *Bt* and non-*Bt* rice grown under same CO_2 level and at same rice growth stage, and between aCO_2 and eCO_2 for same rice cultivar and at same rice growth stage by the Student's *t*-test at $p < 0.05$.

The activity of total protease in *C. suppressalis* larvae fed on *Bt* rice was significantly lower than that in *C. suppressalis* larvae fed on non-*Bt* rice grown under aCO_2 and eCO_2 during seedling stage for 3 and 4 days ($p < 0.05$; Figure 3A,B), and during tillering stage for 2, 3 and 4 days ($p < 0.05$; Figure 3C,D) and during heading stage for 3 days ($p < 0.05$; Figure 3E,F), respectively.

3.3.2. Trypsin-like Enzyme

The activity of trypsin-like enzyme in *C. suppressalis* larvae was significantly affected by rice cultivar ($p < 0.001$), growth stage ($p < 0.001$) and their interaction ($p = 0.003$), and the interactions between CO_2 level and rice cultivar ($p = 0.040$), and among CO_2 level, rice cultivar and growth stage ($p = 0.004$) (Table 2). Compared with aCO_2, eCO_2 significantly

increased the trypsin-like enzyme activity in *C. suppressalis* larvae fed on *Bt* rice during seedling stage for 3 and 4 days ($p < 0.05$; Figure 4A), and significantly decreased that during tillering stage for 3 and 4 days ($p < 0.05$; Figure 4C) and during heading stage for 4 days, respectively ($p < 0.05$; Figure 4E). Moreover, the trypsin-like enzyme activity in *C. suppressalis* larvae fed on *Bt* rice was significantly lower than that in *C. suppressalis* larvae fed on non-*Bt* rice during seedling stage for 4 days under aCO_2 ($p< 0.05$, Figure 4A,B), and during tillering stage for 3 and 4 days under eCO_2 ($p < 0.05$, Figure 4C,D).

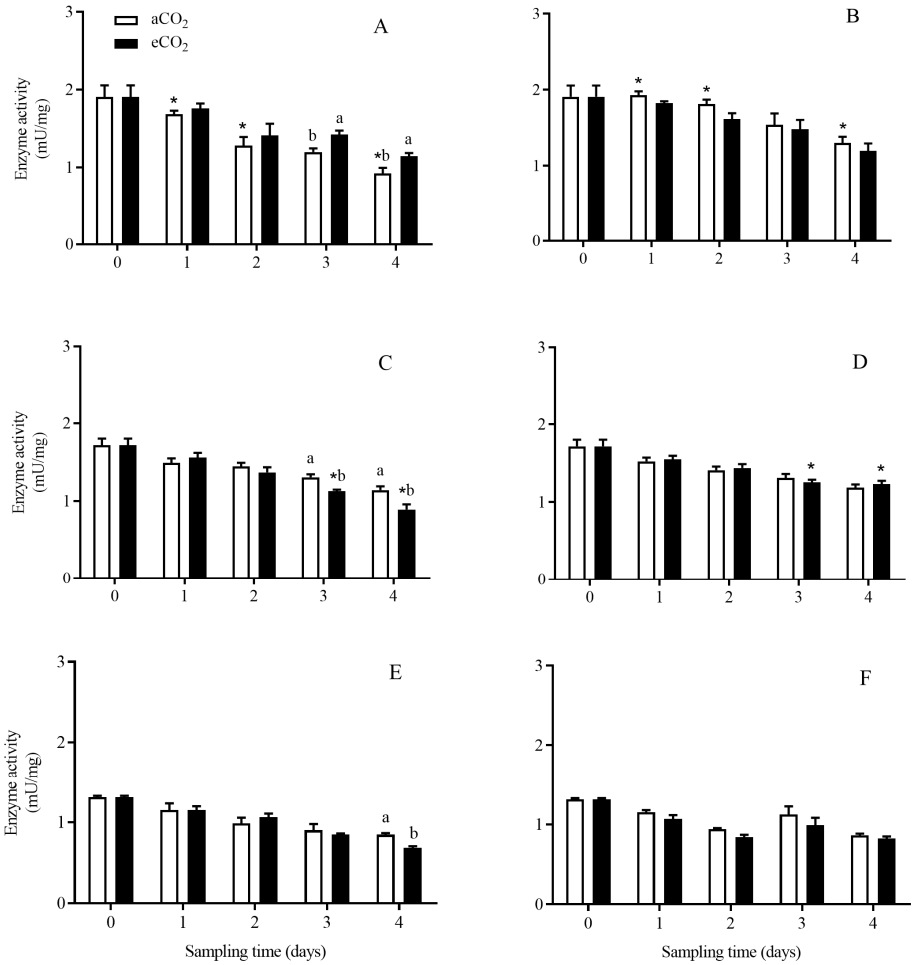

Figure 4. The activity of trypsin-like enzyme in *C. suppressalis* larvae fed on *Bt* and non-*Bt* rice plants grown under aCO_2 and eCO_2 for 0, 1, 2, 3 and 4 days, during seedling, tillering and heading stages. Note: (**A**,**B**)—seedling stage; (C,D)—tillering stage; (**E**,**F**)—heading stage; (**A**,**C**,**E**)—*Bt* rice, (**B**,**D**,**F**)—non-*Bt* rice. Each value represents means ± SE. *, different lowercase letters indicate significant difference between *Bt* and non-*Bt* rice grown under same CO_2 level and at same rice growth stage, and between aCO_2 and eCO_2 for same rice cultivar and at same rice growth stage by the Student's *t*-test at $p < 0.05$.

3.3.3. Chymotrypsin-like Enzyme

The activity of chymotrypsin-like enzyme in *C. suppressalis* larvae was significantly affected by rice cultivar ($p < 0.001$) and growth stage ($p < 0.001$; Table 2). Compared with aCO_2, eCO_2 significantly decreased the chymotrypsin-like enzyme activity in *C. suppressalis* larvae fed on *Bt* rice during seedling stage for 4 days ($p < 0.05$, Figure 5A). The activity of chymotrypsin-like enzyme in *C. suppressalis* larvae fed on *Bt* rice was significantly lower than that in *C. suppressalis* larvae fed on non-*Bt* rice during tillering stage for 2 days ($p < 0.05$; Figure 5C,D).

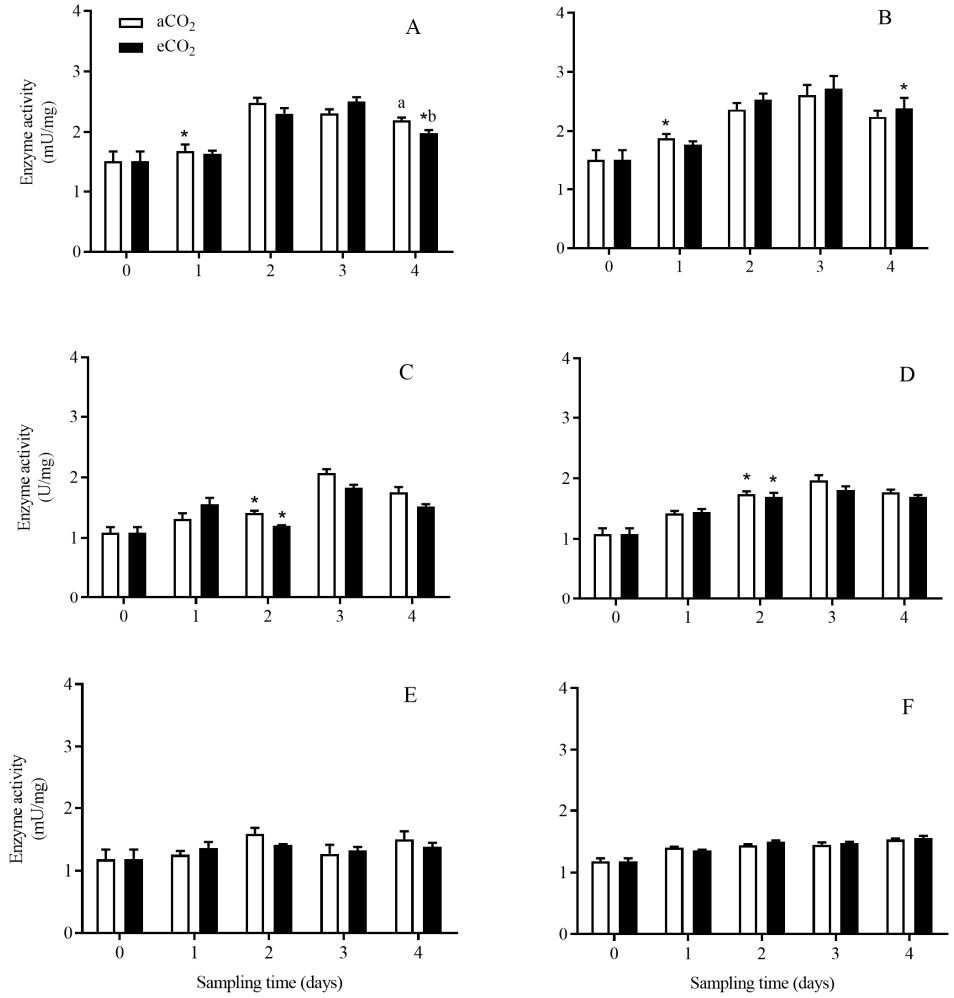

Figure 5. The activity of chymotrypsin-like enzyme in *C. suppressalis* larvae fed on *Bt* and non-*Bt* rice plants grown under aCO_2 and eCO_2 for 0, 1, 2, 3 and 4 days, during seedling, tillering and heading stages. Note: (**A**,**B**)—seedling stage; (**C**,**D**)—tillering stage; (**E**,**F**)—heading stage; (**A**,**C**,**E**)—*Bt* rice, (**B**,**D**,**F**)—non-*Bt* rice. Each value represents means ± SE. *, different lowercase letters indicate significant difference between *Bt* and non-*Bt* rice grown under same CO_2 level and at same rice growth stage, and between aCO_2 and eCO_2 for same rice cultivar and at same rice growth stage by the Student's *t*-test at $p < 0.05$.

3.3.4. Aminopeptidase

The activity of aminopeptidase in *C. suppressalis* larvae was significantly affected by rice cultivar ($p < 0.001$), growth stage ($p < 0.001$) and their interaction ($p < 0.001$), and the interaction among CO_2 level, rice cultivar and growth stage ($p = 0.022$) (Table 2). Compared with aCO_2, eCO_2 significantly increased the aminopeptidase activity in *C. suppressalis* larvae fed on *Bt* rice during seedling stage for 2 and 4 days ($p < 0.05$; Figure 6A), and significantly decreased that during tillering stage for 3 and 4 days ($p < 0.05$; Figure 6C) and that during heading stage for 4 days ($p < 0.05$; Figure 6E), respectively. The activity of aminopeptidase in *C. suppressalis* larvae fed on *Bt* rice grown under aCO_2 and eCO_2 during seedling and heading stage for 4 days, and that during tillering stage for 2 days was significantly higher than that in *C. suppressalis* larvae fed on non-*Bt* rice, respectively ($p < 0.05$; Figure 6). While the aminopeptidase activity in *C. suppressalis* larvae fed on *Bt* rice was significantly lower than that in *C. suppressalis* larvae fed on non-*Bt* rice grown under eCO_2 during tillering stage for 4 days ($p < 0.05$; Figure 6C,D).

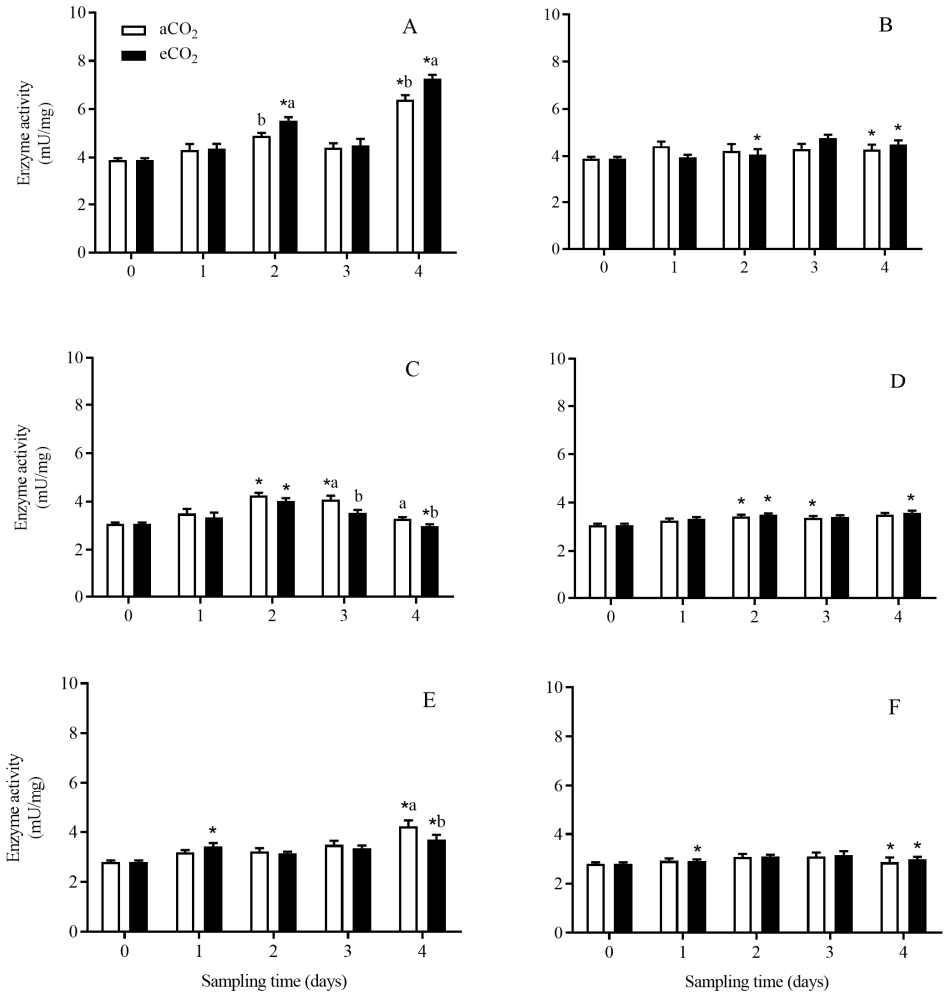

Figure 6. The activity of aminopeptidase enzyme in *C. suppressalis* larvae fed on *Bt* and non-*Bt* rice plants grown under aCO_2 and eCO_2 for 0, 1, 2, 3 and 4 days, during seedling, tillering and heading stages. Note: (**A**,**B**)—seedling stage; (**C**,**D**)—tillering stage; (**E**,**F**)—heading stage; (**A**,**C**,**E**)—*Bt* rice, (**B**,**D**,**F**)—non-*Bt* rice. Each value represents means ± SE. *, different lowercase letters indicate significant difference between *Bt* and non-*Bt* rice grown under same CO_2 level and at same rice growth stage, and between aCO_2 and eCO_2 for same rice cultivar and at same rice growth stage by the Student's *t*-test at $p < 0.05$.

3.4. The Transcript Expression Level of APNs in C. suppressalis Larvae Fed on the Stems of Bt and Non-Bt Rice Plants Grown under aCO_2 and eCO_2

3.4.1. APN1

The expression level of *APN1* in *C. suppressalis* larvae was significantly affected by rice cultivar ($p < 0.001$), growth stage ($p < 0.001$) and their interaction ($p < 0.001$), and the interaction among CO_2 level, rice cultivar and growth stage ($p = 0.020$) (Table 2). Compared with aCO_2, eCO_2 significantly increased the expression level of *APN1* in *C. suppressalis* larvae fed on *Bt* rice during seedling stage for 4 days ($p < 0.05$, Figure 7A) and significantly decreased that during tillering stage for 4 days ($p < 0.05$, Figure 7C). The *APN1* expression level in *C. suppressalis* larvae fed on *Bt* rice was significantly higher than that in *C. suppressalis* larvae fed on non-*Bt* rice during seedling stage (aCO_2: 3 d; eCO_2: 1, 2, 3 and 4 d; $p < 0.05$, Figure 7A,B), tillering stage under aCO_2 for 4 days ($p < 0.05$; Figure 7C,D) and heading stage under aCO_2 and eCO_2 for 4 days ($p < 0.05$; Figure 7E,F), respectively.

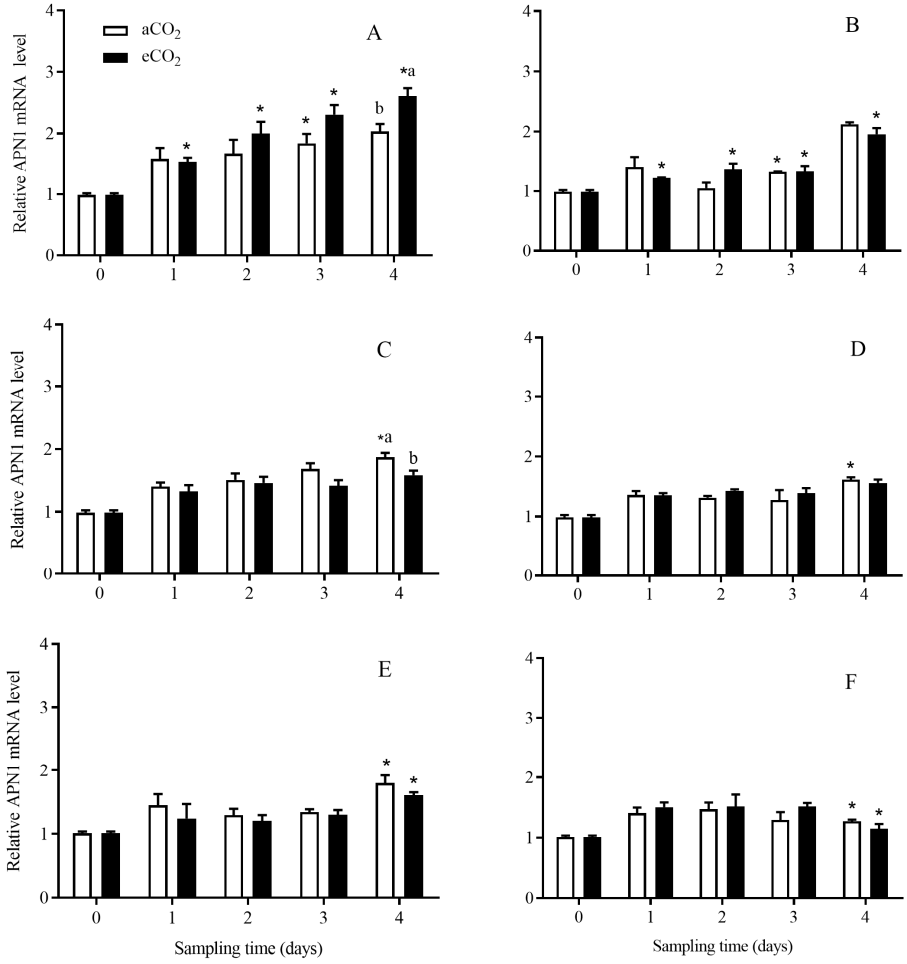

Figure 7. The APN1 expression level in *C. suppressalis* larvae fed on *Bt* and non-*Bt* rice plants grown under aCO_2 and eCO_2 for 0, 1, 2, 3 and 4 days, during seedling, tillering and heading stages. Note: (**A**,**B**)—seedling stage; (**C**,**D**)—tillering stage; (**E**,**F**)—heading stage; (**A**,**C**,**E**)—*Bt* rice, (**B**,**D**,**F**)—non-*Bt* rice. Each value represents means ± SE. *, different lowercase letters indicate significant difference between *Bt* and non-*Bt* rice grown under same CO_2 level and at same rice growth stage, and between aCO_2 and eCO_2 for same rice cultivar and at same rice growth stage by the Student's *t*-test at $p < 0.05$.

3.4.2. APN3a

The expression level of *APN3a* in *C. suppressalis* larvae was significantly affected by rice cultivar ($p = 0.040$), growth stage ($p = 0.005$) and their interaction ($p = 0.049$) (Table 2). Compared with aCO_2, eCO_2 significantly reduced the expression level of *APN3a* in *C. suppressalis* larvae fed on non-*Bt* rice during seedling and tillering stage for 4 days, respectively ($p < 0.05$; Figure 8B,D). The APN3a expression level in *C. suppressalis* larvae fed on *Bt* rice was significantly higher than that in *C. suppressalis* larvae fed on non-*Bt* rice during seedling stage for 1 day under eCO_2 (Figure 8A,B), tillering stage for 4 days under eCO_2 ($p < 0.05$; Figure 8C,D) and heading stage for 4 days under aCO_2 ($p < 0.05$; Figure 8E,F), respectively.

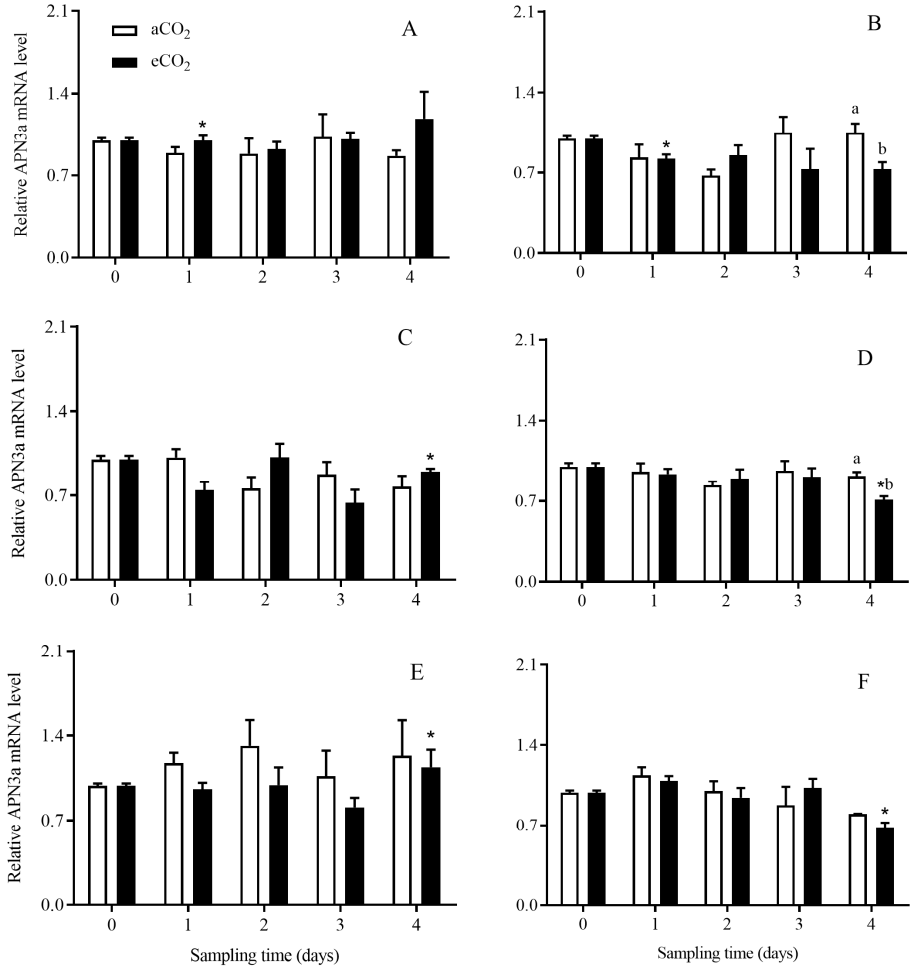

Figure 8. The *APN3a* expression level in *C. suppressalis* larvae fed on *Bt* and non-*Bt* rice plants grown under aCO_2 and eCO_2 for 0, 1, 2, 3 and 4 days, during seedling, tillering and heading stages. Note: (**A**,**B**)—seedling stage; (**C**,**D**)—tillering stage; (**E**,**F**)—heading stage; (**A**,**C**,**E**)—*Bt* rice, (**B**,**D**,**F**)—non-*Bt* rice. Each value represents means ± SE. *, different lowercase letters indicate significant difference between *Bt* and non-*Bt* rice grown under same CO_2 level and at same rice growth stage, and between aCO_2 and eCO_2 for same rice cultivar and at same rice growth stage by the Student's *t*-test at $p < 0.05$.

3.4.3. APN3b

The expression level of *APN3b* in *C. suppressalis* larvae was significantly affected by rice cultivar ($p < 0.001$; Table 2). The *APN3b* expression level in *C. suppressalis* larvae fed on *Bt* rice was significantly lower than that in *C. suppressalis* larvae fed on non-*Bt* rice during seedling stage for 4 days under aCO_2 ($p < 0.05$; Figure 9A), tillering stage for 3 days under eCO_2 ($p < 0.05$, Figure 9C,D) and heading stage for 4 days under eCO_2 ($p < 0.05$; Figure 9E), respectively.

3.4.4. APN4

The expression level of *APN4* in *C. suppressalis* larvae was significantly affected by rice cultivar ($p = 0.023$), growth stage ($p < 0.001$), and the interaction among CO_2 level, rice cultivar and growth stage ($p = 0.037$) (Table 2). Compared with aCO_2, eCO_2 significantly increased the *APN4* expression level in *C. suppressalis* larvae fed on *Bt* rice during seedling stage for 3 days ($p < 0.05$; Figure 10A), and significantly decreased that during heading stage for 3 days ($p < 0.05$; Figure 10E). The *APN4* expression level in *C. suppressalis* larvae fed on *Bt* rice was significantly lower than that in *C. suppressalis* larvae fed on non-*Bt* rice grown under aCO_2 during seedling stage for 1, 3 and 4 days, respectively ($p < 0.05$; Figure 10A,B).

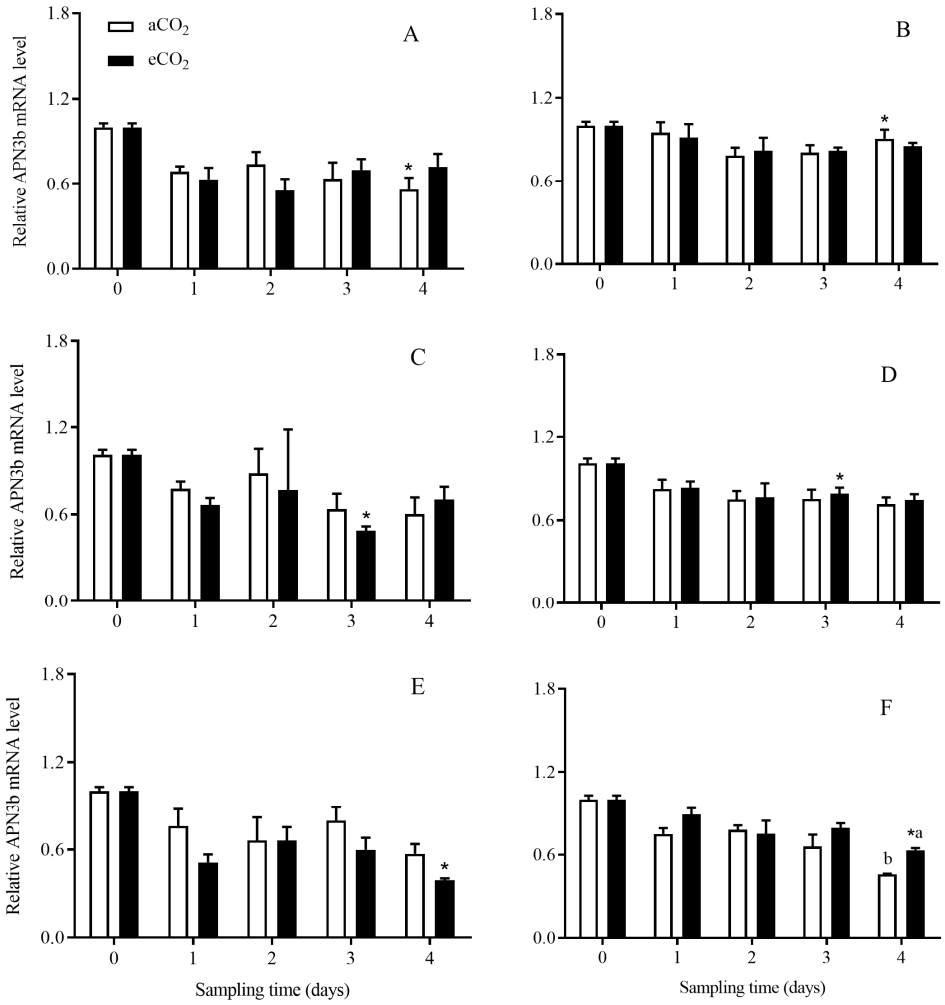

Figure 9. The *APN3b* expression level in *C. suppressalis* larvae fed on *Bt* and non-*Bt* rice plants grown under aCO_2 and eCO_2 for 0, 1, 2, 3 and 4 days, during seedling, tillering and heading stages. Note: (**A**,**B**)—seedling stage; (**C**,**D**)—tillering stage; (**E**,**F**)—heading stage; (**A**,**C**,**E**)—*Bt* rice, (**B**,**D**,**F**)—non-*Bt* rice. Each value represents means ± SE. *, different lowercase letters indicate significant difference between *Bt* and non-*Bt* rice grown under same CO_2 level and at same rice growth stage, and between aCO_2 and eCO_2 for same rice cultivar and at same rice growth stage by the Student's *t*-test at $p < 0.05$.

3.4.5. APN5

The expression level of *APN5* in *C. suppressalis* larvae was significantly affected by CO_2 level ($p = 0.003$), rice cultivar ($p < 0.001$), growth stage ($p = 0.014$), and the interactions between CO_2 level and rice cultivar ($p = 0.004$), between CO_2 level and growth stage ($p < 0.001$), between rice cultivar and growth stage ($p < 0.001$), and among CO_2 level, rice cultivar and growth stage ($p = 0.003$) (Table 2). Compared with aCO_2, eCO_2 significantly enhanced the *APN5* expression level in *C. suppressalis* larvae fed on *Bt* rice for 3 and 4 days during seedling stage ($p < 0.05$; Figure 11A), and significantly decreased that during tillering stage for 3 and 4 days ($p < 0.05$; Figure 11C) and heading stage for 4 days ($p < 0.05$; Figure 11E), respectively. The expression level of *APN5* in *C. suppressalis* larvae fed on *Bt* rice was significantly higher than that in *C. suppressalis* larvae fed on non-*Bt* rice during seedling stage for 1, 2, 3 and 4 days under aCO_2 and eCO_2 ($p < 0.05$; Figure 11A,D), during tillering stage (aCO_2: 1, 2, 3 and 4 d; eCO_2: 1, 2, 3 d; $p < 0.05$, Figure 11C,D) and heading stage (aCO_2: 1 and 4 d; eCO_2:1, 3 and 4 d; $p < 0.05$, Figure 11E,F), respectively.

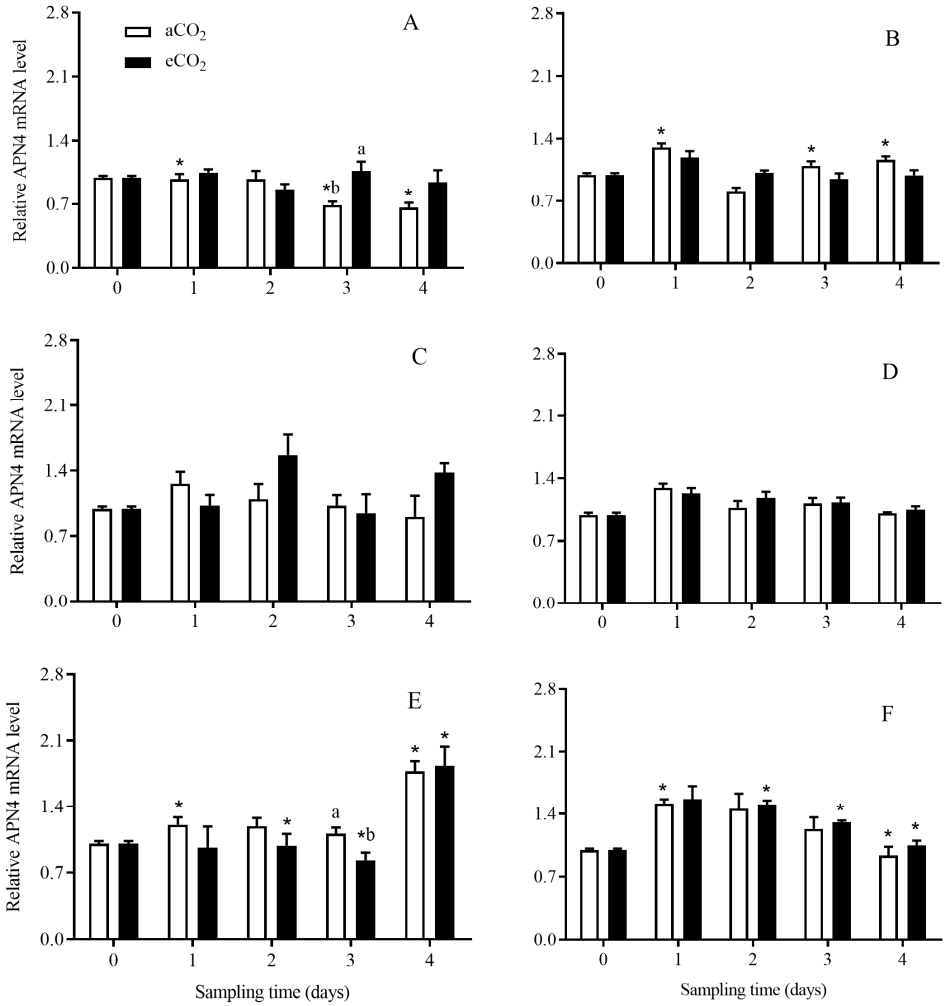

Figure 10. The *APN4* expression level in *C. suppressalis* larvae fed on *Bt* and non-*Bt* rice plants grown under aCO_2 and eCO_2 for 0, 1, 2, 3 and 4 days, during seedling, tillering and heading stages. Note: (**A**,**B**)—seedling stage; (**C**,**D**)—tillering stage; (**E**,**F**)—heading stage; (**A**,**C**,**E**)—*Bt* rice, (**B**,**D**,**F**)—non-*Bt* rice. Each value represents means ± SE. *, different lowercase letters indicate significant difference between *Bt* and non-*Bt* rice grown under same CO_2 level and at same rice growth stage, and between aCO_2 and eCO_2 for same rice cultivar and at same rice growth stage by the Student's *t*-test at $p < 0.05$.

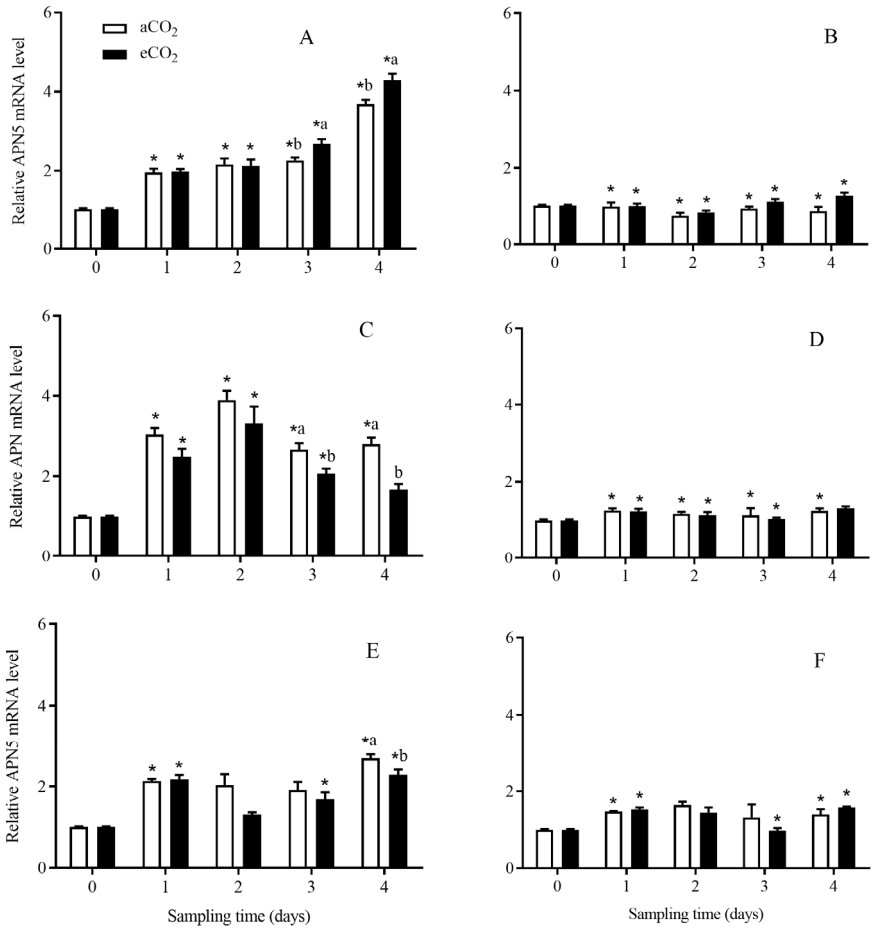

Figure 11. The *APN5* expression level in *C. suppressalis* larvae fed on *Bt* and non-*Bt* rice plants grown under aCO_2 and eCO_2 for 0, 1, 2, 3 and 4 days, during seedling, tillering and heading stages. Note: (**A**,**B**)—seedling stage; (**C**,**D**)—tillering stage; (**E**,**F**)—heading stage; (**A**,**C**,**E**)—*Bt* rice, (**B**,**D**,**F**)—non-*Bt* rice. Each value represents means ± SE. *, different lowercase letters indicate significant difference between *Bt* and non-*Bt* rice grown under same CO_2 level and at same rice growth stage, and between aCO_2 and eCO_2 for same rice cultivar and at same rice growth stage by the Student's *t*-test at $p < 0.05$.

4. Discussion

Previous studies showed that Bt-toxin content in plant tissues fluctuates during the growing season of transgenic *Bt* crops [35,36]. Our results showed that the Bt-toxin content in *Bt* rice stems were high at seedling and tillering stages, but relatively low at heading stage, which is consistent with the results of Chen et al. [35] and Wang et al. [4], that eCO_2 significantly enhanced exogenous toxin amount per stem in seedlings of *Bt* rice in contrast to aCO_2. The decreased Cry protein content in later growth stages may be related to that most of the energy materials in the late stage of *Bt* rice growth, which were mainly used to synthesize yield materials instead of just Bt protein [37]. In addition, our laboratory bioassay showed that the *Bt* rice (cv. HH1) had high resistance to the target stemborers, *C. suppressalis*. The high resistance of *Bt* rice to *C. suppressalis* was also indicated in the laboratory and field experiments [4,6]. Thus, our findings confirmed that although the Bt-toxin content in *Bt* rice stems was relatively lower at the heading stage, it was relatively high during all rice growth stages, which guarantees a high efficiency for consistently controlling of target insect pests throughout the whole growth season.

In this study, the results indicated that eCO_2 significantly reduced Bt-toxin content in *Bt* rice stems during tillering stage compared with aCO_2,. In our previous study, we found that the foliar Bt-toxin content of *Bt* rice grown under eCO_2 during tillering stage was significantly lower than that under aCO_2 [29]. So the response of *Bt* rice to eCO_2

way be specific in different plant tissues under global climate change. For transgenic *Bt* cotton, there are many reports describing the depletion of Bt toxin in response to eCO_2 levels [38,39]. However, the specific mechanism behind the lower Bt-toxin content in transgneic *Bt* plants exposed to eCO_2 remains unclear [40]. Previous studies showed that the target insect-resistance of *Bt* rice was positively correlated with the Bt-toxin contents in plant tissues [35,36,41,42]. The mortality of *C. suppressalis* larvae fed on *Bt* rice stems during tillering stage grown under eCO_2 was lower than that under aCO_2, and simultaneously lower Bt-toxin content in *Bt* rice stems was also detected under eCO_2.

Serine proteinases are some common luminal midgut digestive enzymes of dietary nutriens in many species of herbivorous insects including lepidopterans [43]. Protoxins are activated by midgut proteases in caterpillar midgut [44]. Among the insect serine proteinases, midgut trypsin and chymotrypsin are important in both solubilization and activation of Bt toxins [45]. In this study, the results showed that the activity of total protease and tryptase-like enzyme in *C. suppressalis* larvae fed on *Bt* rice stems were lower than that in *C. suppressalis* larvae fed on non-*Bt* rice stems regardless of CO_2 level. The tryptase-like enzyme activity in *Mythimna separata* larvae fed on Cry1Ac diet was significantly lower than that in *M. separata* larvae fed on the control diet [46]. The total protease activity of Asian corn borer *Ostrinia furnacalis* fed on *Bt* corn was significantly lower than that in *O. furnacalis* larvae fed on non-*Bt* corn, and the tryptase-like enzyme activity was significantly higher than that fed on non-*Bt* corn [47]. The changing trend of proteinase activity may be related to the insects faced with different stress of insecticidal proteins [46]. In addition, some studies focus on the resistance of target insects to Cry toxins, which showed that the resistance can be reduced by the decreased proteinase activity, and could lead to low activation of Bt protoxins to active toxins [48–52]. So it can be presumed that the resistance change of target insects to Bt toxins can also be alterred by the upregulation of midgut proteinases, which causes of the increased Bt-toxin degradation [53].

A key step for the toxicity of Cry toxins is the binding of Cry-toxin core to specific receptors at midgut epithelium of target insects. One recent study shown that the Bt resistance of target insects largely focused on the reduced binding of Bt toxin to protein receptors in the midgut of insects and the reduced binding was resulting from mutation or altered expression of genes encoding aminopeptidase N, cadherin or alkaline phosphatase [44]. In this study, the aminopeptidase activity of *C. suppressalis* larvae fed on *Bt* rice increased with the fed time prolonged, and it was higher than that in *C. suppressalis* larvae fed on non-Bt rice. Similarly, the activity of aminopeptidase activity in *Cnaphalocrocis medinalis* fed on *Bt* rice was higher than that in *C. medinalis* fed on non-*Bt* rice [54]. Valaitis (2008) found that massive APNs of *Lymantria dispar* larvae rapidly shed from midgut epithelial cells into the luminal fluid after force-feeding *B. thuringiensis*, and the shedding APNs might have function as competitive inhibitors, preventing toxin interaction with the cell surface receptors [15]. In this study, the increased aminopeptidase activity of *C. suppressalis* larvae fed on *Bt* rice stems may protect themselves by preventing the combination of Bt toxin and aminopeptidase in midgut epithelial cells.

As a type of Bt-toxin receptor, APN is a key factor for *Bt*-resistance mechanism of target insects. Our result showed that eCO_2 significantly increased the expression level of APN5 in *C. suppressails* larvae fed on *Bt* rice during seedling stage, and significantly reduced the expression level of APN5 of *C. suppressalis* larvae fed on *Bt* rice during tillering and heading stages. The different expression trend of APN5 in *C. suppressalis* larvae detected as fed on rice plants during different growth stages in this study may be a result of adaptation to the different Bt-toxin content of *Bt* rice during different growth stage especially under elevated CO_2 condition. In addition, APN5 expression level was up-regulated at 24 h after fed on *Bt* rice during seedling, tillering and heading stages regardless of CO_2 level. Consistent with the result of Zhang et al. (2017) that HcAPN3 in *Hyphantria cunea* was up-regulated at the 24 h after Cry1Ab35 infection [55]. Our results also showed that the gene expression levels varied for the five APNs in *C. suppressalis* larvae. Yang et al. (2010) founded that the expression level of APN1, 2 and 3 in *Diatraea saccharalis* was different [56]. The APN1, 2,

3 and 4 expression level of *H. armigera* was approximately 1000-fold higher than that of APN5 [57].

5. Conclusions

In summary, eCO_2 can enhance the Bt-toxin content in *Bt* rice during seedling stage, and accordingly might enhance the resistance of *Bt* rice to the target insect pest, *C. suppressails* during this plant growth stage. During the tillering and heading stages, eCO_2 decreased the resistance of *Bt* rice to *C. suppressails* due to decreasing of Bt-toxin content in *Bt* rice stems grown under eCO_2 during these plant growth stages. In addition, the activity of tryptase-like enzyme and aminopeptidase, and the expression level of *APNs* of *C. suppressails* larvae were changed when they fed on the stems of *Bt* rice grown under eCO_2. Under eCO_2, the resistance of *Bt* rice to *C. suppressails* showed a decreased trend during tillering and heading stages. It's necessary to strength monitor and control of these target insect pests especially during the later growth stages of *B*t rice under global climate change.

Author Contributions: Methodology, Y.L. and F.C.; Formal analysis, Y.W. and Y.L.; Investigation, Y.L., Y.W., G.C., S.L. and B.X.; Writing-original draft, Y.L. and G.C.; Writing-review and editing, Y.L. and F.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by National Nature Science Foundations of China (NSFC) (31871963), the National Key Research and Development Program of China (2023YFC2604900; 2023YFD1400800), the Special Program for New Transgenic Variety Breeding of the Ministry of Science and Technology, China (2016ZX08012005), the Fundamental Research Funds for the Central Universities (KYZ201818), the Qing-Lan Project of Jiangsu Province of China.

Data Availability Statement: Data are contained within the article.

Conflicts of Interest: The authors declare no conflict of interest.

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