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Geraniol treatment enhances sulforaphane content through modulation of the glucosinolate biosynthetic pathway in kale (*Brassica oleracea*)

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Abstract

Kale (*Brassica oleracea*) is a cruciferous vegetable known for health benefits, primarily due to sulforaphane, a compound with notable anticancer properties. Recently, we reported that the application of geraniol can significantly increase sulforaphane content in kale. However, the molecular mechanisms underlying this enhancement remain unexplored. In this study, we aimed to elucidate the metabolic pathways by which geraniol influences sulforaphane biosynthesis. Geraniol was applied as a 500-ppm solution, sprayed on the leaves of kale cultivated in an indoor aeroponic farm, once daily for two consecutive days, one week prior to harvest. As a result, we found that the sulforaphane content in the geraniol-treated group was 2.2 times higher than in the control group. Moreover, gene expression analysis demonstrated significant upregulation of *CYP83*, *SUR1*, and *UGT*, which are key genes involved in sulforaphane biosynthesis, with increases of 1.7, 1.3, and 1.1-fold, respectively, in the geraniol-treated group compared to the control group. These findings suggest that geraniol enhances sulforaphane content by upregulating genes critical to its biosynthesis.

Keywords Sulforaphane, Kale, Aeroponics, CYP83, Geraniol

Introduction

Cruciferous vegetables, such as kale (*Brassica oleracea*), are integral components of a healthy diet and have been extensively studied for rich nutritional benefits. Kale produces a variety of bioactive compounds and secondary metabolites central to intrinsic defense systems [1]. Among these, the glucosinolate family is particularly significant, undergoing hydrolysis to form potent bioactive compounds such as isothiocyanates [2]. Sulforaphane, a prominent isothiocyanate derived from the precursor glucosinolate, is known for significant antioxidant, anti-inflammatory, and anticancer properties [3].

For instance, sulforaphane modulates detoxification pathways by inducing enzymes that eliminate xenobiotics and carcinogens and by promoting the accumulation of

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glutathione, a molecule crucial for antioxidation, immune response, and cancer prevention [4]. At the cellular level, sulforaphane activates the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway, reducing oxidative stress and inflammation [5]. Sulforaphane also induces apoptosis and cell cycle arrest in cancer cells, thereby preventing the proliferation of damaged or mutated cells [6]. Furthermore, sulforaphane impacts epigenetic modifications, acting as a histone deacetylase inhibitor and promoting demethylation, which can reverse abnormal gene transcription associated with various cancers [7]. In addition to these effects, sulforaphane exhibits therapeutic potential in managing diabetes, enhancing cardiovascular health by regulating blood pressure and cholesterol levels, and providing neuroprotective benefits [8, 9]. These diverse biological activities highlight the importance of understanding and enhancing sulforaphane content in kale.

Meanwhile, efforts to enhance the production of functional bioactive compounds in kale have increasingly focused on metabolite farming [10]. Elicitation, a key strategy in this approach, involves the application of specific substances to stimulate secondary metabolite production [11]. Various elicitors, including methyl jasmonate, salicylic acid, sodium chloride, and methionine, have been shown to significantly increase sulforaphane content in cruciferous vegetables such as kale and broccoli [12–14]. Furthermore, geraniol, a volatile monoterpene, has emerged as a promising elicitor in plant systems [15]. Known for its role in plant defense signaling, geraniol can induce the production of various secondary metabolites, including terpenoids and phenolic compounds, by activating plant stress responses [16]. Its potential to modulate defense mechanisms aligns with the action of phytohormone elicitors known to upregulate glucosinolate production in *Brassicaceae*. Recently, we reported that geraniol treatment further enhances sulforaphane biosynthesis in kale, indicating its potential as an effective elicitor [17]. However, the molecular mechanisms by which geraniol enhances sulforaphane content in kale have not been previously explored. In this study, in order to elucidate these mechanisms in kale, we analyzed the gene expression in the biosynthetic pathway of sulforaphane precursors.

Materials and methods

Kale cultivation and growth conditions

The nutrient composition and cultivation conditions followed those previously described in our previous report [17]. Briefly, kale seedlings were initially germinated on a water-soaked sponge at 23 °C and 80% humidity without light for 2–3 days. Following germination, seedlings were exposed to LED light until transplantation, which occurred approximately four weeks after sowing when

roots were sufficiently developed. During the initial week post-planting, a nutrient solution was supplied with an electrical conductivity (EC) of 0.5 mS/cm and a pH of 6.0. The cultivation condition was maintained at 23 °C with 70% humidity, and a 16-h light/8-h dark cycle was implemented using LED lights at 290 $\mu\text{mol}/\text{m}^2/\text{s}$ as photosynthetic photon flux density. The EC was gradually adjusted to 0.7 mS/cm in the second week, 1.0 mS/cm in the third week, and 1.5 mS/cm in the fourth week post-planting. The plants were cultivated using an aeroponics system (Insungtech, Korea) in a culture room (Woori Technology Inc., Korea).

Geraniol treatment and sulforaphane quantification

Geraniol (food grade; Dotter Co., Korea) was applied to the kale leaves as a solution of 500 ppm, prepared by diluting with 10% ethanol in water, after 60 days of growth [17]. The leaves were treated once each day over a period of two consecutive days, and harvested for analysis seven days after the initial treatment. Sulforaphane content was quantified using LC–MS/MS at the Green Bio Research Facility Center (Pyeongchang, Korea), and presented as mean with standard deviation. Statistical analysis was performed using the Student's *t*-test to determine significant differences between groups.

Gene expression analysis by qRT-PCR

To analyze mRNA expression levels associated with sulforaphane precursor biosynthesis in kale, total RNA was extracted from kale samples using the IQeasy™ plus Plant RNA Extraction Kit (iNtRON Biotechnology, Korea) [18]. The concentration and purity of the extracted RNA were assessed with a BioPhotometer D30 (Eppendorf, Germany), ensuring that only high-quality RNA samples were selected for further analysis. Complementary DNA was synthesized from the extracted RNA using the AccuPower® RT PreMix & Master Mix (Bioneer, Korea) according to the manufacturer's instructions. Quantitative real-time polymerase chain reaction (qRT-PCR) was then performed on a QuantStudio 1 instrument (Applied Biosystems, USA) using SYBR green qPCR premix (Prime Q-Mastermix, Daejeon, Korea) to quantify RNA expression levels [19–21]. GAPDH was employed as the reference gene to normalize gene expression levels in the kale samples. Gene expression levels were analyzed following the same statistical methods used for sulforaphane content analysis, using the Students *t*-test. The specific primers used for the qRT-PCR analysis are listed in Table 1.

Results and discussion

Enhanced sulforaphane content with geraniol treatment

In this work, we sought to confirm whether geraniol treatment increases sulforaphane content in kale before

Table 1 Primers sequences used for qRT-PCR analysis

Gene	Direction	Primer sequences (5 → 3)
GAPDH	Forward	GAT TCC CTT TAG CTT GCC TTC TGA
	Reverse	ACG GAA AGT TGA CCG GAA TG
UGT	Forward	GCT CGA AGT CGC GAG ATC TAA
	Reverse	GGC AAG CCA CGG ACG AGA TA
CYP79	Forward	TGG CCC ATC ATC GGA ATG
	Reverse	TGC TAT CTT AGG GCA TGT GAC GG
CYP83	Forward	TGG TTA ACC TAT TCA GCC CAA ATC
	Reverse	CAC AGT TGG TGA ATG ACA AGA GAA GC
SUR1	Forward	CCC CTC GGC CAT GGA
	Reverse	ATC GGC GAC GGC TCT TCT G

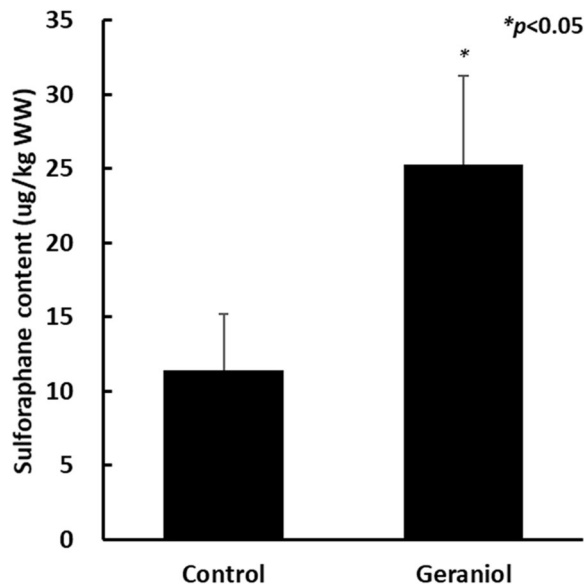


Fig. 1 Sulforaphane content in control and geraniol-treated kale

analyzing gene expression in the biosynthetic pathway of sulforaphane precursors. Sulforaphane content in harvested kale leaves showed 25.25 µg/kg in the geraniol-treated group, compared to 11.41 µg/kg in the control, indicating a 2.2-fold increase (Fig. 1). This significant difference is consistent with our previous findings and demonstrates the reproducibility of geraniol treatment in enhancing sulforaphane content in kale.

Transcriptional upregulation of key genes in the sulforaphane biosynthetic pathway

To understand the increase in sulforaphane content observed in the geraniol-treated group, we analyzed the expression of four critical genes involved in sulforaphane biosynthesis: *CYP79*, *CYP83*, *SUR1*, and *UGT* (Fig. 2). The expression of *CYP83*, *SUR1*, and *UGT* genes was significantly upregulated in the geraniol-treated group (Fig. 2). Specifically, the expression of *CYP83* increased by 1.66-fold, *SUR1* by 1.3-fold, and *UGT* by 1.1-fold compared to the control group (Fig. 2A). These results suggest that geraniol treatment enhances sulforaphane

biosynthesis by upregulating these critical genes within the biosynthetic pathway.

Metabolic insights into geraniol-induced sulforaphane enhancement in kale

The biosynthesis of sulforaphane is a complex, multi-step metabolic pathway that begins with the amino acid precursor methionine (Fig. 2B). This pathway can be divided into three primary stages: side chain elongation (yellow), core structure formation (green), and sulforaphane formation (red; Fig. 2B). In the initial phase, side chain elongation, methionine undergoes a series of reactions to form dihomomethionine, with methylthioalkylmalate synthases catalyzing the addition of methylene groups to extend the methionine side chain (Fig. 2B). Following elongation, the core structure formation involves the conversion of intermediates into glucosinolates, such as 4-methylthiobutyl-desulfoglucosinolate and its sulfated form, 4-methylthiobutyl glucosinolate (Fig. 2B). This stage is catalyzed by a series of enzymes encoded by the *CYP79*, *CYP83*, *SUR1*, and *UGT* genes, which play crucial roles in transforming dihomomethionine into the glucosinolate structure (Fig. 2B). The final stage involves the conversion of glucosinolates into sulforaphane, a bioactive compound, through cascade reactions by enzymes such as myrosinase. In this study, we demonstrated that geraniol treatment led to a 2.2-fold increase in sulforaphane content in kale, resulting from significant upregulation of critical genes involved in core structure formation (Figs. 1 and 2). Specifically, the expression of *CYP83* increased by 1.66-fold, *SUR1* by 1.3-fold, and *UGT* by 1.1-fold compared to the control group. These findings provide a molecular basis for the observed enhancement in sulforaphane levels.

The observed upregulation of *CYP83*, *SUR1*, and *UGT* in our study is partially consistent with previous findings regarding elicitor-mediated sulforaphane (or glucosinolates) enhancement in cruciferous vegetables [12, 13, 22]. While specific elicitors and their mechanisms can vary, there is a consistency in the increase of *SUR1* and *UGT* genes induced by methyl jasmonate, brassinolide, and geraniol, with the exception of NaCl (Table 2). However, the up-regulation of *CYP79* and *CYP83* genes exhibit different depending on the type of elicitors and the specific cruciferous species (Table 2). Notably, expression levels of *SUR1* and *UGT* were upregulated in both broccoli and Chinese kale, with *CYP79* and *CYP83* overexpressed, respectively; methyl jasmonate markedly induced these genes, correlating with increased sulforaphane accumulation [13, 22]. Brassinolide has also been shown to enhance glucosinolates accumulation, potentially by extending its impact to the upregulation of *CYP83*, *SUR1*, and *UGT* gene expression in Chinese kale [13]. Although in this study, we observed upregulation of

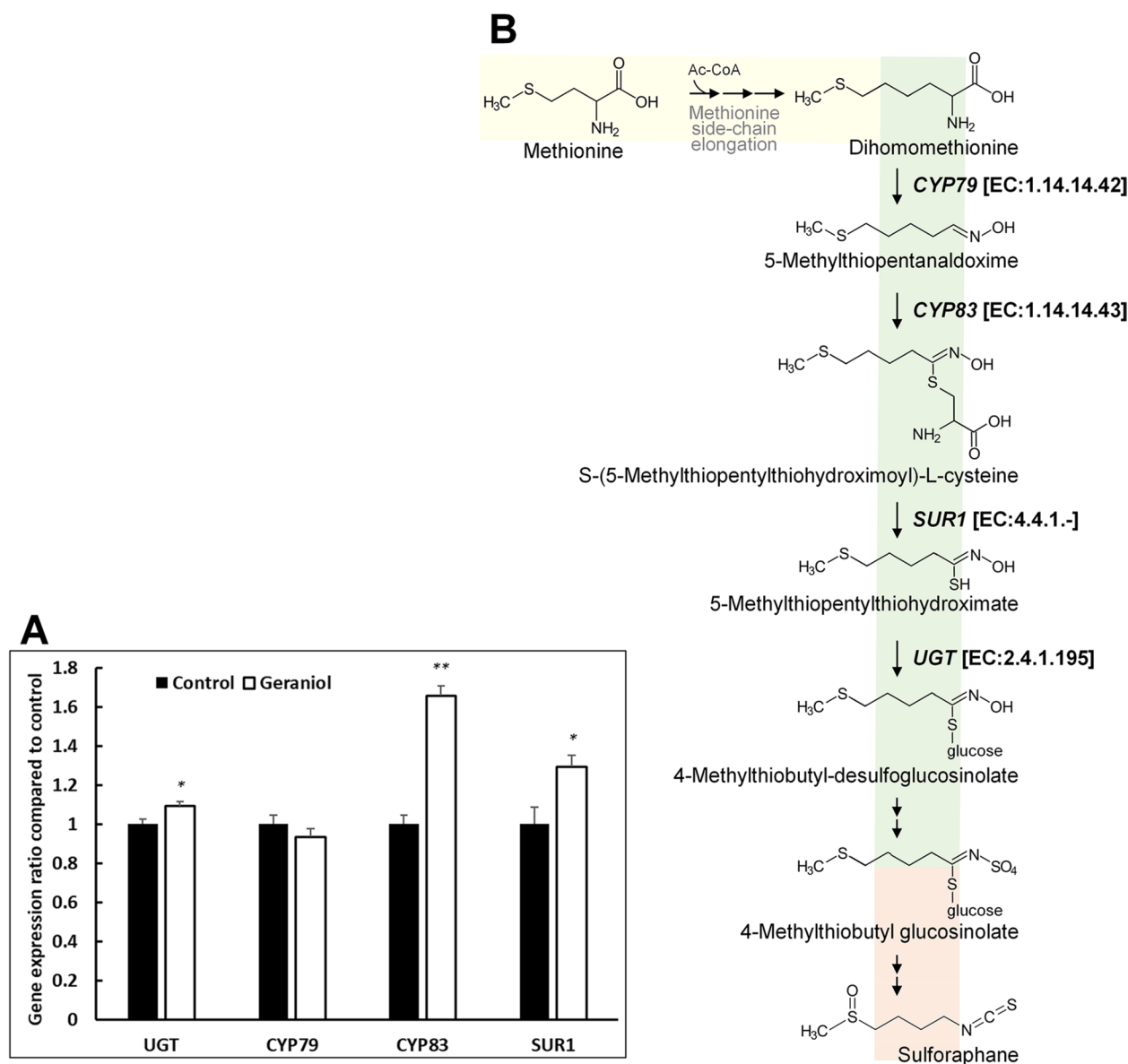


Fig. 2 Effects of geraniol treatment on the expression of genes involved in sulforaphane biosynthesis. **(A)** Expression ratio of *UGT*, *CYP79*, *CYP83*, and *SUR1* genes, normalized to the *GAPDH* gene. Black and white bars represent the control and geraniol-treated groups, respectively. Asterisks indicate statistically significant differences compared to the control (*, $p < 0.05$; **, $p < 0.01$). **(B)** Schematic representation of the sulforaphane biosynthetic pathway from methionine. Yellow, green, and red shading indicate the side chain elongation, core structure formation, and sulforaphane formation stages, respectively. Gene abbreviations: *CYP79* (homomethionine N-monooxygenase, EC:1.14.14.42), *CYP83* (methylthioalkanaldoxime N-monooxygenase, EC:1.14.14.43), *SUR1* (S-alkyl-thiohydroximate lyase EC:4.4.1.-), and *UGT* (N-hydroxythioamide S-beta-glucosyltransferase, EC:2.4.1.195). The sulforaphane formation stage involves enzymes such as myrosinase

genes critical to sulforaphane biosynthesis in *B. oleracea*, further research is needed to elucidate the signal transduction pathways between geraniol and these genes.

In conclusion, our findings demonstrate that geraniol treatment significantly enhances sulforaphane content in kale by upregulating key genes, including *CYP83*, *SUR1*, and *UGT*, within the glucosinolate biosynthetic pathway. These results provide valuable insights into the molecular mechanisms underlying geraniols elicitation effect and support its potential application as a natural method for

increasing the nutritional value of kale and other cruciferous vegetables.

Table 2 Elicitor-specific effects on gene expression and metabolite profiles in the sulforaphane biosynthetic pathway^a

Elicitor	Gene expressions		Metabolites		Cruciferous vegetables	References
	Core structure formation pathways	Sulforaphane formation pathways	Glucosinolates	Sulforaphane		
NaCl	CYP79↓, CYP83↓	Myrosinase↑	Decrease	Increase	Broccoli	[12]
NaCl	CYP83b1↓, SUR1↓, UGT74b1↓	N/A	Decrease	Decrease	Chinese kale	[13]
Methyl jasmonate	CYP79b2↑, UGT74b1↑, SUR1↑	TGG↑	Increase	N/A	Broccoli	[22]
Methyl jasmonate	CYP83b1↑, SUR1↑, UGT74b1↑	N/A	Increase	Increase	Chinese kale	[13]
Brassinolide	CYP83b1↑, SUR1↑, UGT74b1↑	N/A	Increase	No change	Chinese kale	[13]
Geraniol	CYP83↑, SUR1↑, UGT↑	N/A	N/A	Increase	Kale	This study

^a N/A indicates ‘not available’ or ‘not determined’, or ‘unclear’

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Author contributions

JMJ: Investigation, Data analysis, Writing – original draft. HL: Investigation, Data analysis. YSJ: Conceptualization, Supervision, Data analysis, Writing – review & editing. JB: Conceptualization, Supervision, Data analysis, Writing – review & editing. YSJ is an associate editor of Applied Biological Chemistry. Associate editor status has no bearing on editorial consideration.

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Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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