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$CaCl_2$–HCl electrolyzed water promotes glucosinolate metabolism in broccoli sprouts via calcium signalling

Cui Li[1], Shuhui Song[2], Yanan He[1], Siyu Han[1] and Haijie Liu[1*]

Abstract

Background Electrolyzed water, a green chemistry invention, is a novel disinfectant that has been widely used in the food field. Previous studies have found that slightly acidic electrolyzed water not only has a bactericidal effect but also promotes the accumulation of active substances in many types of sprouts. We developed a new type of electrolyzed water, $CaCl_2$–HCl electrolyzed water (CHEW), which can effectively enhance the formation of glucosinolates and isothiocyanates in broccoli sprouts. The molecular mechanism by which $CaCl_2$–HCl electrolyzed water promotes glucosinolate metabolism in broccoli sprouts during growth was investigated in this study.

Results On the 4th day, the total glucosinolate content in broccoli sprouts reached 195.50 μmol/g DW under CHEW treatment. On the 8th day, compared to treatment with tap water, the contents of three isothiocyanates (sulforaphane, erucin, and 4-isothiocyanato-1-butene) in broccoli sprouts under CHEW treatment were significantly increased by 45.93, 34.04 and 185.15%, respectively. The results of calcium distribution and qRT–PCR assessments indicated that CHEW treatment activated calcium signalling in broccoli sprouts and promoted the relative expression of glucosinolate biosynthesis-related genes through calcium sensors [calmodulin (CaM), calmodulin-like protein (CML), and calcium-dependent protein kinase (CDPK)] and the calcium-binding nuclear protein IQD1.

Conclusion Our research provides new insights into the molecular mechanism of glucosinolate metabolism and a theoretical basis for cultivating high-quality broccoli sprouts.

Keywords $CaCl_2$–HCl electrolyzed water (CHEW), Broccoli sprouts, Glucosinolate metabolism, Calcium signal, Molecular mechanisms

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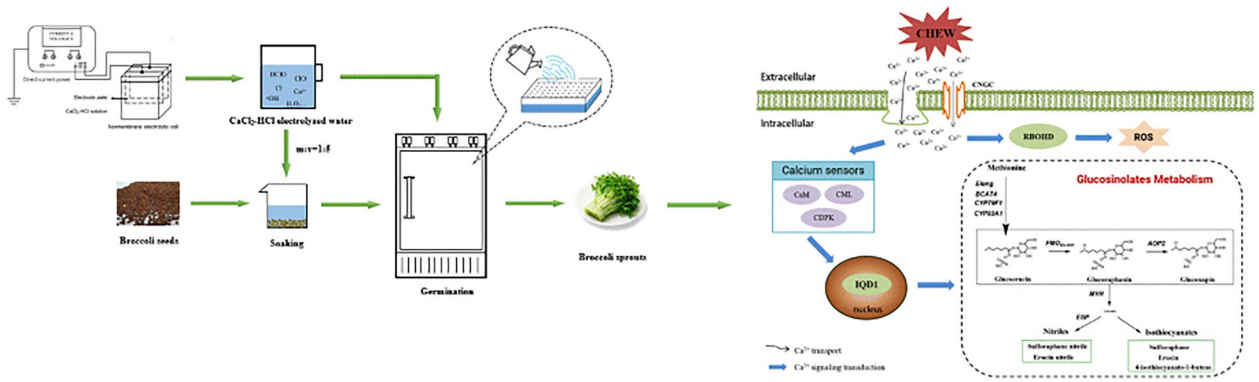
Graphical Abstract



Introduction

Electrolyzed water, a green chemistry invention, is a novel disinfectant that has been widely used in the food field [1]. Slightly acidic electrolyzed water (SAEW) with an available chlorine concentration (ACC) of 10–60 mg/L and pH 5.0–6.5 is obtained by the electrolysis of dilute HCl solution in a nonmembrane device [2]. Previous studies have found that SAEW not only has a bactericidal effect but also promotes the accumulation of active substances in many types of sprouts. For example, SAEW promotes the production of γ-aminobutyric acid through oxidative stress and alters mung bean sprout endogenous hormone levels [3]. SAEW not only controlled natural microbial population growth during buckwheat germination but also promoted rutin accumulation [4, 5].

Seed germination is a complex biochemical process accompanied by various metabolic reactions and changes in chemical compounds [6]. For cruciferous brassica vegetables such as broccoli, the glucosinolate content in seeds increases after germination. Interestingly, the broccoli sprout glucosinolate content has been found to be ten times higher than that in broccoli plants [7]. Therefore, increasing research has focused on broccoli sprouts. Glucosinolates can be hydrolysed under the action of endogenous myrosinase to produce certain hydrolysates: isothiocyanates, thiocyanates, and nitriles [8]. Among glucosinolate hydrolysates, nitriles have no biological activity, while 4-methylsulfinylbutyl isothiocyanate (sulforaphane), with the highest anticancer activity among them, is the most abundant isothiocyanate in broccoli sprouts [9, 10]. In addition, other benefits of sulforaphane have been identified, such as anti-inflammatory effects by inhibiting the expression of proinflammatory factors [11]; reducing insulin resistance by blocking serine palmitoyltransferase-3-mediated ceramide biosynthesis [12]; and reducing obesity by affecting fatty acid oxidation pathways such as the AMPK transcription pathway, the triacylglyceride synthesis pathway and adipose tissue browning [13, 14]. Thus, how to further enhance the formation of glucosinolates and sulforaphane in broccoli sprouts by exogenous treatments has been a research hotspot.

Our previous study found that SAEW treatment increased the contents of secondary metabolites, sulforaphane, and phenolic compounds in broccoli sprouts and increased its antioxidant capacity [15]. However, the oxidative components (HClO, ClO− etc.) in SAEW will produce oxidative stress, which is not conducive to the growth of broccoli sprouts [16]. In addition, studies have also shown that calcium not only enhances the formation of glucosinolates in broccoli sprouts but also improves broccoli sprout productivity, postharvest quality and shelf life [17, 18]. Calcium, a second messenger in cell signalling, is involved in various plant metabolism pathways [19]. Calcium can bind to calmodulin to form Ca^{2+}/CaM, which conducts extracellular signals into the intracellular space through concentration changes to initiate biochemical processes [20]. Therefore, we combined SAEW and calcium to obtain $CaCl_2$–HCl electrolyzed water (CHEW, pH 5.5, 10 mg/L ACC) and found that the sulforaphane content in broccoli sprouts with 5CHEW (5 mM $CaCl_2$) treatment was further improved to 40.03 mg/g DW [21]. This result indicated that calcium plays an important role in glucosinolate metabolism in broccoli sprouts.

Therefore, the objective of this study was to investigate the molecular mechanism by which CHEW promotes the growth and glucosinolate metabolism of broccoli sprouts during growth via calcium signalling. We not only evaluated the contents of ROS (H_2O_2, O_2^-), malondialdehyde

(MDA), glucosinolates and their hydrolysates in broccoli sprouts during growth under CHEW treatment but also detected the activities of myrosinase and epithiospecifier protein (ESP), calcium distribution, and the relative expression of genes related to glucosinolate metabolism and calcium signalling.

Results and discussion

The processes of $CaCl_2$–HCl electrolyzed water production and broccoli sprout cultivation are shown in Fig. 1. Details can be seen in "Materials and methods".

Change in calcium, ROS and MDA contents in broccoli sprouts

As shown in Fig. 2a, the calcium content in broccoli sprouts gradually increased during growth. CHEW significantly improved the calcium level in broccoli sprouts compared to tap water. On the 2nd, 4th, 6th and 8th days of growth, compared to tap water, the calcium contents in broccoli sprouts under CHEW treatment were significantly increased by 11.3, 22.9, 52.8 and 50.2%, respectively. This result indicated that CHEW treatment further promotes calcium accumulation in broccoli sprouts during growth.

To investigate the effects of CHEW on the physiological changes in broccoli sprouts, we found that the H_2O_2 content in broccoli sprouts gradually decreased during growth, while the O_2^- and MDA contents first increased and then decreased (Fig. 2b–d). In plants, ROS are key signalling molecules in abiotic and biotic stress sensing that enable cells to rapidly respond to different stimuli and establish defence mechanisms [22]. Plants with oxidative stress would produce a large amount of ROS, while excessive ROS would disrupt the oxidation–reduction balance of cells and had a toxic effect [23]. As a second messenger in cell signalling, calcium could regulate the balance of intracellular ROS via the calcium –NADPH oxidase signaling pathway: calcium enhanced the activity of a respiratory burst oxidase–nicotinamide–adenine dinucleotide phosphate (NADPH) oxidase (the key enzyme for ROS production) through directly binds to the EF hand unit of NADPH [24, 25]. MDA can reflect the response of broccoli sprouts to oxidative stress. Compared to tap water, CHEW treatment significantly reduced the contents of ROS and MDA in broccoli sprouts during growth. On the second day of growth, compared to tap water, the contents of H_2O_2, O_2^- and MDA in broccoli sprouts under CHEW treatment were significantly decreased by 8.8, 25.7 and 48.8%, respectively. These results indicated that CHEW treatment regulated ROS levels in broccoli sprouts during growth and alleviated membrane lipid peroxidation in comparison with tap water via calcium signalling.

Glucosinolate biosynthesis in broccoli sprouts

The species of glucosinolates identified in broccoli sprouts are listed in Additional file 1: Fig. S1. There are 3 aliphatic glucosinolates (glucoraphanin, glucoerucin, and glucoalyssin) and 4 indole glucosinolates (4-hydroxyglucobrassicin, 4-methoxyglucobrassicin, glucobrassicin, and neoglucobrassicin) in broccoli sprouts. As shown in Table 1, glucoraphanin and glucoerucin are the main glucosinolates in broccoli sprouts. In addition, glucoraphanin is produced by the desulfurization of glucoerucin and can be further converted to gluconapin (which was present in a concentration that was too low to

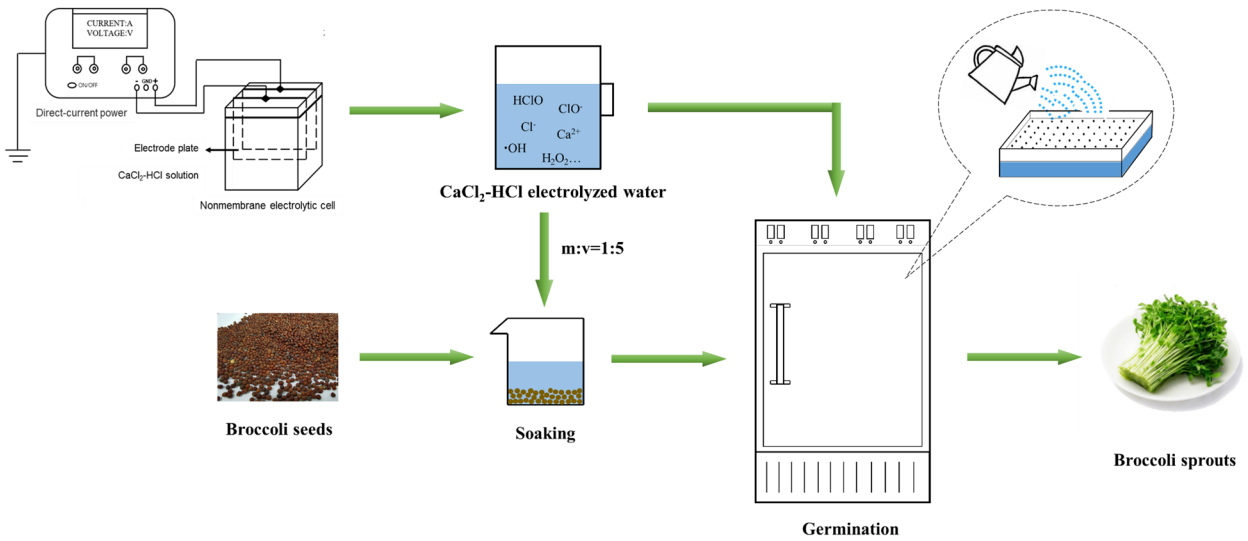


Fig. 1 The process of $CaCl_2$–HCl electrolyzed water's production and broccoli sprouts' cultivation

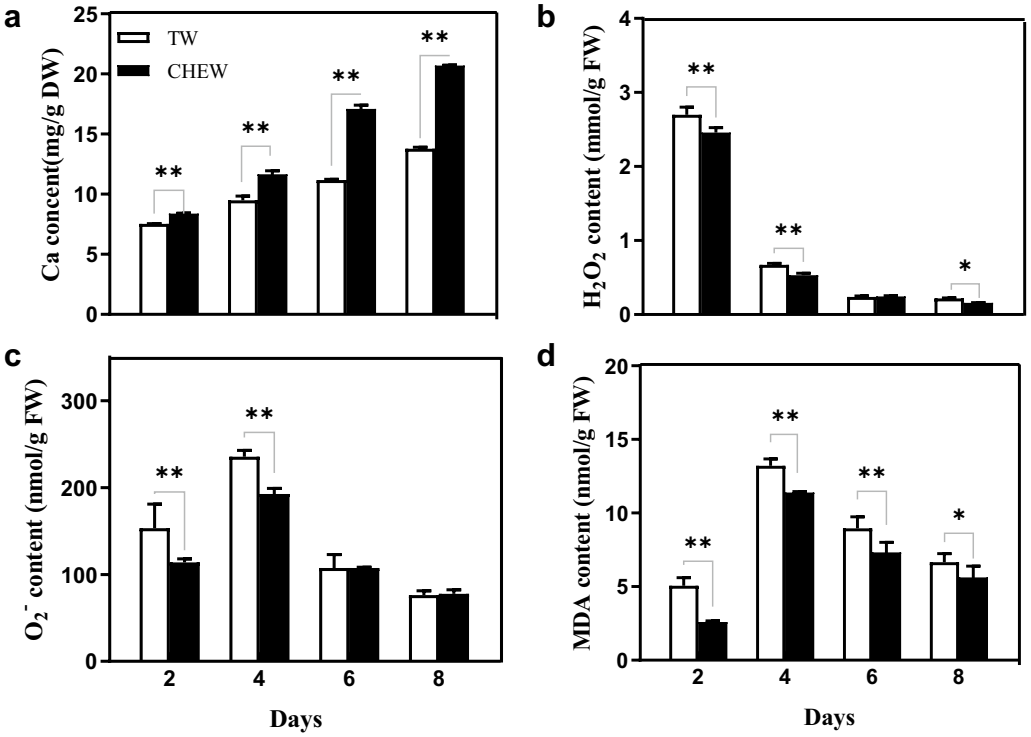


Fig. 2 Effects of $CaCl_2$–HCl electrolyzed water on physiological properties of broccoli sprouts. *TW* tap water, *CHEW* $CaCl_2$–HCl electrolyzed water. Asterisk (*) indicates a significant difference between the tap water and $CaCl_2$–HCl electrolyzed water treatment groups at $P < 0.05$ while double asterisk (**) indicates significance at $P < 0.01$

Table 1 The total and individual glucosinolate contents (μmol/g DW) in broccoli sprouts under different treatments

Groups		Aliphatic glucosinolate			Indole glucosinolates				Total glucosinolates
		GRA	GER	Aly	4OHGBS	4MOGBS	GBS	nGBS	
2d	TW	110.08 ± 1.96^{ef}	38.47 ± 0.17^{d}	2.59 ± 0.01^{b}	2.95 ± 0.08^{b}	0.42 ± 0.01^{f}	1.31 ± 0.01^{e}	0.51 ± 0.01^{f}	156.34 ± 1.87^{e}
	CHEW	115.84 ± 2.18^{cd}	38.61 ± 0.13^{cd}	2.59 ± 0.13^{b}	2.71 ± 0.08^{b}	0.35 ± 0.01^{f}	1.27 ± 0.01^{e}	0.45 ± 0.01^{f}	161.83 ± 2.34^{d}
4d	TW	120.38 ± 1.89^{bc}	45.16 ± 0.28^{a}	2.71 ± 0.03^{ab}	3.47 ± 0.02^{a}	0.78 ± 0.01^{e}	2.39 ± 0.05^{d}	1.57 ± 0.04^{e}	176.46 ± 1.69^{b}
	CHEW	141.24 ± 0.57^{a}	43.31 ± 0.50^{b}	2.79 ± 0.04^{a}	2.78 ± 0.01^{b}	0.91 ± 0.02^{d}	2.49 ± 0.01^{cd}	1.97 ± 0.04^{d}	195.50 ± 1.05^{a}
6d	TW	105.52 ± 1.02^{fg}	33.85 ± 1.46^{e}	1.89 ± 0.13^{e}	2.06 ± 0.18^{c}	1.50 ± 0.10^{c}	2.52 ± 0.08^{c}	2.33 ± 0.30^{c}	150.16 ± 0.94^{f}
	CHEW	122.01 ± 1.29^{b}	37.30 ± 0.46^{d}	2.11 ± 0.02^{d}	2.14 ± 0.03^{c}	1.51 ± 0.03^{c}	2.64 ± 0.12^{b}	2.60 ± 0.13^{b}	170.80 ± 1.03^{c}
8d	TW	103.43 ± 1.58^{g}	37.23 ± 0.22^{d}	2.21 ± 0.02^{cd}	2.05 ± 0.05^{c}	2.14 ± 0.01^{b}	2.53 ± 0.03^{c}	2.74 ± 0.06^{b}	152.35 ± 1.54^{ef}
	CHEW	114.93 ± 1.92^{de}	40.37 ± 0.19^{c}	2.30 ± 0.06^{c}	1.95 ± 0.05^{c}	2.50 ± 0.04^{a}	3.27 ± 0.08^{a}	3.20 ± 0.06^{a}	168.54 ± 1.96^{c}

Data are expressed as the mean ± standard deviation (SD), and values were obtained by three replicate measurements

TW tap water, *CHEW* $CaCl_2$–HCl electrolyzed water, *GRA* glucoraphanin, *GER* glucoerucin, *Aly* glucoalyssin, *4OHGBS* 4-hydroxyglucobrassicin, *4MOGBS* 4-methoxyglucobrassicin, *GBS* glucobrassicin, *nGBS* neoglucobrassicin

Different letters indicate significant differences ($P < 0.05$) within a column

be detected in this study). During growth, the total glucosinolate content in broccoli sprouts increased and then decreased. On the 4th day, the contents of glucoraphanin and total glucosinolates reached maximum values of 120.38 and 176.46 μmol/g DW, respectively. This may be because seed germination activates a number of physiological activities, such as glucosinolate biosynthesis, in plants, resulting in an increase in glucosinolate content in broccoli sprouts [2]. In addition, with the extension of growth time, the glucosinolates in broccoli sprouts were gradually decomposed and used to synthesize other sulfur-containing substances; therefore, the content of glucosinolates gradually decreased [26]. Under CHEW treatment, the glucosinolate content in broccoli sprouts

during growth was always significantly higher than that under tap water treatment. On the 4th day, the contents of glucoraphanin and total glucosinolates in broccoli sprouts treated with CHEW reached maximum values of 141.24 and 195.50 μmol/g DW, respectively, and were significantly increased by 17.32 and 10.79%, respectively, compared to tap water. These results indicated that CHEW could change the glucosinolate biosynthesis and metabolism in broccoli sprouts during growth and further increase the glucosinolate content.

Table 2 GC–MS analysis result of the glucosinolate' hydrolyzates in broccoli sprouts

No.	Compound	CAS	Retention time (min)	Formula
1	4-Isothiocyanato-1-butene	3386-97-8	5.759	C_5H_7NS
2	Erucin nitrile	59121-25-4	9.202	$C_6H_{11}NS$
3	Erucin	4430-36-8	12.437	$C_6H_{11}NS_2$
4	Sulforaphane nitrile	61121-66-2	13.599	$C_6H_{11}NOS$
5	Sulforaphane	4478-93-7	16.443	$C_6H_{11}NOS_2$

Glucosinolate hydrolysis in broccoli sprouts

According to the TIC map of glucosinolate hydrolysates, we detected 5 hydrolysis products in broccoli sprouts (Fig. 3a). After further identification, they were 3 isothiocyanates (sulforaphane, 4-isothiocyanato-1-butene, and erucin) and 2 nitriles (sulforaphane nitrile and erucin nitrile) (Table 2). Among the isothiocyanates, the content of sulforaphane was the highest, followed by erucin. The hydrolysis products of glucoraphanin are sulforaphane and sulforaphane nitrile; erucin and erucin nitrile are the hydrolysis products of glucoerucin; and 4-isothiocyanato-1-butene is generated by gluconapin hydrolysis.

As shown in Fig. 3b–f, the contents of isothiocyanates gradually increased during broccoli sprout growth, and the content of nitriles first increased and then decreased. On the 8th day, the contents of sulforaphane, erucin and 4-isothiocyanato-1-butene in broccoli sprouts under CHEW treatment reached maximum values of 34.78, 5.43 and 59.65 μg/g DW, respectively. Compared to tap water, the contents of the three isothiocyanates in broccoli sprouts under CHEW treatment on the 8th day were significantly increased by 45.93, 34.04 and 185.15%, respectively. CHEW increased the isothiocyanate content significantly during broccoli sprout growth while also reducing nitrile formation. On the 8th day, the contents of sulforaphane nitrile and erucin nitrile in broccoli sprouts with CHEW treatment were significantly decreased by 18.38 and 77.08%, respectively, compared to tap water treatment. In addition, the activities of myrosinase and ESP increased gradually during broccoli sprout growth (Fig. 3g, h). The activity value of myrosinase was much greater than that of ESP. On the 8th day, compared to tap water, the myrosinase activity in broccoli sprouts under CHEW treatment was significantly increased by 52.46%. The above results indicated that CHEW treatment could affect the hydrolysis direction of glucosinolates during broccoli sprout growth and promote the formation of isothiocyanates mainly by improving myrosinase activity.

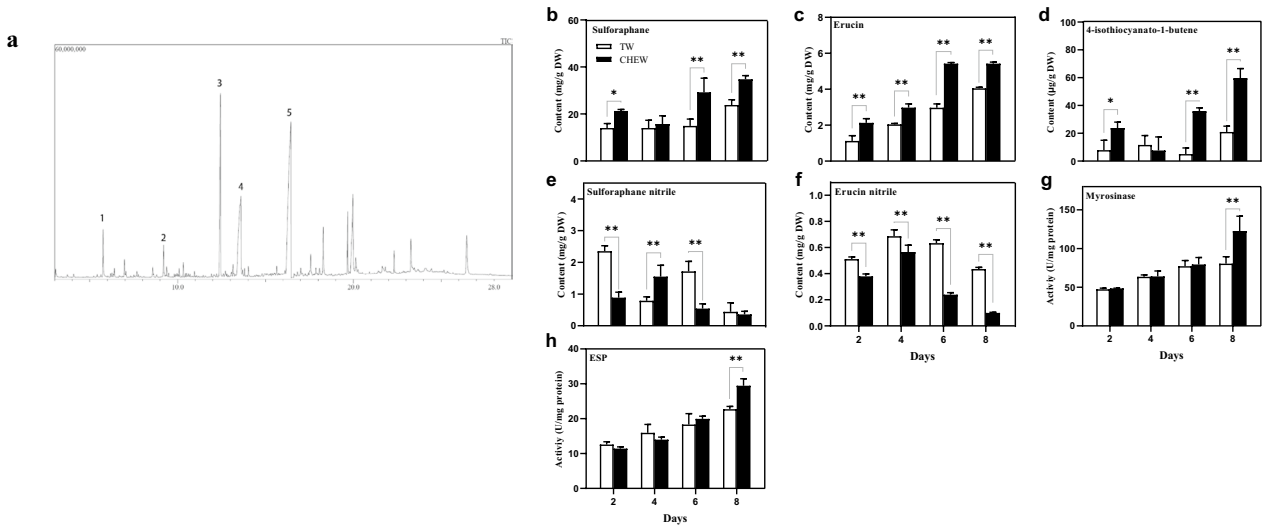


Fig. 3 Effects of $CaCl_2$–HCl electrolyzed water on the glucosinolate hydrolysate of broccoli sprouts. *TW* tap water, *CHEW* $CaCl_2$–HCl electrolyzed water. Asterisk (*) indicates a significant difference between the tap water and $CaCl_2$-HCl electrolyzed water treatment groups at $P < 0.05$ while double asterisk (**) indicates significance at $P < 0.01$

Effect of CHEW on the expression of genes related to glucosinolate metabolism

The molecular mechanism of aliphatic glucosinolate biosynthesis has been elucidated, and mainly includes the following three stages: (1) extension of the methionine side chain; (2) formation of the glucosinolate core structure; and (3) dimeric glucosinolate side chain secondary modification [27, 28]. *Elong* and *BCAT4* are the main genes involved in the first stage. The second stage is common during the formation of all types of glucosinolates and is also a key step in glucosinolate biosynthesis. *CYP79F1* and *CYP83A1* are the main genes involved in this stage. In the third stage, under flavin-containing monooxygenase (FMO) catalysis, the side chain of glucoerucin undergoes oxidation to form glucoraphanin. Then, glucoraphanin is catalysed by 2-oxoglutarate-dependent dioxygenases (AOPs) to form gluconapin. Genes involved in this stage include $FMO_{GS\text{-}OX1}$ and *AOP2*.

The expression of glucosinolate biosynthesis-related genes during the growth of broccoli sprouts is shown in Fig. 4a–f. Different genes display their highest expression levels during different broccoli sprout growth periods. The relative expression levels of *Elong*, *BCAT4*, *CYP79F1*, *CYP83A1* and $FMO_{GS\text{-}OX1}$ increased first and then decreased in broccoli sprouts during growth. On the 6th day, the relative expression levels of *Elong*, *BCAT4*, *CYP79F1*, *CYP83A1*, and $FMO_{GS\text{-}OX1}$ in broccoli sprouts reached their maximum value. This is consistent with the changing trend of glucosinolates during broccoli sprout growth (Table 1). The relative expression levels of *Elong*, *BCAT4*, *CYP79F1* and $FMO_{GS\text{-}OX1}$ in broccoli sprouts under CHEW treatment were 2.55-, 3.21-, 1.71- and 1.96-fold higher than those in the tap water treatment on the 6th day, respectively. The relative expression of *AOP2* increased gradually in broccoli sprouts during growth. The relative expression levels of *CYP83A1* and *AOP2* in broccoli sprouts treated with CHEW reached their maximum on the 8th day. On the 8th day, compared to tap water, the relative expression of *CYP83A1* and *AOP2* of broccoli sprouts with CHEW treatment significantly increased by 47.27 and 113.84%, respectively. This result indicated that CHEW treatment promoted glucosinolate biosynthesis by upregulating

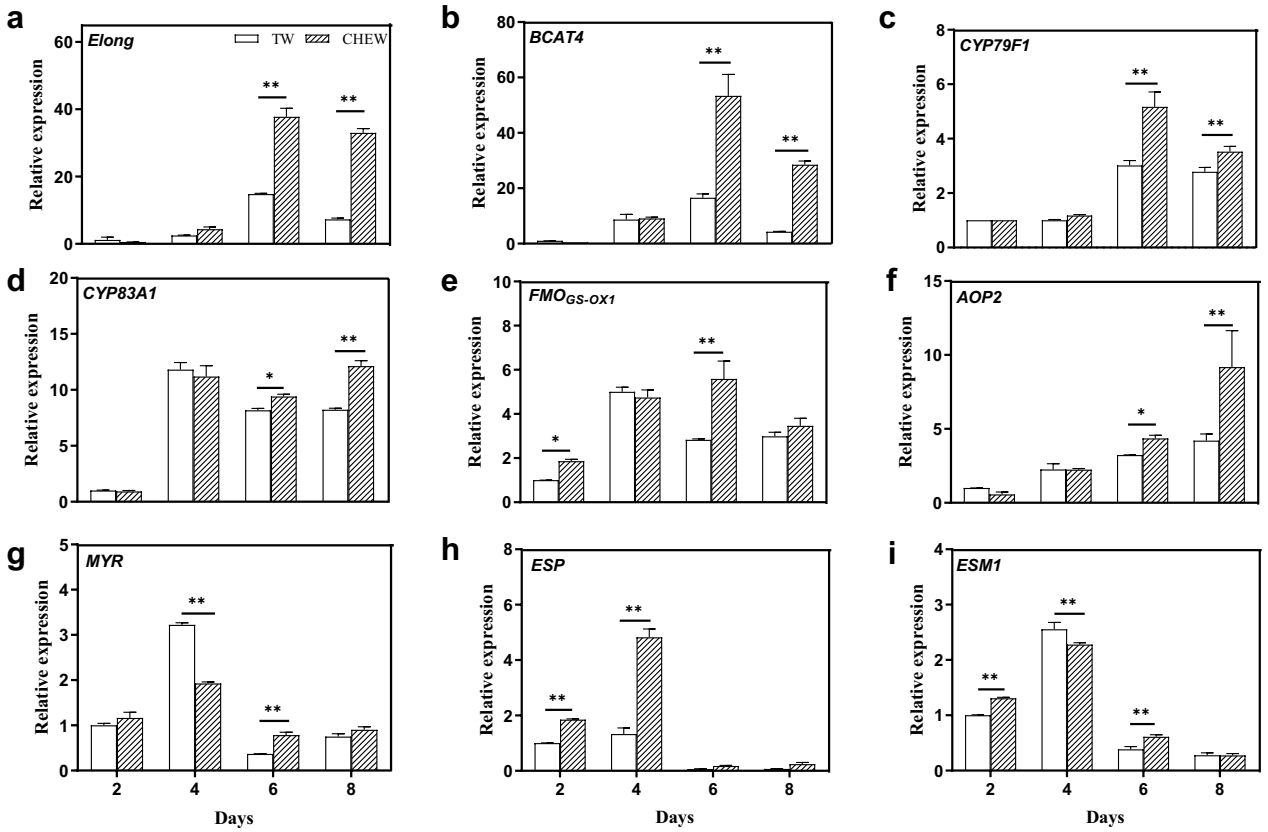


Fig. 4 Effects of $CaCl_2$–HCl electrolyzed water on the relative expression of genes in broccoli sprouts. *TW* tap water, *CHEW* $CaCl_2$–HCl electrolyzed water. Asterisk (*) indicates a significant difference between the tap water and $CaCl_2$–HCl electrolyzed water treatment groups at $P < 0.05$ while double asterisk (**) indicates significance at $P < 0.01$

glucosinolate synthesis-related genes in broccoli sprouts during growth.

Figure 4g–i shows that the relative expression of the glucosinolate hydrolysis-related genes *MYR, ESP* and *ESM1* increased first and then decreased during the growth of broccoli sprouts. The genes related to glucosinolate hydrolysis all reached their highest expression levels in broccoli sprouts on the 4th day. On the 4th day, compared to tap water, the relative expression of *MYR* and *ESM1* in broccoli sprouts under CHEW treatment decreased by 66.84 and 10.59%, respectively, while the relative expression of *ESP* was 3.65-fold that in the tap water-treated sprouts. This may be because the glucosinolate content reached the maximum value on the 4th day, which triggered negative feedback regulation to promote the expression of glucosinolate hydrolysis-related genes to maintain the balance of glucosinolate metabolism in broccoli sprouts.

The above finding may be a result of the glucosinolate content reaching the maximum value on the 4th day, which negatively regulates the glucosinolate metabolic balance in broccoli sprouts by promoting glucosinolate hydrolysis-related genes. In addition, the change trends in the expression of *MYR* and *ESP* were inconsistent with the change trends of the activities of myrosinase and ESP (Fig. 3g, h). On the one hand, myrosinase activity is not only regulated by the MYR gene but also influenced by various factors, such as the coenzyme factor ascorbic acid, pH, metal ions, water activity and temperature [29]. On the other hand, the epithiospecifier modifier protein ESM1 also interferes with the activity of ESP [30]. The above results showed that CHEW mainly promoted the hydrolysis of glucosinolates to generate more isothiocyanates through negative feedback when the glucosinolate content changes during broccoli sprout growth.

Localization of calcium and calcium signal transduction in broccoli sprouts

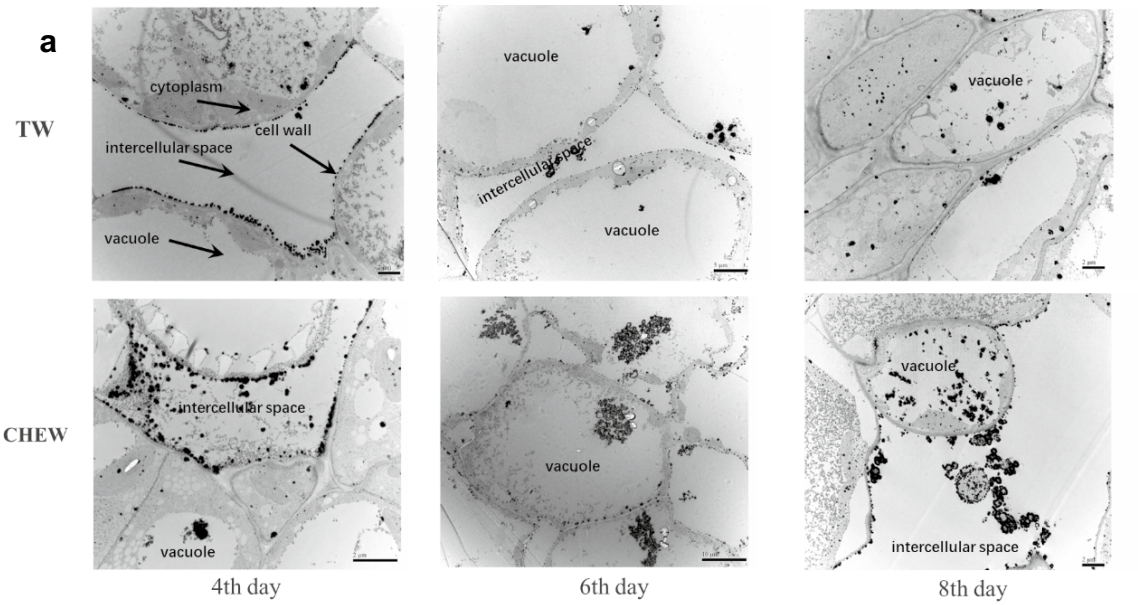
Calcium exists in broccoli sprout cells in three forms: free calcium, bound calcium and stored calcium [31]. The free calcium content in broccoli sprouts is not high (below 10^{-6} mol/L). Bound calcium can bind to specific strong-affinity structural components. Organelles and cell walls usually contain high levels of stored calcium, above 10^{-6} mol/L, accounting for most of the total intracellular calcium. These forms of calcium can be interconverted to meet the needs of the physiological responses of plants. Since the leaves of the broccoli sprouts on the 2nd day were too small to be detected, the calcium distribution on the 4th, 6th, and 8th days only is discussed. The dynamic distribution of calcium in broccoli sprouts is shown in Fig. 5a. During growth, a small amount of calcium was distributed in the cell walls and vacuoles in the broccoli sprouts. In plant cell walls, calcium binds to pectin in the gum layer in the cell wall to form pectin calcium. Pectin calcium can not only bind the cell wall material but also regulate membrane permeability and increase the strength of the cell wall. In the CHEW treatment group, it was clearly observed that on the 4th day, calcium entered the cells through endocytosis. Large amounts of calcium accumulated in the vacuoles on the 6th day. On the 8th day, the calcium that had aggregated within the vacuoles gradually dispersed. The type of calcium in the vacuoles was mainly calcium oxalate, which is formed by combining with organic acids produced by secondary metabolism in broccoli sprouts. The formation of calcium oxalate is reversible, can regulate intracellular ion balance and cell membrane permeability and is affected by intracellular calcium concentration and calmodulin activity.

Figure 5b shows that the relative expression of the respiratory burst oxidase—NADPH oxidase gene *RBOHD* in broccoli sprouts gradually decreased during growth. Compared to the tap water group, the expression of *RBOHD* under CHEW treatment was significantly reduced by 23 and 66% on the 4th and 8th days, respectively. On the 2nd day, the expression level of *RBOHD* was significantly higher in the CHEW treatment group than in the tap water treatment group, which may be due to the main role of the oxidative components in CHEW, such as HClO and ClO^{-}. RBOHD is an enzyme involved in ROS generation and can act as an electron donor to reduce O_2 to $O_2^{\cdot}$, which is then rapidly converted to H_2O_2 [32, 33]. Calcium regulates the activity of RBOHD by binding to it and changing its conformation. The change trend of *RBOHD* relative expression is consistent with the results in Fig. 1 in that CHEW significantly reduced ROS contents during broccoli sprout growth. This indicated that CHEW treatment reduced the ROS contents in broccoli sprouts by regulating the Ca-RBOHD enzyme signalling pathway, thereby alleviating oxidative stress compared to tap water.

The relative expression of the calcium ion channel gene *CNGC* increased first and then decreased during the growth of broccoli sprouts (Fig. 5c), which represents the change in the influx rate of calcium. This result is consistent with the dynamic change processes of calcium observed in Figs. 2d and 4a. On the 8th day, the rate

(See figure on next page.)

Fig. 5 Effects of $CaCl_2$–HCl electrolyzed water on the calcium signal transduction in broccoli sprouts. *TW* tap water, *CHEW* $CaCl_2$–HCl electrolyzed water. Asterisk (*) indicates a significant difference between the tap water and $CaCl_2$–HCl electrolyzed water treatments at $P < 0.05$ while double asterisk (**) indicates significance at $P < 0.01$



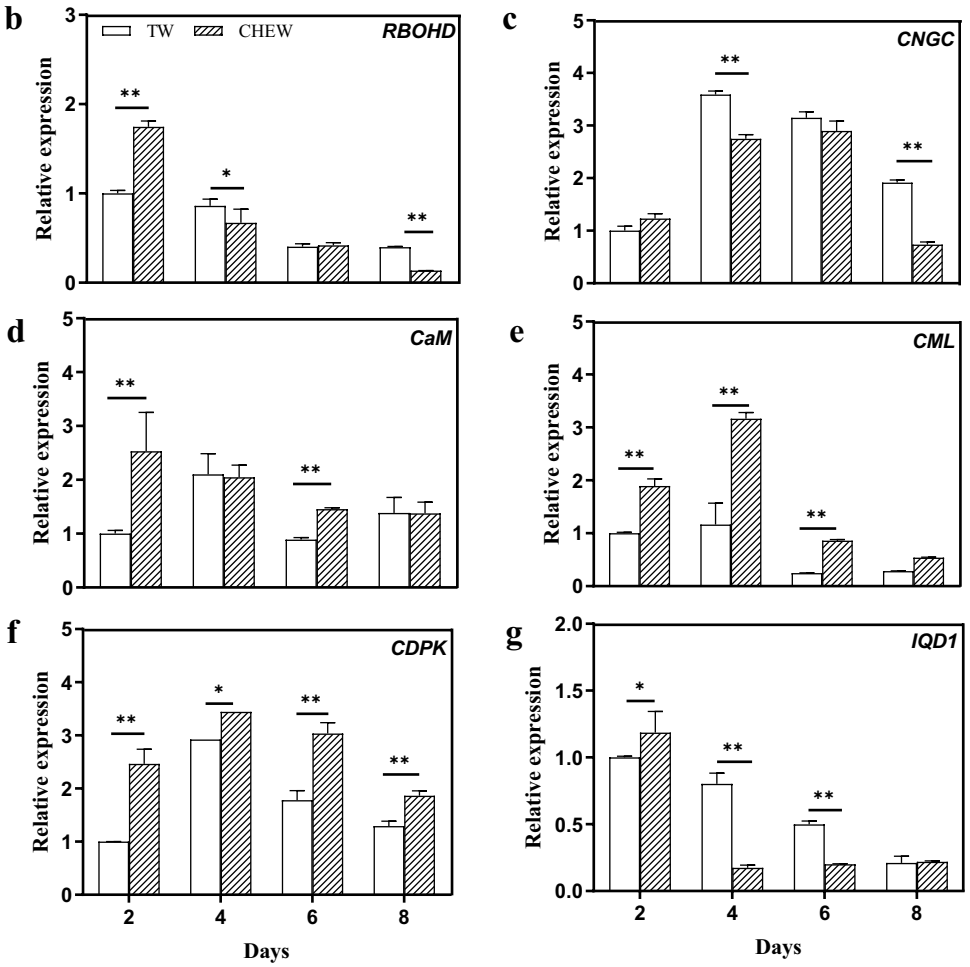


Fig. 5 (See legend on previous page.)

of increase in the calcium content in broccoli sprouts decreased from 52.8 to 50.2% (Fig. 2d). During growth, the intracellular calcium concentration in broccoli sprouts gradually increased, and mesophyll cells maintained the intracellular calcium balance by regulating the influx rate of calcium. In addition, the relative expression of the calcium sensor (calmodulin, calmodulin-like protein, and calcium-dependent protein kinase) genes *CaM*, *CML* and *CDPK* increased first and then decreased during broccoli sprout growth, and their expression in the CHEW treatment group was always higher than that in the tap water group (Fig. 5d–f). Compared to that in tap water-treated sprouts, the expression levels of *CaM*, *CML* and *CDPK* in broccoli sprouts under CHEW treatment on the 2nd day were significantly increased by 146, 89 and 145%, respectively. This result indicated that CHEW treatment activated the calcium signalling pathway in broccoli sprouts, which was mainly transmitted downstream through the calcium sensors CaM, CML and CDPK.

Figure 4G shows that the relative expression of *IQD1* in broccoli sprouts gradually decreased during growth. On the 4th and 6th days, the relative expression of *IQD1* in the CHEW treatment group was significantly lower than that in the tap water treatment group. IQD1 is a nuclear calcium-binding protein that regulates glucosinolate metabolism by integrating intracellular calcium signals. Previous research has found that overexpression of *IQD1* can promote the accumulation of aliphatic glucosinolates in *Arabidopsis thaliana*, while when aliphatic glucosinolates accumulation reaches a certain level, IQD1 will inhibit the expression of the glucosinolate biosynthesis-related gene *CYP79F1* and the activity of myrosinase through negative feedback regulation [34]. In this study, the trend of *IQD1* expression during broccoli sprout growth was consistent with changes in glucosinolate content. This result indicated that calcium signalling could promote glucosinolate metabolism in broccoli sprouts through IQD1.

The mechanism by which CHEW treatment regulates glucosinolate biosynthesis in broccoli sprouts

The molecular mechanism by which CHEW regulates glucosinolate metabolism in broccoli sprouts during growth via calcium signalling is shown in Fig. 6. The calcium in CHEW enters cells mainly through endocytosis and calcium ion channels (CNGCs) on the cell membrane to generate calcium signals. On the one hand, calcium signalling regulates the activity of the NADPH oxidase RBOHD by changing its conformation, thereby affecting the level of reactive oxygen species in broccoli sprouts and relieving the oxidative stress caused by tap water. On the other hand, the calcium sensors CaM, CML, and CDPK in broccoli sprouts receive the calcium signal and transmit it to the nuclear calcium-binding protein IQD1. A previous study showed that IQD1 affected the relative expression of glucosinolate biosynthesis-related genes and promoted glucosinolate accumulation in watercress [35]. However, when the glucosinolate content in plants increases to a certain extent, IQD1 inhibits the biosynthesis of glucosinolates through negative feedback [34]. Overall, CHEW activated calcium signalling in broccoli sprouts and promoted glucosinolate metabolism by increasing the relative expression of glucosinolate biosynthesis-related genes and inducing the negative feedback of glucosinolates.

Conclusion

CHEW mainly regulates the growth and metabolism of glucosinolates by activating calcium signalling in broccoli sprouts. On the one hand, CHEW treatment reduced the contents of ROS and MDA in broccoli sprouts during growth via calcium signalling (affecting the relative expression of *RBOHD*), thereby alleviating the oxidative stress and broccoli sprouts could grow better. On the second day, compared to tap water, the contents of H_2O_2, O_2^- and MDA in broccoli sprouts treated with CHEW were reduced by 8.8, 25.7 and 48.8%, respectively, which alleviated the membrane lipid peroxidation caused by tap water. On the other hand, calcium signals transmit information to the nuclear factor IQD1 via calcium sensors (CaM, CML, and CDPK). Ultimately, IQD1 played an important role in regulating glucosinolate metabolism in broccoli sprouts during growth. On the 4th day, CHEW treatment improved the glucoraphanin content in broccoli sprouts by 17.32% compared to tap water by promoting the relative expression of glucosinolate biosynthesis-related genes. On the 8th day, compared to tap water, the contents of isothiocyanates (sulforaphane, erucin, and 4-isothiocyanato-1-butene) in broccoli sprouts under CHEW treatment were significantly increased by 45.93, 34.04 and 185.15%, respectively. This result indicated that CHEW efficiently enhanced glucosinolate and isothiocyanate formation in broccoli sprouts. In addition, this study elucidated that calcium signalling is the molecular mechanism by which CHEW regulates glucosinolate metabolism in broccoli sprouts during growth, which provides a theoretical basis for improving the nutritional quality of plant-derived foods and the molecular breeding of broccoli.

Materials and methods

Preparation of CHEW and broccoli sprout cultivation

As shown in Fig. 1, CHEW was produced by electrolyzing a $CaCl_2$–HCl solution (5 mM $CaCl_2$, adjusted to pH 4.40 ± 0.05 with concentrated hydrochloric acid). The

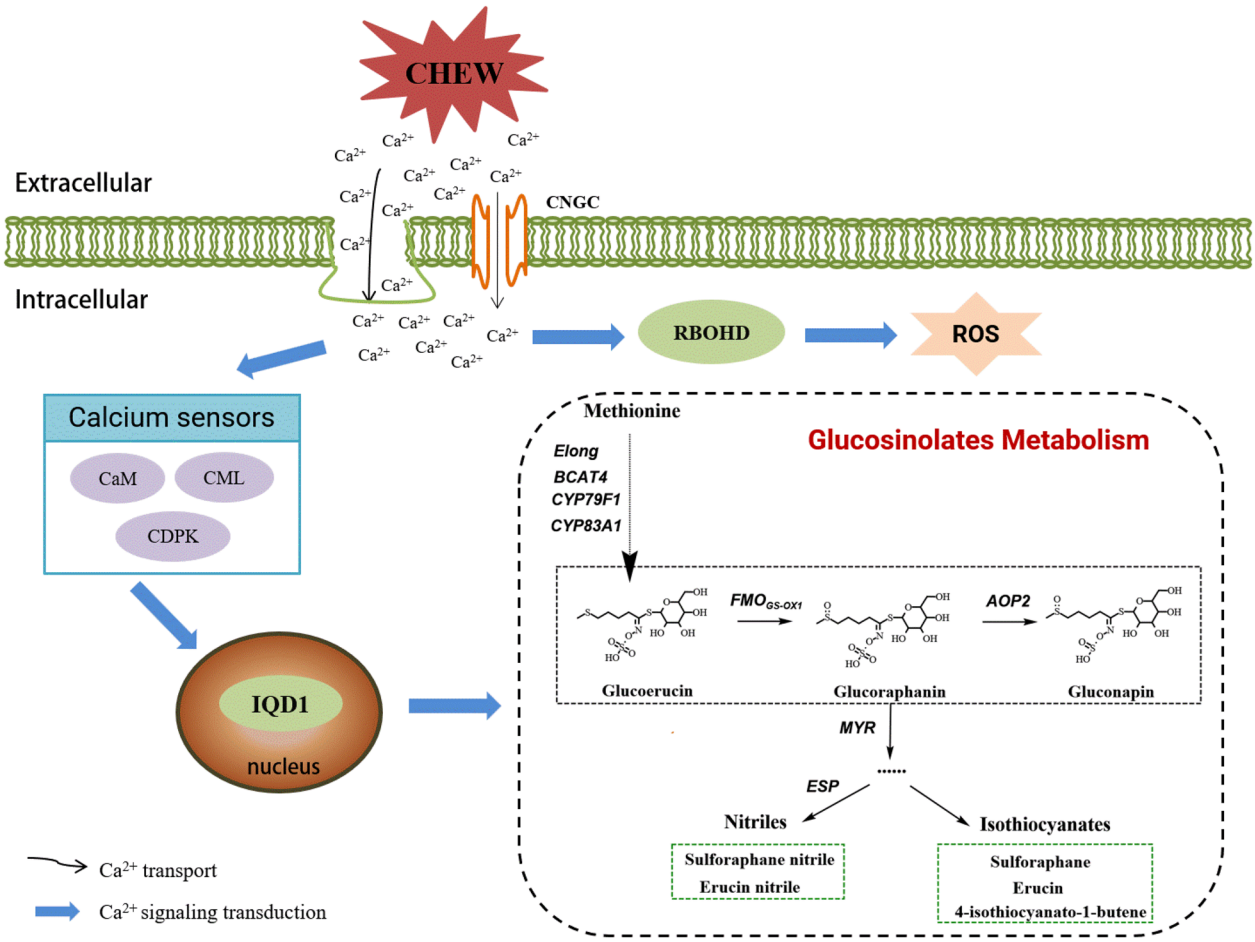


Fig. 6 Schematic overview of the glucosinolates metabolism in broccoli sprouts

final pH and available chlorine concentration (ACC) of CHEW were 5.5 and 10 mg/L, respectively. The main substances in CHEW include Ca^{2+}, HClO, ClO^{-}, Cl^{-}, H_2O_2 and ·OH.

Zhongqing 12 broccoli seeds were soaked in tap water (control) and CHEW (m:v = 1:5) for 3 h. Then, they were evenly distributed in a polypropylene box covered with sterile cheesecloth and placed in an incubator (PRx-450C, Saifu Co., Ltd, China) at 25 °C and 80% relative humidity and received 24 h of light. Broccoli sprouts were carefully taken on the 2nd, 4th, 6th and 8th days of growth. A portion of the broccoli sprouts was freeze-dried and then preserved at − 20 °C.

Determination of ROS and MDA contents

Reactive oxygen species (ROS) in plants mainly include H_2O_2 and O_2^{-}. H_2O_2 and MDA contents were determined by kits (A064, Jiancheng, Nanjing, China and BC0025, Solarbio, Beijing, China, respectively) according to methods described previously [21].

The O_2^{-} content was determined by a kit (BC1290, Solarbio, Beijing, China). One millilitre of extraction solution was used to homogenize 100 mg of fresh broccoli sprouts. The mixture was centrifuged, and then the supernatant was mixed with the reaction solution according to the manual. Finally, the absorbance of the solution was measured at a wavelength of 530 nm.

Calcium content and location assessments

The calcium content was determined according to a previous method [21].

The distribution of calcium in the leaves of broccoli sprouts was assessed using a transmission electron microscope following the method described by Tan et al. [36] with minor modifications. Broccoli sprout leaves were cut into slices of approximately 3 × 3 mm and immediately immersed in a fixative (0.1 M pH 7.6 potassium phosphate buffer, 3% glutaraldehyde and 2% potassium pyroantimonate). A vacuum pump was used to remove the air, and then the samples were fixed at 25 °C for 24 h.

The fixed samples were then rinsed with fixative, dehydrated in a series of ethanol solutions, underwent solvent replacement with anhydrous acetone, embedded and sectioned with an ultramicrotome (LEICA EM UC6, LEICA, Germany). After negative staining with 1% uranyl acetate, the samples were observed using a transmission electron microscope (H-7500, HITACHI, Japan).

Analysis of glucosinolate contents

The glucosinolate contents in broccoli sprouts were determined according to previous studies [37]. Five millilitres of 100% methanol were used to homogenize 200 mg solution broccoli sprout dry powder, and the mixture was extracted three times (65 °C, 10 min). The resulting mixture was centrifuged (3000 rpm, 10 min), and then the glucosinolates were purified by a DEAE Sephadex A-25 column. An HPLC system with a UV detector set to a detection wavelength of 229 nm was used to analyse the glucosinolates. The chromatogram and glucosinolate species are shown in Additional file 1: Fig. S1.

Glucosinolate hydrolysate assay

The composition and contents of glucosinolate hydrolysates were determined according to previous studies [20]. Two hundred milligrams of broccoli sprout dry powder was hydrolysed with 0.8 mL of distilled water for 8 h. Afterwards, 1.75 g of terminator (sodium chloride:sodium sulfate = 4:3) and 4 mL of methylene chloride were added. The mixture was extracted three times (25 °C, 30 min) and then centrifuged (10,000×*g*, 15 min). The dichloromethane layer of the mixture was then filtered and collected. The composition and contents of isothiocyanates (ITCs) and nitriles were detected by GC–MS (QP2010 ultra, Shimadzu, Kyoto, Japan) with an HP 5-ms column (30 m × 0.25 mm × 0.25 μm, Agilent). The TIC and specific information on the glucosinolate hydrolysates are shown in Fig. 3a and Additional file 1: Table S1, respectively.

Activities of myrosinase and epithiospecifier protein (ESP)

The method reported by Li et al. [16] was used to evaluate the activities of myrosinase and ESP. Six millilitres of sodium phosphate buffer (0.1 M, pH 6.5) was used to homogenize 1 g of fresh broccoli sprouts, which were then centrifuged (10,000×*g*, 15 min). The supernatant was collected for follow-up experiments. The total protein content in the supernatant was determined according to the Bradford method [38]. The absorbance of glucose was measured at a wavelength of 505 nm using a kit (BC2500, Solarbio, Beijing, China). One myrosinase unit corresponded to 1 nmol of glucose formed per minute.

ESP activity assays were conducted with 200 μL of the above supernatant and 800 μL of 0.5 mg/mL glucoraphanin (25 °C, 1 h). Afterwards, 1.75 g of terminator (sodium chloride: sodium sulfate = 4:3) and 4 mL of methylene chloride were added. The sulforaphane nitrile content in the mixture was determined according to the previous extraction and detection methods. One ESP unit corresponded to 1 nmol of sulforaphane nitrile formed per minute.

Quantitative real-time PCR (qRT–PCR) analysis

Total RNA of broccoli sprouts was extracted by a kit (R1200, Solarbio). Then, cDNA was synthesized with a kit (RP1105, Solarbio) in a thermocycler (Bio-Rad Laboratories, CA, USA). Finally, PCR was initiated with TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) (RR820A, Takara) in a CFX Connect Real-Time System according to the following program: 95 °C/60 s, followed by 45 cycles of 95 °C/30 s, 60 °C/30 s and 72 °C/60 s, after which the gene fragments were completely melted at the appropriate temperature. The primers used are listed in Additional file 1: Table S2, and *Actin* was used as an internal reference gene. Relative expression levels were calculated with the $2^{-\triangle\triangle CT}$ method.

Statistical analysis

Assays were conducted in triplicate, and the results are expressed as the means ± standard errors. The data were analysed by GraphPad Prism version 9 (GraphPad Software, Inc., USA) and IBM SPSS Statistics 19 (SPSS Inc., USA). One-way ANOVA and Duncan's multiple range test were used to evaluate the statistical significance of differences ($P < 0.05$).

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40538-023-00416-5.

Additional file 1: Figure S1. HPLC analysis results of the glucosinolates in broccoli sprouts: the species of glucosinolates (A); glucosinolates chemical name (B). **Table S1.** GC–MS analysis result of the glucosinolate' hydrolyzates. **Table S2.** Primer sequences used for qRT-PCR.

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Author contributions

CL participated in validation, data curation, writing—original draft, writing—review and editing. SS performed the glucosinolate content assay. YH performed the experimental work of broccoli sprout cultivation. SH performed the preparation of $CaCl_2$–HCl electrolyzed water. HL participated in writing-review, supervision and funding acquisition. All authors read and approved the final manuscript.

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Availability of data and materials
The authors declare that the data and materials supporting the findings of this study are available within the article.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
All authors listed have read the complete manuscript and have approved submission of the paper.

Competing interests
All authors declare no financial or nonfinancial competing interests.

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