A DISSERTATION FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Improvement of secondary metabolite production with anti-inflammatory activity via adventitious root culture of *Aloe vera*

By

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FEBRUARY, 2014

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UNDER THE DIRECTION OF DR. TAE-JIN YANG
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF SEOUL NATIONAL UNIVERSITY

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APPROVED AS A QUALIFIED DISSERTATION OF YUN SUN LEE

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GENERAL ABSTRACT

Aloe vera (Asphodeaceae) is a medicinal plant in which useful secondary metabolites are plentiful. Among the representative secondary metabolites of *Aloe vera* are the anthraquinones including aloe emodin and chrysophanol, which are tricyclic aromatic quinones synthesized via a plant-specific type III polyketide biosynthesis pathway. However, it is not yet clear which cellular responses can induce the pathway, leading to production of tricyclic aromatic quinones. In this study, I established optimized culture condition for *Aloe vera* adventitious roots culture. Then I examined the effect of endogenous elicitors on the type III polyketide biosynthesis pathway and identified the metabolic changes induced in elicitor-treated *Aloe vera* adventitious roots. The examination on culture condition revealed

that MS solid media supplemented with 0.5 mg/L NAA was best condition to induce adventitious roots from leaf explants of *Aloe vera*. Optimal suspension culture condition for mass production of adventitious roots was observed in MS media with 0.3 mg/L IBA. Then, salicylic acid (SA), methyl jasmonate (MJ), and ethephon was treated to the adventitious roots as elicitors is capable of activating type III polyketide biosynthesis pathway. SA elicitation resulted in 10-11 and 5-13 folds increase in production of aloe emodin and chrysophanol compared to untreated control, respectively, suggesting that SA might activate the biosynthetic pathway for tricyclic aromatic quinones including aloe emodin and chrysophanol. Ultra-performance liquid chromatography-electrospray ionization mass spectrometry analysis identified a total of 37 SA-induced compounds, including aloe emodin and chrysophanol, and 3 of the compounds were tentatively identified as tricyclic aromatic quinones. Transcript accumulation analysis of polyketide synthase genes and gas chromatography mass spectrometry showed that these secondary metabolic changes resulted from increased expression of octaketide synthase genes and decreases in malonyl-CoA, which is the precursor for the tricyclic aromatic quinone biosynthesis pathway. In addition, anti-inflammatory activity was enhanced in extracts of SA-treated adventitious roots. The results suggest that SA has an important role in activation of the plant specific-type III polyketide biosynthetic pathway, and therefore that the efficacy of *Aloe vera* as medicinal agent can be improved through SA treatment.

Key words: Salicylic acid; *Aloe vera* adventitious root; Metabolomics; Anti-inflammatory activity

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LIST OF ABBREVIATIONS

BA	6-Benzyl aminopurine
B5 media	Gamborg B5 medium
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
MS media	Murashige & Skoog medium
NAA	1-naphthalene acetic acid
HPLC	High performance liquid chromatography
PVP	Polyvinylphyrollidine
SH media	Schenk & Hildebrandt medium
2,4-D	2,4-Dichlorophenoxyacetic acid
PKS	Polyketide synthase
OKS	Octaketide synthase
HPLC-ESI-MS	High-performance liquid chromatography-electrospray ionization-mass spectrometry
LC-ESI-MS/MS	Liquid chromatography-electrospray ionization- tandem mass spectrometry
GC-IT-MS	Gas chromatography-ion trap-mass spectrometry
UPLC-QTOF-MS	Ultra-performance liquid chromatography-quadruple time of flight-mass spectrometry
SA	Salicylic acid
MJ	Methyl jasmonate
Ethephon	2-Chloroethylphosphonic acid

DW	Dry weight
PTFE	Polytetrafluoroethylene
MSTFA	N-methyl-N-(trimethylsilyl) trifluoroacetamide
TMCS	Trimethylchlorosilane
RT	Retention time
PCA	Principal component analysis
PLS-DA	Partial least square discriminant analysis
OPLS-DA	Orthogonal partial least squares-discriminant analysis
VIP	Variable importance in the project
HSD	Honestly Significant Difference
NIST	National Institute of Standards and Technology
RT-qPCR	Reverse transcription quantitative PCR
MEM	Minimal essential medium
FBS	Fetal bovine serum
TCA	Tricarboxylic acid cycle
ORF	Open reading frame
CHS	Chalcone synthase
ANOVA	Analysis of variance
G6P	Glucose-6-phosphate
F6P	Fructose 6-phosphate
PEP	Phosphoenolpyruvate

GENERAL INTRODUCTION

Aloe vera, belongs to Asphodeloideae family within Asparagale, is a perennial succulent plant. The extracts obtained from pulpy leaves of *A. vera* has been widely used as a folk medicine for wounding healing, burn treatment, laxative, and cosmetic material. Recently, several useful metabolites contained in *Aloe vera* is isolated, and their biological effects are evaluated. Aloe emodin and chrysophanol, anthraquinone derivatives, are representative compounds in *Aloe vera*. Several reports have revealed that those compounds possess anti-bacterial, genetic toxicity, anti-inflammation, and anti-cancer as regards chemopreventive agents. However, the studies on secondary metabolites are still limited in *Aloe vera*, because heavy browning makes it difficult suspension culture required for mass production.

Plant cell tissue and organ culture is alternative approach not only to obtain useful metabolites but also to elucidate metabolite biosynthesis mechanism. Based on the totipotential of plants which is ability of single cell to express the full genome by cell division, diverse tissue culture technique is developed e.g. organ culture, embryo culture, genetic transformation, protoplast fusion, haploid production. Among the organ culture, micropropagation is useful tool to conserve excellent genotypes, and adventitious roots culture is applicable to obtain useful secondary metabolites. *Agrobacterium*-mediated genetic transformation technique is

most widely used to understand gene function.

Aim of this study was development of optimized tissue culture for *Aloe vera in vitro*. Moreover, I tried to understand regulation mechanism of anthraquinone biosynthesis pathway, and verify potential as chemopreventive agents of *Aloe vera.*

LITERATURAL REVIEWS

Plant molecular farming

Plant molecular farming means production of pharmacologically valuable proteins or secondary metabolites in plant (Obembe *et al.,* 2011). Mainly, mammalian cell or microbial fermentation have been used to produce biopharmaceutical products, however, these technologies have a couple of disadvantages such as cost or safety (Fischer *et al.,* 2004). As alternative to overcome these problems, production of recombinant protein or secondary metabolites using plant is emerging (Fischer *et al*., 2004). In 1997, commercial production of recombinant protein, avidin, from transgenic maize was succeeded (Hood *et al.,* 1997). Commercial scale production of secondary metabolites was achieved in *Panax gingseng* (Hibino and Ushiyama 1999), *Catharanthus roseus* (Zhao *et al.,* 2001), and *Taxus brevifolia* (Yukimune *et al.,* 1996; Luo *et al.,* 2001).

Plant-based system has several benefits compared with mammalian or microbial cell culture system. First, plant cell is more economical than widely used industrial fermentation. Second, purification step can be eliminated when plant cells with useful metabolites or protein are used as food. Third, contamination of human pathogen or toxin is able to minimize (Daniell *et al.,* 2001).

With several benefits, plant-based system still has limitation factors such

as low yield or protein instability, non-mammalian glycosylation, leading to getting low awareness (Obembe *et al*., 2011). If current technical drawbacks are to be conquered and commercial attentions are to be received, plant-derived drugs could be achieved.

Promotion of secondary metabolite production through elicitation

Plant cell culture system has been superb alternative to produce secondary metabolite. However, accumulated secondary metabolites are low in normal condition, because secondary metabolites play a role to protect plant from various environmental stresses. Many abiotic and biotic elicitors have been used as one of the strategies for inducing or enhancing the synthesis of secondary metabolites through to stimulate cellular stress response in plant (DiCosmo and Misawa 1985; Ramachandra Rao and Ravishankar 2002). The number of elicitors has been identified, including the plant-originated signal molecules such as salicylic acid (SA), methyl jasmonate (MJ), and ethylene as well as microbe-derived molecules such as polysaccharides, glycoproteins, and inactivated enzymes (Benhamou 1996; Bennett and Wallsgrove 1994).

SA and MJ, which is plant-originated signal molecules, have been known to representative compounds involved in plant defense mechanism. Previous results reported that SA triggered plant systematic acquired resistance mechanism (Zhao *et al*., 2005). MJ is a representative signal messenger which is involved in wounding response caused by insect infection and environmental stress, flowering and senescence (Cheong and Choi 2003; Ali *et al.,* 2006). Recent studies, exogenously treated SA and MJ provoked production of intracellular signal molecules, resulting to perception of several binding sites located in plasma membrane or cytoplasm and activation of receptors for effectors such as G protein, protein kinase, ion channels.

Then these cascades triggered the release of the messenger molecules such as NO, H_2O_2, O_2^-, and intracellular calcium etc., and eventually promoted the expression of defense-related genes including genes involved in secondary metabolites biosynthesis pathway (Ali *et al*., 2006; Zhao *et al*., 2005).

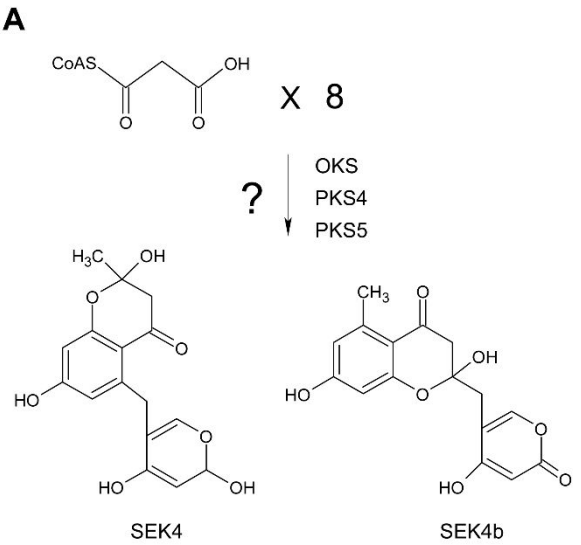
By supplying SA, accumulation effects of useful secondary metabolites were observed in various cultured plant cell: soluble phenolic compounds in *Matricaria chamomilla* plants and *Salvia miltiorrhiza* cell culture (Dong *et al*., 2010; Kováčik *et al*., 2009), podophyllotoxin in cell cultures of *Linum albums* (Yousefzadi *et al*., 2010), artemisinin in *Artemisia annua L* (Pu *et al*., 2009) and hypericins in shoot culture of *Hypericum* spp. (Coste *et al*., 2011). Exogenous treatment of MJ had an effect on accumulation of wide range of secondary metabolite such as ginsenosides in *Panax ginseng* (Yu *et al*., 2002), paclitaxel and baccatin III in *Taxus spp*.(Yukimune *et al*., 1996), and flavonoid in *Rubus spp*. (Wang *et al*., 2008).

Tricyclic aromatic quinone biosynthesis pathway

Anthraquinones are natural products which are detected in bacterial, fungi, and higher plants (Han *et al*., 2001). In plants, it have been reported that anthraquinone derivative compounds were detected from Rubiacaceae, Rhamnaceae, Polygonaceae, Leguminosae, Rhamnaceae, Polygonaceae, and Asphodeloideae family. Two major biosynthetic pathways for anthraquinone suggested that one is type III plant specific polyketide biosynthesis pathway found in Leguminosae, Rhamnaceae, Polygonaceae, and Asphodeloideae family and the other is chorismate/o-succinylbenzoic acid pathway found in Rubiacaceae, Rhamnaceae, and Polygonaceae family (Han *et al*., 2001). In this review, type III plant-specific polyketide biosynthesis pathway of Aloe species within Asphodeloidea are dealt with.

The type III polyketide biosynthesis pathway of aloe species is initiated through the condensation of malonyl-CoA, catalyzed by type III polyketide synthases (PKS) of chalcone synthase superfamily. Based on the type of starter molecules, the number of the malonyl-CoA condensations, and the mechanisms of cyclization reactions, a number of functionally divergent plant type III PKSs were characterized, including stilbene synthase, acridone synthase, benzalacetone synthase, 2-pyrone synthase, and aloesone synthase. Recently, novel plant-specific type III polyketide synthase (PKS), octaketide synthase (OKS), PKS4, and PKS5 were isolated from *Aloe arborescens*, and those possible functions were examined

in *Escherichia coli* (Abe *et al*., 2005; Mizuuchi *et al*., 2009). In the study the researchers speculated that the type III PKSs belonging to the chalcone synthase superfamily would catalyze the condensation of eight molecules of malonyl-CoA, resulting in yield of tricyclic aromatic quinones.



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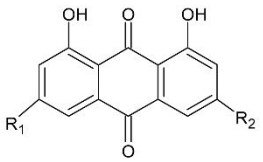

	R1	R2
Aloe emodin	H	CH_2OH
Chrysophanol	H	CH_3
Rhein	H	COOH
Emodin	OH	CH_3

Figure 1. Plant specific type III polyketide biosynthesis pathway

Indeed *E. coli* which were expressed the enzymes produced octaketides of SEK and SEK4b from eight malonyl-CoAs, but SEK and SEK4b were the shunt products of type II PKS that have not been detected in plant (Figure 1). It is suggests that the enzymes might be potentially associated with biosynthesis of natural tricyclic aromatic quinones in aloe. But it unclearly remains that the enzymes can produce the end products such as aloe emodin and chrysophanol actually *in vivo*.

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CHAPTER I

Induction and proliferation of adventitious roots from *Aloe vera* leaf tissues for *in vitro* production of aloe emodin

ABSTACT

I have inspected aloe-emodin and aloin compounds in different tissues of *Aloe vera* which were grown in Aloe farm for three years. Surprisingly, aloe emodin contents were much richer in the roots (574.8 ± 92.4 µg/dry weight) than in leaves (5.52 ± 0.32 µg/dry weight) that encouraged us to establish adventitious root culture system of the plant. The optimal condition for induction and proliferation of adventitious roots using young leaves was established by treatments of variety of conditional media and auxin supplements. Adventitious root induction was suitable by enrichment of 0.5 mg/L 1-naphthalene acetic acid (NAA) and 0.2 mg/L 6-benzylaminopurine (BA) in Murashige & Skoog (MS) medium. However, root proliferation was hindered by accumulation of phenolic compounds in the media that was overcome by pre-washing of the adventitious roots with more than 4 g/L of polyvinylphyrollidine (PVP) analogs increasing the survival rate (up to 60%). Inspection of aloe-emodin contents in various adventitious roots grown different basal medium revealed that aloe-emodin accumulation is much higher on B5 medium (133.08 ± 0.12 µg/dry weight) than on MS medium (3.56 ± 0.26 µg/dry weight).

INTRODUCTION

Aloe belongs to the Asphodeaceae family and is a perennial tropical plant originated from Africa. The genus Aloe includes approximately 500 species. Among them, only five species, *Aloe vera*, *Aloe arborescens*, *Aloe perryi*, *Aloe ferox,* and *Aloe saponaria* are mainly used for medicinal purposes. Among them, *A. vera* is the most popular for commercial and therapeutic purposes (Park and Lee, 2006).

The representative compound in *Aloe vera* is an anthraquinone, aloe emodin and chrysophanol, synthesized via the polyketide pathway (Han *et al.,* 2001). Aloe emodin and chrysophanol has been shown to exhibit efficacy for anti-inflammatory and genetic toxicity properties (Park and Lee, 2006). The leaves contain more than 98.5-99.5% water that indicated trace amounts of secondary metabolites residing in the leaf tissues, even after three years of cultivation for commercial harvest (Femenia *et al.,* 1999). Moreover, the medicinal composition and activity can be easily decreased by different environmental and physiological conditions, especially those that hinder the stable productions of certain compounds (Beppu *et al.,* 2004).

Plant cell cultures are an alternative way to obtain useful secondary metabolites and have been studied in many plant species. The differentiated organ culture, especially adventitious root cultures, have been applied in many medicinal plants due to its rapid growth and stable mass production ability of secondary

metabolites (Murthy *et al.,* 2008). Adventitious root systems have been established for production of anthraquinone in *Morinda citrifolia* (Baque *et al.,* 2010) and for an anthraquinone compound, lucidin-3-*O*-primeveroside, in *Rubia tinctorum* (Sato *et al.,* 1997).

Our inspection of aloe emodin and chrysophanol contents revealed that roots contain much higher amounts than leaves of *Aloe vera*. No aloe adventitious root culture system had yet been reported. Thus, we established an optimized condition for adventitious root induction and proliferation using young leaf of *Aloe vera*.

MATERIAL AND METHODS

Explants preparation and culture conditions

All the Aloe plants were kindly provided from Kim Jeong Moon Aloe Co. Ltd (Jeju, Korea). Young leaves of approximately 10-cm-tall shoots emerged from suckers of 3 to 5-year-old *Aloe vera* plants were used as explants for adventitious root induction. The leaves were surface-sterilized with 70% ethanol for 30 s, immersed in 4% sodium hypochlorite for 5 min and rinsed three times with sterile distilled water. The pH of the medium was adjusted to 5.8 before autoclaving at 121 ℃ for 15 min. Cultures were maintained at 28 ± 1 ℃ in the dark.

Adventitious roots induction

The leaf explants were cultured on full-strength MS medium (Murashige and Skoog, 1962) supplemented with various concentrations (0, 0.1, 0.5, 1, 2, 4, and 6 mg/L) of 1-naphthalene acetic acid (NAA), Indole-3 acetic acid (IAA), Indole-3-butyric acid (IBA), and 2,4-dichlorophenoxyacetic acid (2,4-D) in order to determine the optimal auxin and their concentration. Different concentrations (0.1, 0.2, and 0.3 mg/L) of 6-benzyl aminopurine (BAP) supplements were compared in combination with 0.5 mg/L NAA. Different kinds of media, such as SH (Schenk

and Hildebrandt, 1972), B5 (Gamborg *et al.,* 1968), 2 MS, MS, and 1/2 MS, were compared to identify the best conditions for growth and aloe emodin accumulation in adventitious roots. Growth patterns of each treatment were measured every week for six or seven weeks of culture duration. All experiments included eight replicates per treatment, and the experiment was repeated ten times.

Quantification of metabolites in adventitious roots

Contents of aloe emodin, aloin, and chrysophanol were verified based on t he method of Park *et al.,* (1998). Leaves and roots of three-year-old *Aloe vera* plants and seven-week-old adventitious roots induced from *Aloe vera* leaf were analyzed using high performance liquid chromatography (HPLC). Freeze-dried mat erial (0.5 g) was prepared in ethanol (10 mL) for 1 hr at 50 ℃ using a JAC ultrasonic 2010 sonicator (Jinwoo Engineering, Korea) and suspensions were centrifuged at 2330 x g for 15 min. The supernatants were filtered through 0.45 μm (Whatman) membrane filters.

Hewlett Packard Series 1100 HPLC and a Shiseido capcell pak C18 column (4.6 x 250 mm, 5 μm) were used for analysis. The separation was conducted using 25-30% methanol for 5 min, 30-35% methanol for 10 min, 35-70% methanol for 35 min and 70% methanol for 10 min at 0.7 mL/min. The peaks were monitored using a diode array detector (DAD) at 293 nm at room temperature. The data

represent the means of three replicates and all experiments were repeated twice.

Estimation of adventitious root growth

The adventitious roots were harvested every 7 d up to 42 d during growth. Fresh weight was determined to 0.05 g accuracy, and then dry weight (DW) was measured after lyophilization. The growth ratio was calculated as follows: DW of harvested roots - DW of inoculated adventitious roots (initial DW) / initial DW as previously reported (Jung *et al*., 2002)

Statistical analysis

Analysis of variance (ANOVA) was used to ascertain significant differences between treatments using the Statistical Analysis System (SAS 9.1). Multiple comparisons of treatment means were conducted using Duncan's test or the Least Significant Distribution (LSD) test at $p < 0.05$.

RESULTS

Quantification of aloe emodin and aloin in different aloe tissues and adventitious roots

I compared the aloe emodin and aloin contents in different tissues. Leaves and roots of three-year-old *Aloe vera* which were normally grown in Aloe farm for three years and adventitious roots initially induced by various combinations of hormones were analyzed in order to determine the potential of *in vitro* mass production. Interestingly, aloe emodin and aloin were found not only in leaves, but also in roots of three-year-old *Aloe vera* (Table 1-1). The aloin content was 1,499.1 ± 904.0 μg/DW in leaves. A small amount of aloin (3.9 ± 0.2 μg/DW) was detected in roots of three-year-old aloe and nothing was detected in the adventitious roots. Meanwhile, the content of aloe emodin was 574.8 ± 92.4 μg/DW in roots, remarkably higher than that of leaves (5.5 ± 0.3 μg/DW). Furthermore, 3.6 ± 0.3 μg/DW of aloe emodin was detected in adventitious roots grown for seven weeks in basal media including 0.5 mg/L NAA + 0.2 mg/L BAP. The concentration was similar to that of the three-year-old leaves, indicating that adventitious root culture has strong potential to produce aloe secondary metabolites via *in vitro* culture of adventitious roots.

Table 1-1 Contents of aloin and aloe emodin in adventitious roots, leaves, and roots of *Aloe vera*.

Sample	Aloin (μg/dry weight)	Aloe emodin (μg/dry weight)
Adventitious roots	0	3.6 ± 0.3^{b}
Roots	3.9 ± 0.2^{a}	574.8 ± 92.4^{a}
Leaves	1499.1 ± 904.0^{b}	5.5 ± 0.3^{b}

Adventitious root were grown for six weeks in MS media including 0.5 mg/L NAA + 0.2 mg/L BAP, and roots and leaves were collected from plants grown for three years in an aloe farm field.

Induction of *Aloe vera* adventitious roots in solid media

In order to determine the optimal conditions of *Aloe vera* for adventitious root induction, I tested various conditions of auxin supplements (2,4-D, NAA, IAA, and IBA) on MS media. NAA was the only phytohormone to induce adventitious roots from leaf explants after three weeks of culture. No adventitious roots were initiated in auxin-free media. Old leaves and shoots greater than 10 cm in size did not induce adventitious roots under any conditions. Supplementation with IBA and IAA showed no or very little adventitious root induction, respectively. An easily friable callus was induced by supplementation of 2,4-D in basal MS media.

Significant effects of NAA concentration were detected for adventitious root induction. Supplement of lower concentrations, such as 0.1, 0.5, and 1.0 mg/L was good for adventitious root induction, while increasing to higher concentrations, 2.0, 4.0, and 6.0 mg/L resulted in induction of a friable callus. Collectively, 0.5 mg/L NAA was the most effective for bringing about improvements in induction rate, number of adventitious roots per explant, and root length during six weeks of culture (Figure I-1A, Table 1-2). Although DW was greater at 1 mg/L NAA (0.13 ± 0.03 g) in MS media than at 0.5 mg/L NAA (0.058 ± 0.01 g), the root was not suitable for mass production caused by callus-like, short, and fragile phenotype (Figure I-1B).

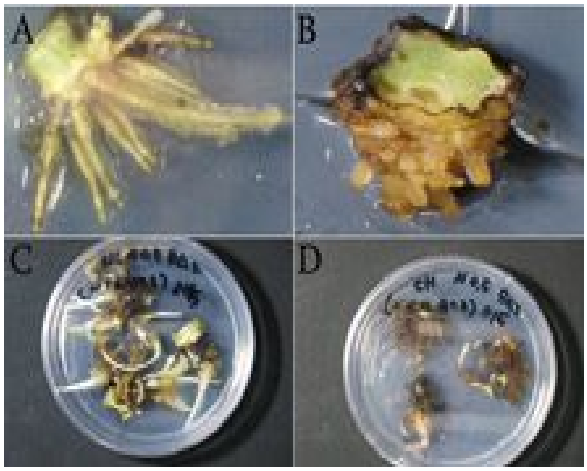

Figure I-1 Different features of *Aloe vera* adventitious roots based on different NAA concentrations (A and B) and different media (C and D). A and B, adventitous roots cultured for three weeks on MS media supplemented with different concentrations of NAA. 0.5 mg/L NAA (A) and 1.0 mg/L NAA (B) supplementation resulted in normal and callus-like adventitous roots, respectively. C and D, adventitous roots cultured for six weeks on different media, MS (C) and SH media (D) with the same hormone 0.5 mg/L NAA + 0.2 mg/L BA.

Proliferation of adventitious roots

Even though NAA 0.5 mg/L supplementation was the best condition to induce adventitious roots, biomass increase was not best under this condition. Therefore, I determined the optimal condition for adventitious root growth. Adventitious roots induced for three weeks on MS media culture with 0.5 mg/L NAA supplementation were transferred to media with additional supplementation of 0.1, 0.2, or 0.3 mg/L BAP (Figure I-2). Time course analysis revealed that DW was increased for the first five weeks of culture and decreased thereafter in most treatments. DW was increased gradually for the first six weeks of supplementation of BAP 0.2 mg/L in addition to NAA 0.5 mg/L (0.106 ± 0.001 g) (Figure I-2).

I also compared biomass increases based on different kinds of media, 2 MS, MS, half strength-MS (1/2 MS), SH, and B5, with the same supplementation of 0.5 mg/L NAA + 0.2 mg/L BAP. The best adventitious root growth was observed in MS media (0.123 ± 0.002 g) (Figure I-1C, Figure I-3), which was twice as great as that in SH media (0.061 ± 0.002 g) (Figure I-1D, Figure I-3).

Table I-2 Effects of NAA concentration on adventitious root induction from leaf explants of *Aloe vera*.

NAA concentration (mg/L)	Induction rate (%)	Number of roots per explant	Root length (cm)
0.1	23	6.5 ± 0.3^{b}	2.7 ± 0.2^{b}
0.5	52	10.1 ± 0.2^{a}	4.1 ± 0.3^{a}
1.0	27	5.1 ± 0.4^{c}	1.9 ± 0.1^{c}

Significantly different at a 5% level

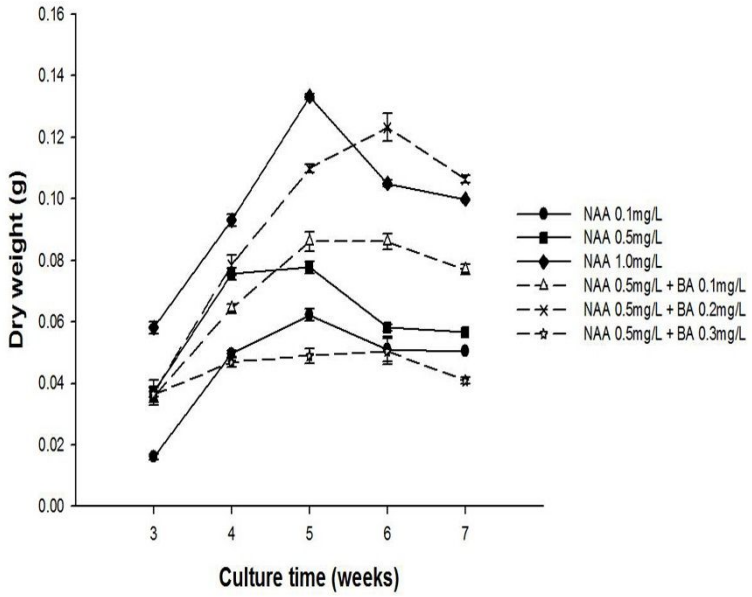

Figure I-2 Effects of different hormone supplements on dry weight increase in *Aloe vera* adventitious root culture for the first seven weeks. Six hormone combinations, 0.1, 0.5, 1.0 mg/L NAA, 0.5 mg/L NAA + 0.1 mg/L BA , 0.5 mg/L NAA + 0.2 mg/L BA, 0.5 mg/L NAA + 0.3 mg/L BA, were supplemented on MS media.

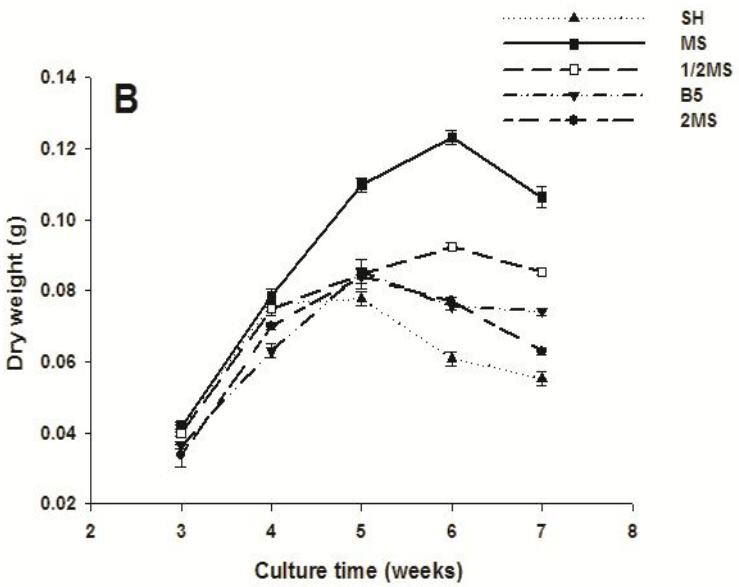

Figure I-3 Effects of basal media on dry weight increase in *Aloe vera* adventitious root culture for the first seven weeks. Three different media, 2 MS, MS, and 1/2 MS, were compared in a supplement mixture of 0.5 mg/L NAA + 0.2 mg/L BA.

Inhibition of browning

When adventitious roots were separated from the primary induction media and cultured individually, the roots severely browned and died. This was the critical problem for tissue culture of aloe species. Therefore, it is necessary to prevent explants browning to achieve successful mass production of *Aloe vera* adventitious roots. Roy *et al.,* (1991) reported that supplementation of PVP in the media inhibited browning in aloe tissue culture. However, I did not obtain any significant improvements with these additions.

A significant delay in browning was observed after pre-washing the adventitious roots in PVP analogues prior to the transfer to the second growth media. Adventitious roots pre-washed with various PVP analogous survived, regardless of PVP analog type, washing time or pH value. However, PVP concentration substantially affected survival rate, indicating that viability increased by pre-washing tissue with a solution containing more than PVP 4.0 g/L of analogs (Figure I-4).

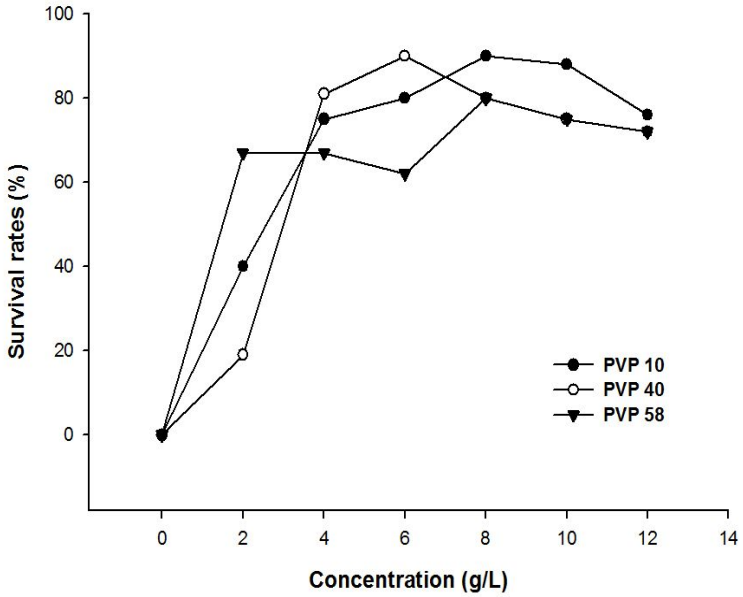

Figure I-4 Effects of various concentrations of PVP analogs during pre-washing of the adventitious roots. Same stage adventitious roots were separated from the primary adventitious root induction media and were washed via agitation in the PVP solution in order to inhibit browning and were sub-cultured on MS media without growth hormone.

Comparison of aloe emodin contents in adventitious roots grown different growth media

I compared aloe emodin contents in adventitious roots grown on different basal media (Table I-3). No significant difference in aloe emodin content was detected between two different adventitious roots when they were grown on the same MS media with different hormone combinations, such as 0.5 mg/L NAA + 0.1 mg/L BAP and 0.5 mg/L NAA + 0.3 mg/L BAP. Therefore, I compared five basal media with the same hormone combinations to clarify the effects of increasing aloe emodin content. The results showed that aloe emodin content was dramatically increased (133.08 ± 0.12 μg/dry weight) in the adventitious roots grown in B5 media to 20-40 folds higher than that those in the other media (Table I-3).

Table I-3 Effects of basal media on aloe emodin content in aloe adventitious roots.

Media	Dry weight/ explants (g)	Aloe emodin (μg/g)
MS	0.1	3.6 ± 0.3^{c}
1/2MS	0.09	4.3 ± 0.5^{b}
SH	0.05	5.7 ± 0.1^{b}
2MS	0.06	6.4 ± 0.03^{b}
B5	0.07	133.1 ± 0.1^{a}

Each medium has the same hormone combination, NAA 0.5 mg/L + BAP 0.2 mg/L.

DISCUSSION

In this study, *in vitro* culture system for *Aloe vera* was established to retain adventitious roots lines in controlled environment. Previous studies have been attempted to optimize induction of callus on solid media in *Aloe saponaria* (Yagi *et al*., 1983), *Aloe arborescencs* (Kawai *et al*., 1993), and *Aloe vera* (Yagi *et al*., 1998). However suspension culture of aloe species had been limited, because releasing phenolic compounds from cultured cell eventually led to cell death (Roy and Sarkar 1991).

First, solid culture condition for *Aloe vera* adventitious roots was optimized. The results revealed that MS media supplemented with 0.5 mg/L NAA was the best condition for induction of adventitious roots from leaves explants. Many studies have reported that NAA activates cell division and root induction. In *Lycopersicon esculentum,* NAA treatments induced adventitious roots and activated elongation of lateral roots (Taylor *et al.,* 1998). On the other hand, a high concentration of NAA induced callus-like, short adventitious roots in *Karwinskia humboldtiana* (Kollarova *et al.,* 2004), as in my results for supplementation with more than 1 mg/L NAA, indicating that an overdose of NAA may reduce organogenesis.

Then media types of MS, 1/2 MS, 2 MS, B5, and SH, including 0.5 mg/L were examined, resulting that MS media was most effect on biomass production of

Aloe vera adventitious roots. Ammonium nitrogen in MS media is quickly consumed in plant tissues and is directly metabolized. However, excess ammonium is very toxic, inhibiting cell metabolism. Previous results revealed that low ammonium nitrogen vs. nitrate nitrogen ratio was shown to be important for the induction of adventitious roots in ginseng root culture (Han *et al.,* 2006). Similarly, *Echinaceae angustifolia* root growth rate was best in 1/2 MS media with a 5:25 ratio of ammonium nitrogen vs. nitrate nitrogen (Wu *et al.,* 2006). However, aloe adventitious roots showed a higher growth rate in MS media, rather than in other media with lower ammonium nitrogen concentrations, implying that ammonium nitrogen is not critically inhibitive for aloe adventitious root culture, even though more a precise ammonium nitrogen vs. nitrate nitrogen ratio should be determined to conclude this assumption.

Moreover, I examined that significant delay in browning was observed after pre-washing the adventitious roots in PVP analogues prior to the transfer to the second growth media (Figure I-4). According to the results, survival rate was increased when adventitious roots were pre-washed with solution containing more than PVP 4.0 g/L (Figure I-4). Pre-washing mango explants via agitation in PVP solution was very efficient not only for inhibition of browning, but also for prevention of leakage of phenolic compound in excised explants (Krishna *et al.,* 2008). Pre-washing, including with glutathione, also reduced total phenolic compounds and PAL activity in pistachio, resulting in significant growth increases

(Tabiyeh *et al.,* 2005). We assumed that tissue pre-washing with PVP removed phenolic compounds secreted from excised explant surfaces to aid in the adaptation of the explants in the new media for second growth. Therefore, the aloe roots transferred after pre-washing in PVP solution survived, and lateral roots were induced in MS media without growth hormone.

Meanwhile, adventitious roots were not induced when PVP was directly supplemented on root induction media. Many phenolic compounds released from excised surfaces might induce cell necrosis. Pre-washing of the excised explants with PVP removes these compounds and results in reduced cell damage, thus promoting root initiation. However, addition of PVP in initial root induction media resulted in absorption of phenolic compounds that might negatively affect the initial induction of adventitious roots in *Aloe vera*.

Finally, I analyzed aloe emodin content in *Aloe vera* adventitious roots cultured on MS, 1/2MS, 2MS, B5, or SH media including 0.5 mg/L NAA and 0.2 mg/L BA, showing that B5 media was most effects on production of aloe emodin (Table I-3). Increased ammonium nitrogen concentration not only inhibited biomass increase but also accumulated useful phytochemicals, even though preferences for nitrate nitrogen and ammonium nitrogen components vary by species (Kronzucker *et al.,* 1999). Adventitious root growth and phenolic compound accumulation in *Echinacea angustifolia* was maximized in modified MS media, which had 5 and 25 mM of ammonium nitrogen and nitrate nitrogen contents, respectively (Wu *et al.,*

2006). Adjustment to a 1 : 5 (w/w) ammonium nitrogen : nitrate nitrogen ratio in MS media was shown to be optimal for artemisinin accumulation in *Artemisia annua* L. hairy root culture, but ammonium itself inhibited biomass and artemisinin production (Wang and Tan, 2002). Our experiments showed slight difference for *Aloe vera* adventitious root growth dependent on basal media. However, a critical increase in aloe emodin was detected in the adventitious root grown in B5 medium which had the lowest ammonium concentration.

Hairy root growth of *Lobelia inflate* occurred in the order of MS > 1/2 MS > B5 media, but NN medium (Nitsch and Nitsch, 1967) showed more lobeline synthesis in fresh weight (Yonemitsu *et al.,* 1990). Even though B5 showed the best aloe-emodin production in our experiment, further conditions should be inspected. Providing two-step cultures that differ for adventitious root growth and useful chemical production may be considered because the observed growth was the best in ammonium-rich MS medium, but aloe emodin content was the best in ammonium-free B5 medium. Further experiments should be performed to determine the possibility of mass production of adventitious roots in adventitious roots suspension culture. Further experiments for adjustment of nitrogen sources and optimal secondary metabolite production upon treatment of various elicitors will contribute to establish an efficient root culture system for *in vitro* mass production of useful metabolites using *Aloe vera* adventitious roots.

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CHAPTER II

Exogenous polyamine promotes *in vitro* propagation of *Aloe vera*

ABSTRACT

I established an advanced protocol for *in vitro* propagation of *Aloe vera* via comparison of basal media, sucrose contents, growth hormone combinations, and additional supplementation with various polyamines. The maximal number and growth of shoots after 5 weeks was obtained using MS media including 30 g/L sucrose supplemented with 1.0 mg/L BA and 0.1 mg/L NAA. To improve shoot production, various concentrations of putrescine, spermidine, and spermine were added under the optimal growth hormone condition (MS media supplemented with 30 g/L sucrose, 1.0 mg/L BA, and 0.1 mg/L NAA). Maximal shoot number and growth after 5 weeks were achieved with supplementation of 50 mg/L spermidine. Regenerated plants were successfully acclimatized in soil with 100% efficiency. Cytogenetic inspection revealed that the regenerated plants maintained intact chromosomes identical to those of plants grown in field conditions. This protocol provides a valuable alternative for mass production of elite *Aloe vera.*

INTRODUCTION

Aloe vera belongs to the Asphodeloideae family and originated in tropical Africa (Ahlawat and Khatkar, 2011; Singh and Sood, 2009). The leaves and roots of *Aloe vera* possess pharmacologically useful compounds including anthraquinone derivatives such as aloin, aloe-emodin, chrysophanol, and rhein, and chromone derivatives such as aloesin (Choi and Chung, 2003). *Aloe vera*-derived compounds have been reported to have anticancer, antiprotozoal, antioxidant, and whitening activities (Choi and Chung, 2003). Accordingly, *Aloe vera* is the predominant constituent in several commercial and pharmacological products (Grace *et al.,* 2008).

Aloe vera reproduces through adventitious shoots induced from underground stems, but adventitious shoot production is dependent on the season and occurs with only low efficiency (Meyer and Staden, 1991; Singh and Sood, 2009). Therefore, vegetative propagation is not sufficient to meet increasing commercial demand (Singh and Sood, 2009). To overcome these problems, previous research has aimed to establish effective protocols for micropropagation of *Aloe vera* using shoot tips, meristem including shoot tips, or meristem segments (Abadi and Kaviani, 2010; Hashemabadi and Kaviani, 2008; Liao *et al.,* 2004; Wenping *et al.,* 2004; Singh and Sood, 2009; Meyer and Staden, 1991). However, the development of the previous protocols was focused mainly on optimizing

growth hormone effects or explant preparation, and improved *in vitro* shoot regeneration methods are still requried for mass propagation of *Aloe vera*.

Polyamines such as putrescine, spermidine, and spermine are among the aliphatic compounds detected in all organisms (Kakkar *et al.,* 2000; Wallace *et al.,* 2003). Although the biological activities of polyamines have not been fully discovered, they are known to play major roles in cell division, embryogenesis, and developmental flowering (Kakkar *et al.,* 2000). Exogenous polyamines promote *in vitro* culture of plants such as improvement of shoot and root growth in *Malaxis acuminata* D. Don, *Pinus virginia* Mill., and *Curcuma longa* L.(Cheruvathur *et al.,* 2010; Tang and Newton 2005; Viu *et al.,* 2009), enhancement of somatic embryo production in *Momordica charantia* L. (Paul *et al.,* 2009). Ethylene accumulation was reduced by treatment of polyamine together with aminoethoxyvinylglycine (AVG) in *Prunus armeniaca* L, (Petri *et al.,* 2005). Thus, polyamines have come to be regarded as another plant growth regulator in recent years (Kakkar *et al.,* 2000).

In this paper, I established a regeneration protocol for *Aloe vera* using meristem segments. I optimized the basal medium, including growth hormones, and characterized the effect of polyamines on shoot regeneration and growth of *Aloe vera*. Using our protocol, all of the regenerated plants were successfully transferred onto soil and showed normal growth with intact chromosomes.

MATERIAL AND METHODS

Plant material and culture conditions

Young shoots (under 20 cm) of *Aloe vera* were harvested from the Kim Jeong Moon *Aloe* farm (Jeju, Korea). Meristem segments were sterilized using 70% ethanol for 30 s and 4% sodium hypochlorite for 5 min. The explants were rinsed five times with sterilized water. The sterilized meristem segments were cut into 6 slices, and six explants were placed on each petri dish, with 5 replicates per condition. The explants were cultured in a temperature-controlled room at 25 ± 1 ℃ under a 16 : 8 (light : dark) photoperiod with light intensity of 50 μE/ms from cool white fluorescent tubes.

Optimization of conditions for shoot regeneration and acclimatization

Meristem explants were cultured on MS (Murashige and Skoog 1962), SH (Schenk and Hildebrandt, 1972), or B5 (Gamborg *et al.,* 1968) medium including 30 g/L sucrose. Alternatively, explants were cultured on 0, 10, 20, 30, 40, and 50 g/L sucrose-supplemented MS medium. To find optimal growth hormone conditions, meristem explants were inoculated on MS medium supplemented with 30 g/L sucrose containing 0.1, 0.5, 1.0, 2.0, or 3.0 mg/L BA with or without 0.1, 0.3, or 0.5

mg/L NAA. For improvement of shoot production, different concentrations (0, 10, 30, 50, and 100 mg/L) of spermidine (KisanBio, Korea), spermine (Fluka, USA), or putrecine (KisanBio, Korea) were added to MS media containing 30 g/L sucrose with 1.0 mg/L BA and 0.1 mg/L NAA. All media were adjusted to pH 5.8, solidified with 0.8% plant agar (Duchefa, Netherland) and autoclaved at 121 ℃ for 15 min. Regenerated *Aloe vera* was rooted on MS medium without hormones after 5 weeks. Matured plants were transferred to flowerpots containing a mixture of vermiculite and paddy rice bed (50:50).

Cytogenetic analysis

Root tip segments from regenerated and field-grown *Aloe vera* were dipped into 0.002 M 8-hydroxyquinoline for 6 h at 16 ℃ and then fixed in an acetic acid: ethanol (1:3 v/v) solution for 2~24 h at 4 ℃. Pretreated root tips were thoroughly rinsed with sterilized water and incubated in an enzyme mixture solution (2% cellulose - Sigma, 2% pectolyase - Sigma, 1.5% Macerozume- Yakult) at 37 ℃ for 25 min. Prepared root tips were transferred onto slide glass, squashed with 60% acetic acid and air dried. The samples were stained with 2% aceto-orcein and examined with an Olympus BX51 fluorescence microscope and CCD camera (CoolSNAP ™cf).

Statistical analysis

All statistical analyses were conducted using the Statistical Analysis System (SAS 9.1). The difference among means of each treatment were analyzed by Analysis of Variance (ANOVA) and significant differences among each treatment group were determined by Duncan's test ($p<0.05$).

RESULTS AND DICUSSIONS

Optimization of basal medium for multi-shoot induction of *Aloe vera*

Tissue culture systems have been utilized in medicinal plants for elite germplasm production (Ahmad *et al.,* 2011). Shoot culture using meristem explants has been widely used as the best method to obtain virus-free clonal stocks (Adesoye *et al.,* 2012). In this study, we attempted to achieve mass production of *Aloe vera* starting from meristem explants.

First, meristem segments of *Aloe vera* were divided into 6 slices, and the explants were cultured on SH, B5, or MS basal medium supplemented with 30 g/L sucrose for 5 weeks. All meristem explants successfully regenerated on the basal media after 5 weeks. The maximum shoot regeneration rate and shoot number were obtained on MS medium (Table II-1, Figure II-1A).

As a component of growth medium, sugar serves to supply energy and maintain osmotic pressure (Hartmann and Kester, 1975). Previous results showed that among the various carbon sources, sucrose is the most effective for micropropagation of *Aloe* species, and further, that sucrose is even more important for bud initiation than is growth hormone during micropropagation of *Aloe vera* L. var. *chinensis* (Haw.) Berger (Hashemabadi and Kaviani 2008; Liao *et al.,* 2004; Singh and Sood, 2009; Velcheva *et al.,* 2005).

Our present data also revealed that sucrose is an important factor for

regeneration of *Aloe vera*. Explants cultured on medium without sucrose did not form shoots (Table II-2). Supplementation with sucrose allowed shoot initiation, and maximum shoot initiation efficiency was observed in 30 g/L sucrose after 5 weeks of culture (Table II-2, Figure II-1B), similar to the findings for micropropagation of *Aloe vera* L. var. *chinensis* (Haw.) (Liao *et al.,* 2004). Sucrose concentrations over 40 g/L inhibited initial shoot induction and reduced the shoot number and length. High levels of sucrose can have negative effects on cultured cells, resulting from a low capacity for nutrient uptake caused by low water potential in the medium (Baque *et al.,* 2011; Shim *et al.,* 2003; Shohael *et al.,* 2006). Previous studies showed that excessive sucrose contents led to decreased biomass production in *Anoectochilus formosanus* hayata (Yoon *et al.,* 2007), increased formation of abnormal plantlets in *Calanthe* hybrids (Baque *et al.,* 2011) and oxidative damage in *Eleutherococcus sessiliflorus* (Shohael *et al.,* 2006). Our results suggest that 30 g/L sucrose is optimal to maintain osmotic pressure in the medium and to induce and support growth of multi-shoots of *Aloe vera*.

Table II-1 Effects of different media supplemented with 30 g/L sucrose on *Aloe vera* meristem culture after 5 weeks

Medium	Shoot Induction Rate (%)	No. Shoots per Explant*	Shoot Length (cm)*
MS	81.42	1.94 ± 0.42^{a}	0.51 ± 0.07^{a}
B5	70.09	1.65 ± 0.26^{ab}	0.40 ± 0.09^{a}
SH	65.12	1.04 ± 0.33^{b}	0.18 ± 0.08^{b}

* The different letters indicate that means of each treatment were differences by Duncan test ($p<0.05$)

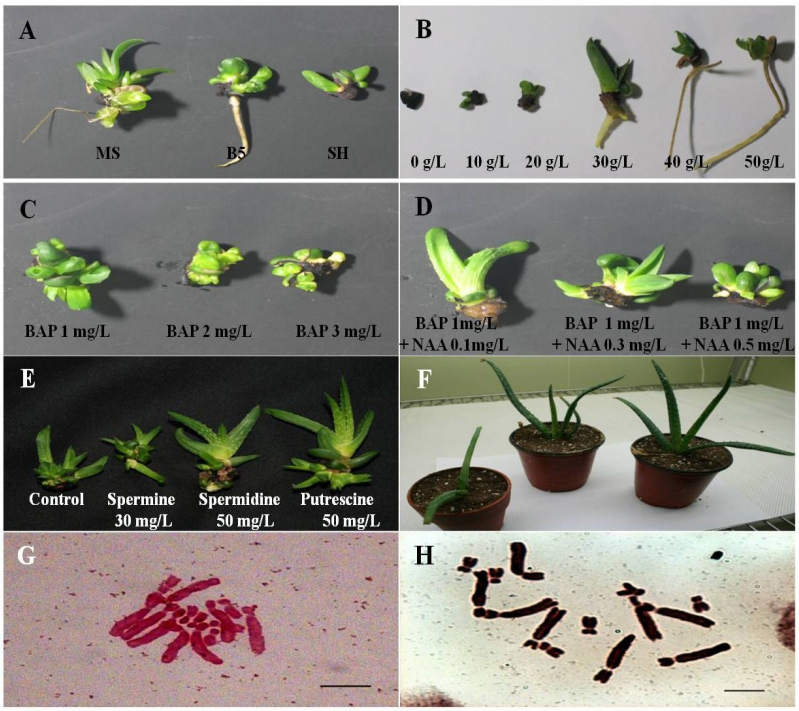

Figure II-1 Micropropagation of *Aloe vera*. (A) Shoot induction on different media. (B) Shoot induction on MS medium containing different sucrose concentrations. (C) Effect of BA concentration in MS medium on shoot induction. (D) Effect of BA and NAA combination supplementation in MS medium on shoot induction. (E) Effects of polyamine supplementation in MS medium with 1.0 mg/L BA + 0.1 mg/L NAA. (A)-(E) were incubated for 5 weeks at each condition. (F) Acclimatization of regenerated plants. (G) Chromosomes of regenerated *Aloe vera.* (H) Chromosomes of field-grown *Aloe vera.* Bar = 10um

Table II-2 Effects of sucrose concentration in MS medium on *Aloe vera* meristem culture

Sucrose (g/L)	Shoot Induction Rate (%)	No. Shoots per Explant*	Shoot Length (cm)*
0	0	0	0
10	21.42	1.16 ± 0.24^{b}	0.20 ± 0.02^{c}
20	41.66	1.58 ± 0.24^{a}	0.60 ± 0.12^{b}
30	81.42	1.82 ± 0.25^{a}	0.93 ± 0.07^{a}
40	57.24	1.00 ± 0.05^{b}	0.51 ± 0.02^{b}
50	37.50	1.00 ± 0.01^{b}	0.30 ± 0.01^{c}

* The different letters indicate that means of each treatment were differences by Duncan test ($p<0.05$)

Effect of phytohormones on multi-shoot production of *Aloe vera*

Plant growth regulators are important components for determining cellular differentiation (Tu and Zheng, 2012). Cytokinin promotes the induction of axillary shoots, and the ratio of cytokinin and auxin in particular is pivotal in shoot production (Nakhooda *et al.,* 2012). BA was previously found to be the most effective phytohormone for promoting biomass production of *Aloe vera* compared to other cytokinins such as zeatin, ribozide (ZR), 6-(c,c-dimethylallyl-amino) purine riboside (2iPR), N-(2-chloro-4-pyridyl)-N′-phenylurea (CPPU), kinetin, and thidiazuron (TDZ) (Hashemabadi and Kaviani, 2008; Velcheva *et al.,* 2010). Furthermore, the combination of NAA and BA has a synergistic effect on shoot production in *Aloe vera* (Abadi and Kaviani, 2010; Hashemabadi and Kaviani, 2008; Liao *et al.,* 2004; Wenping *et al.,* 2004). Based on these previous results, we attempted to determine the optimal combination of BA and NAA for multi-shoot production in *Aloe vera.*

Meristem explants were cultured for 5 weeks on MS medium including 30 g/L sucrose supplemented with 0.1, 0.5, 1.0, 2.0, or 3.0 mg/L BA. Different micro-propagation efficiencies were observed with different BA concentrations. The maximal biomass production occurred with 1.0 mg/L BA (Table II-3, Figure II-1C). Meristem segments were then cultured on MS medium supplemented with 30 g/L sucrose and 1.0 mg/L BA with differential level of NAA, 0.1, 0.3, or 0.5 mg/L, to test for optimal synergistic effects of auxin and cytokinin on multi-shoot production.

The production of multiple shoots was increased in all BA and NAA co-supplementation conditions compared to 1.0 mg/L BA alone, with the highest shoot numbers and growth observed in MS media supplemented with 1.0 mg/L BA and 0.1 mg/L NAA (Table II-3, Figure II-1C). In the case of Chinese aloe, medium supplemented with 2.0 mg/L BA + 0.3 mg/L NAA was reported to be suitable for progressive shoot production (Liao *et al.,* 2004). In *Aloe vera* shoot propagation using shoot tips, MS medium containing 0.5 mg/L BA + 0.5 mg/L NAA was found to be optimal (Hashemabadi and Kaviani, 2008). These differences in optimal conditions might be due to the use of different genotypes and explants, such as suggested by Novero and Jamiri (2012).

Table II-3 Effects of various hormonal conditions on shoot growth in *Aloe vera* meristem culture

Hormone (mg/L)	No. Shoots per Explant*	Shoot Length (cm)*
0	1.82 ± 0.25^{g}	0.93 ± 0.25^{e}
BA 0.1	2.09 ± 0.04^{f}	0.95 ± 0.04^{e}
BA 0.5	2.90 ± 0.02^{e}	1.05 ± 0.04^{cd}
BA 1.0	3.79 ± 0.03^{c}	1.24 ± 0.03^{b}
BA 2.0	3.51 ± 0.04^{d}	0.97 ± 0.04^{f}
BA 3.0	2.89 ± 0.05^{e}	0.82 ± 0.05^{d}
BA 1.0 + NAA 0.1	4.93 ± 0.14^{a}	1.42 ± 0.24^{a}
BA 1.0 + NAA 0.3	4.27 ± 0.09^{b}	1.29 ± 0.11^{b}
BA 1.0 + NAA 0.5	3.89 ± 0.05^{c}	1.08 ± 0.05^{c}

* The different letters indicate that means of each treatment were differences by Duncan test ($p<0.05$)

Effect of polyamine supplementation on multi-shoot production in *Aloe vera*

Accumulating evidence shows that polyamine treatments promote shoot growth in *in vitro* culture (Cheruvathur *et al.,* 2010; Petri *et al.,* 2005; Viu *et al.,* 2009). To examine the effects of polyamine supplementation on shoot production, meristem explants were cultured for 5 weeks on MS medium including various polyamines along with 1.0 mg/L BA and 0.1 mg/L NAA.

Addition of putrescine and spermidine promoted shoot production, whereas treatment with spermine inhibited shoot production (Table II-4). The highest biomass production was found in MS medium supplemented with 50 mg/L spermidine, and resulted in an increase of shoot number up to 1.36-fold and a shoot length increase up to 1.23-fold compared to medium without polyamine (Table II-4, Figure II-1E). The addition of 50 mg/L putrescine also resulted in a 1.29-fold increase in shoot number and a 1.22-fold increase in shoot length compared to medium without polyamine (Table II-4, Figure II-1E). Putrescine similarly promoted the shoot induction rate and biomass production during micropropagation of *Sinningia speciosa* (Park *et al.,* 2012). Furthermore, spermidine enhanced adventitious shoot production of *Malaxis acuminate*, whereas spermine had no effect, in agreement with our results (Cheruvathur *et al.,* 2010). Together, these findings indicate that spermidine and putrescine might act as signaling molecules to promote shoot regeneration in *Aloe vera*, similar to the cases of *Sinningia speciosa,*

Prunus armeniaca L, and *Malaxis acuminate* (Cheruvathur *et al.,* 2010; Park *et al.,* 2012; Petri *et al.,* 2005).

Polyamine is an essential component in cell division and proliferation during various developmental stages. A high concentration of polyamine promotes cell division and proliferation *in vivo* (Cvikrová *et al.,* 1999; Davidonis, 1995). In addition, polyamine has been reported to inhibit ethylene synthesis, which has negative effects on shoot growth (Chen *et al.,* 2013; Park *et al.,* 2012). Supplementation with polyamines together with ethylene inhibitors such as AVG and $AgNO_3$ leads promotion of shoot growth in *Prunus armeniaca L* and *Raphanus sativus* L. var. *longipinnatus* Bailey (Petri *et al.,* 2005; Pua *et al.,* 1996). It is likely that polyamine treatment promotes cell division and inhibits ethylene production in *Aloe vera*, resulting in enhanced shoot growth.

Table II-4 Effect of different concentrations of various polyamines in MS medium containing 1.0 mg/L BA + 0.1 mg/L NAA on *Aloe vera* meristem culture

Polyamine (mg/L)	No. of Shoots per Explant*	Shoot Length (cm)*
0	4.83 ± 0.14^{f}	1.32 ± 0.03^{d}
Putrecine 10	5.05 ± 0.04^{ef}	1.51 ± 0.04^{bdac}
Putrecine 30	5.82 ± 0.13^{bcd}	1.58 ± 0.09^{bac}
Putrecine 50	6.23 ± 0.15^{ab}	1.62 ± 0.07^{ab}
Putrecine 100	5.33 ± 0.24^{de}	1.42 ± 0.24^{bdc}
Spermidine 10	5.17 ± 0.18^{ef}	1.39 ± 0.07^{dc}
Spermidine 30	5.43 ± 0.49^{cde}	1.51 ± 0.08^{bdac}
Spermidine 50	6.56 ± 0.64^{a}	1.63 ± 0.15^{a}
Spermidine 100	5.87 ± 0.35^{bc}	1.47 ± 0.14^{bdac}
Spermine 10	2.02 ± 0.18^{g}	1.01 ± 0.21^{e}
Spermine 30	1.72 ± 0.24^{g}	0.95 ± 0.09^{e}
Spermine 50	1.21 ± 0.12^{h}	0.87 ± 0.07^{e}

* The different letters indicate that means of each treatment were differences by Duncan test ($p<0.05$)

Acclimatization and chromosome analysis

Regenerated shoots of *Aloe vera* were divided and transferred onto MS medium without growth hormones in order to induce rooting. Roots were successfully induced from all regenerated shoots, and the rooted shoots were acclimatized under controlled conditions and transferred to soil in pots. These plants showed a survival rate of 100% and exhibited normal growth. Somaclonal variations are frequently observed in regenerated plants and hinder the micropropagation of plants (Rathore *et al.,* 2011). Variations in regenerated plants can be confirmed through various methods, e.g. morphological, biochemical, molecular, and cytological analysis (Rathore *et al.,* 2011). In this study, I carried out cytogenetic analysis to confirm that the regenerated plants have intact chromosomes similar to those of field-grown *Aloe vera*. Chromosome analysis showed that the regenerated aloe plants had seven pairs of chromosomes consisting of four large and three small chromosome pairs, as found in field-grown *Aloe vera* plants (Figure II-1G, 1H) and in previous reports (Brandham, 1971; Brandham and Johnson, 1977; Vig, 1968). I optimized protocol thus allowed the regeneration of plants that maintained intact diploid cells, although we cannot exclude the possibility that there was minor DNA sequence level somaclonal variation.

In this study, I established a high efficiency micropropagation protocol for *Aloe vera* and improved it even further by adding spermidine. This optimized protocol led to the production of more than 40 plants from one meristem of *Aloe*

vera, demonstrating much higher efficiency than previous reported protocols (Abadi and Kaviani, 2010; Hashemabadi and Kaviani, 2008; Liao *et al.,* 2004; Wenping *et al.,* 2004; Singh and Sood, 2009; Meyer and Staden, 1991). In addition, regenerated plants were easily transferred to soil without any loss and grew normally without chromosome-level somaclonal variation. This optimized protocol provides a valuable alternative for mass propagation of elite *Aloe vera.*

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CHAPTER III

Enhancement of Anti-inflammatory Activity of *Aloe vera* Adventitious Root Extracts through the Alteration of Primary and Secondary Metabolites via Salicylic Acid Elicitation

ABSTACT

Representative secondary metabolite of *Aloe vera* is anthraquinones, including aloe emodin and chrysophanol which has tricyclic aromatic quinones structure. Tricyclic aromatic quinone structures were synthesized via a plant-specific type III polyketide biosynthesis pathway. However, it is not yet clear which cellular responses can induce the pathway, leading to production of tricyclic aromatic quinones. In this study, I examined the effect of endogenous elicitors on the type III polyketide biosynthesis pathway and identified the metabolic changes induced in elicitor-treated *Aloe vera* adventitious roots. Salicylic acid, methyl jasmonate, and ethephon were used to treat *Aloe vera* adventitious roots cultured on MS liquid media with 0.3 mg/L IBA for 35 days. Aloe emodin and chrysophanol were remarkably increased by the SA treatment, more than 10-11 and 5-13 fold as compared with untreated control, respectively. Ultra-performance liquid chromatography-electrospray ionization mass spectrometry analysis identified a total of 37 SA-induced compounds, including aloe emodin and chrysophanol, and 3 of the compounds were tentatively identified as tricyclic aromatic quinones. Transcript accumulation analysis of polyketide synthase genes and gas chromatography mass spectrometry showed that these secondary metabolic changes resulted from increased expression of octaketide synthase genes and decreases in malonyl-CoA, which is the precursor for the tricyclic aromatic quinone biosynthesis pathway. In

addition, anti-inflammatory activity was enhanced in extracts of SA-treated adventitious roots. Our results suggest that SA has an important role in activation of the plant specific-type III polyketide biosynthetic pathway, and therefore that the efficacy of *Aloe vera* as medicinal agent can be improved through SA treatment.

INTRODUCTION

Aloe vera (Asphodeaceae) is a medicinal plant in which useful secondary metabolites are abundant (Reynolds and Dweck,1999; Boudreau and Beland, 2006). Anthraquinones, which represent one class of *Aloe vera* secondary metabolites, are tricyclic aromatic quinones. Among the naturally occurring anthraquinone derivatives, aloe emodin and chrysophanol are the major compounds (Tan *et al*., 2011). The tricyclic aromatic quinones of aloe have been proposed to be synthesized via the type III polyketide biosynthesis pathway. Recently, novel plant-specific type III polyketide synthases (PKSs), octaketide synthase (OKS), PKS4, and PKS5 were isolated from *Aloe arborescens*, and their functions were examined in *E. coli*. The heterologously expressed enzymes produced SEK and SEK4b, which have an octaketide structure, from eight malonyl-CoAs, but SEK and SEK4b were found to be shunt products of the type II polyketide biosynthesis pathway and have not been detected in plants (Abe *et al*., 2005; Mizuuchi *et al*., 2009) (Figure III-1). This suggested that these novel plant enzymes might potentially be associated with biosynthesis of natural tricyclic aromatic quinones in aloe, but it remains unclear whether these enzymes produce end products such as aloe emodin and chrysophanol *in vivo*.

Identification of secondary metabolites and their derivatives in plants is a first step to determine the associated biosynthetic pathways; however, many remain

to be identified because plants are especially rich in secondary metabolites with complex and varied structures (De Vos *et al*., 2007). Metabolomics approaches have ushered in a new era for elucidating the complicated secondary metabolism of plants (Farag *et al*., 2008; Yonekura-Sakakibara *et al*., 2008; Farag *et al*., 2009; Matsuda *et al*., 2009). Among these techniques, mass spectrometry can contribute to deducing the structures of unknown compounds (Sawada *et al*., 2009), and comprehensive profiling of phenolic compounds including anthraquinones has been carried out in *Cassia* and *Rheum* species using high-performance liquid chromatography-electrospray ionization-mass spectrometry (HPLC-ESI-MS) (Xie *et al*., 2007; Ye *et al*., 2007; Püssa *et al*., 2009; Yu *et al*., 2011). In addition, anthraquinone derivatives in rhubarb extract that were biotransformed by rat liver and intestinal bacteria were identified by liquid chromatography-electrospray ionization- tandem mass spectrometry (LC-ESI-MS/MS) (Song *et al*., 2011; Song *et al*., 2009). In aloe, metabolite profiling was recently carried out for *Aloe vera* leaves at different developmental stages using gas chromatography-ion trap-mass spectrometry (GC-IT-MS) and ultra-performance liquid chromatography-quadruple time of flight-mass spectrometry (UPLC-QTOF-MS) (Lee *et al*., 2012).

In this study, I used GC-MS and UPLC-ESI-MS analysis to investigate the changes in primary and secondary metabolites induced by elicitor treatments of *Aloe vera* adventitious roots. Plant cell culture systems using elicitors have been effective alternatives for production of secondary metabolites, and many abiotic and

biotic elicitors have been used to induce or enhance the biosynthesis of secondary metabolites by stimulating plant cellular stress responses (DiCosmo *et al.*, 1985; Ramachandra Rao and Ravishankar, 2002). Plant-originated signaling molecules such as salicylic acid (SA), methyl jasmonate (MJ), and ethylene as well as microbe-derived molecules such as polysaccharides, glycoproteins, and inactivated enzymes have been utilized for elicitation of secondary metabolites (Bennett and Wallsgrove, 1994; Benhamou, 1996)

Here, I tested the abilities of plant-derived elicitors to activate the type III polyketide biosynthesis pathway in order to improve the production of tricyclic aromatic quinones. I analyzed the changes in metabolic profile and anti-inflammatory activity in the extracts of elicitor-treated adventitious roots. This work shows that SA activates the type III polyketide biosynthesis pathway, resulting in improved production of tricyclic aromatic quinones and increased anti-inflammatory activity. It suggests that the plant specific-type III polyketide biosynthetic pathway is regulated by endogenous SA signaling, and that the efficacy of *Aloe vera* as a medicinal agent can be improved through SA treatment.

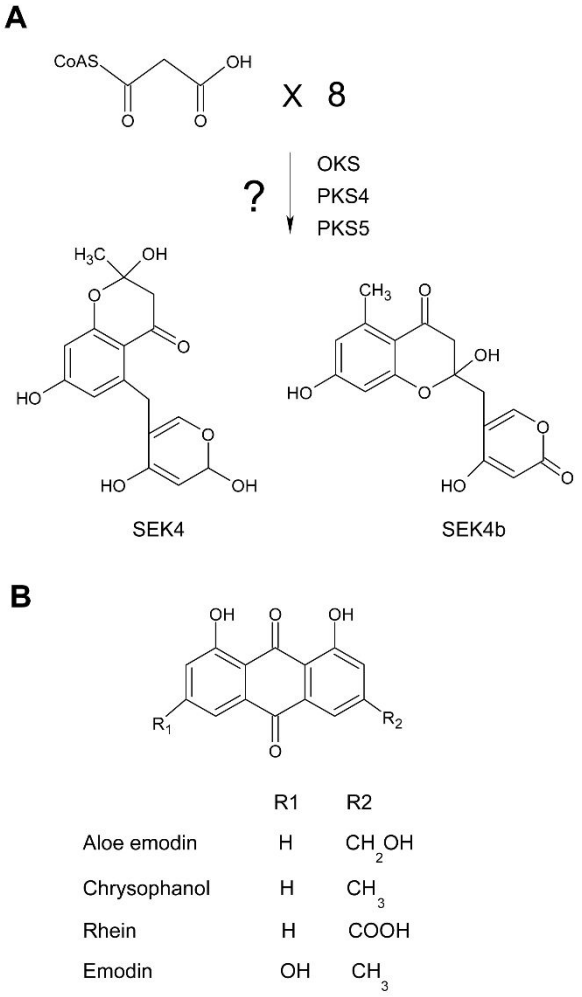

Figure III-1 Tentative biosynthesis mechanism for tricyclic aromatic quinones. (A) Production of SEK4 and SEK4b compounds catalyzed by OKS, PKS4, and PKS5 in *E. coli*. (B) The chemical structures of tricyclic aromatic quinone derivatives.

MATERIAL AND METHODS

Chemicals and reagents

All plant growth media and growth hormones were obtained from Duchefa (Haarlem, The Netherlands). Aloe emodin, chrysophanol, aloin, rhein, and emodin were purchased from Santa Cruz Biotechnology (California, USA). Malonyl-CoA, succinyl-CoA, acetyl-CoA, other chemicals, and solvents were from Sigma-Adrich (St. Louis, USA). All reference standards were dissolved in 100% methanol, and MJ and SA were dissolved in 99.9% (v/v) ethanol. 2-chloroethylphosphonic acid (ethephon) was freshly dissolved in distilled water. Dissolved MJ, SA, and ethephon were sterilized through 0.45 μm membrane filters (Whatman, Tokyo, Japan)

Optimization of suspension culture conditions and elicitor treatments

Young shoots of *Aloe vera* were provided by Kim Jeong Moon Aloe Co. Ltd (Jeju, Korea). Explants of young leaves were inoculated onto MS medium (Murashige and Skoog,1962) supplemented with 30 g/L sucrose, 0.5 mg/L 1-naphthaleneacetic acid (NAA), and 0.7 g/L plant agar to induce adventitious roots as described previously (Lee *et al*., 2011). Three weeks old adventitious roots grown in solid media were transferred into 30 mL liquid media supplemented with various growth hormone conditions (0, 0.1, 03, and 0.5 mg/L of IAA and IBA) and media

(1/2 MS, MS, 2 MS, B5, and SH) in order to find out optimal suspension culture condition of *Aloe vera* adventitious roots. The induced adventitious roots were incubated on an orbital shaker (50 rpm) at 25℃ under constant light conditions (light intensity: 7 μE/m^2s). Various concentrations of MJ, SA, and ethephon were added to the medium to treat 35-d-old adventitious roots.

Estimate of adventitious root growth

The adventitious roots were harvested every 7 d up to 42 d during growth. Fresh weight was determined to 0.05 g accuracy, and then dry weight (DW) was measured after lyophilization. The growth ratio was calculated as follows: DW of harvested roots - DW of inoculated adventitious roots (initial DW) / initial DW as previously reported (Jung *et al*., 2002).

Quantification of aloe emodin and chrysophanol in intracellular and extracellular

The extraction of aloe emodin and chrysophanol from the culture medium was performed as previously reported with some modifications (Chiang *et al*., 2007). Harvested growth medium (30 mL) was supplemented with XAD-4 (0.07 g) and continuously agitated at 125 rpm for 5 days at 25℃. Then, the XAD-4 was collected by vacuum filtration and resuspended in 5 mL 100% ethanol followed by re-

incubation for 5 days at 25℃ with 125 rpm agitation. The extract in ethanol was concentrated to 500 μL. The HPLC analysis conditions for aloe emodin and chrysophanol within the adventitious roots and culture media were described previously (Lee *et al*., 2011). All samples obtained from the same condition were analyzed with three independent replicates and all experiments were repeated twice.

Sample preparation for UPLC-ESI-MS

For analysis of secondary and primary metabolites in cultured adventitious roots, lyophilized *Aloe vera* adventitious roots that were treated with 0, 500, 1000, and 2000 μM SA for 24 h (180 mg, n = 5) were extracted with 70% ethanol under sonication for 30 min at room temperature. The extracts were centrifuged at 14000 x g for 10 min at room temperature and then completely dried under nitrogen gas. The dried residue was resuspended in 200 μL 70% methanol and filtered through a 0.2 μm polytetrafluoroethylene (PTFE) membrane filter (Whatman, Tokyo, Japan). Each sample was collected in quintuplicate from independent culture flasks and experiments were performed twice.

Sample preparation for GC-MS

The 50 μL ethanolic extracts were completely dried under nitrogen gas, and agitated with N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) + 1%

trimethylchlorosilane (TMCS) and pyridine (1:2) at 60 °C for 15 min. The solutions were then transferred into 2 mL glass vials coupled with micro-inserts (Agilent, Santa Clara, CA) and capped immediately.

UPLC-ESI-MS analysis

The extracts were analyzed with a Waters Alliance LC coupled to Quattro microMS on AQUITY UPLC BEH C_{18} columns (2.1 x 100 mm, 1.7 μm). The UPLC-ESI-MS was carried out using mixtures of 0.1% aqueous formic acid (solvent A) and 100% acetonitrile (solvent B) at 0.1 mL/min flow rate and maintained at 40 °C. The elution program was as follows: 30% B at 0 min, 30% B at 5 min, 35% B at 10 min, 70% B at 35 min, 70% B at 45 min, and 100% B at 50 min. The injection volume was 10 μL and capillary voltages were adjusted to +4.0 kV for positive mode and to -4.0 kV for negative mode. MS/MS analysis was performed using collision energy from 5 to 60 eV in positive and negative modes.

GC-MS analysis

The primary metabolites in aloe extracts were analyzed using a 6890 gas chromatograph (Agilent Technologies, CA, USA) coupled with a JMS-GC mate (Jeol, Tokyo, Japan). A DB-5 column (30 m × 0.25 mm I.D., 0.25 μm film thickness, HP) was used with helium (99.9999% He) as a carrier gas at a constant

flow of 1 mL/min. The oven temperature was held at 60 °C for 5 min, ramped to 320 °C at a rate of 10 °C/min and held for 10 min. One microliter sample was injected in split mode (10:1). The ionization energy was 70 eV in electron impact mode. The transfer line and ion source temperatures were set at 300 °C and 300 °C, respectively. After a 300 sec solvent delay, mass spectra were obtained at 20 scans per second with a mass range of 55-600 m/z.

Data processing and multivariate analysis

All raw data obtained from GC-MS were converted to ASCII format. The raw data were reduced into 6 sec buckets and normalized by the total sum of intensities as previously reported (Ong *et al*., 2008). Raw data files obtained from UPLC-ESI-MS were exported to MZ-mine software version 2.1 and filtered through the Savitzky-Golay filter method to remove the noise. The baseline was then corrected and peaks were detected. Drifted retention time (RT) between replicated samples was adjusted through peak alignment. Finally RT was normalized to reduce the deviation of RT between peak lists, and data alignment was performed using RANSAC aligner.

The peak lists resulting from GC-MS and UPLC-ESI-MS were evaluated using multivariate analysis with SIMCA-P 12.0 (Umetrics, Umeå, Sweden). Unsupervised principal component analysis (PCA) was performed, and supervised partial least square discriminant analysis (PLS-DA) and orthogonal partial least

squares-discriminant analysis (OPLS-DA) were processed to compare each elicitor-treated condition and obtain differential metabolites from the elicitor conditions. The major metabolites that were differential between the elicitor conditions were regarded as variable importance in the project (VIP) list and m/z having a cutoff score above the 1 was selected in the PLS-DA model.

Statistical analysis of raw files obtained from GC-MS, UPLC-ESI-MS, and results of other experiments were carried out using Statistica Version 10 (StatSoft Inc., OK, USA), and the Tukey Honestly Significant Difference (HSD) and Least Significant Difference (LSD) test were performed at probability level of 0.05.

Metabolite identification

The compounds were identified based on mass spectra and retention time of authentic compounds. Peaks obtained from GC-MS were identified by comparison with spectra of the National Institute of Standards and Technology (NIST) library on the basis of MS spectra. Among the m/z from UPLC-ESI/MS, major compounds obtained from multivariate or statistical analysis were tentatively identified by MS/MS spectra.

Isolation and RNA expression analysis of octaketide synthase genes in *Aloe vera*

Total RNA was extracted by a modified lithium chloride method according to previous reports (Altenbach *et al*., 1981). The cDNA synthesis was conducted using the Maxime^{TM} RT PreMix (Oligo $(dT)_{15}$ Kit (iNtRON, Sungnam, Korea) according to the manufacturer's protocol. Synthesized cDNA was diluted 1/5 and used as template to isolate *OKS* genes and for real-time PCR.

Full length *OKS* genes were isolated using primers designed from *AaOKS* (Accession number: AY567707) and *AaPKS4* (Accession number: FJ536166.1). PCR was performed as follows: 94℃ for 5 min and 35 cycles of 95℃ for 30 s, 45℃ for 30 s, and 72℃ for 1 min 30 s. The obtained PCR products were cloned into T-blunt cloning vector (Solgent, Daejeon, Korea) and sequenced using an ABI 3730 XL DNA Analyzer (Applied Biosystems, CA, USA) with M13 Forward and reverse primers. The inserts of positive clones were amplified to obtain *OKS* candidate genes.

To carry out real time PCR, the *Ubiquitin* gene isolated from *Aloe vera* was used to normalize the C_T values of target genes (Accession No. EF539181). Gene-specific primers were designed using the Primer 3 program (Rozen *et al*., 2000) and specificity was confirmed by sequencing with an ABI 3730 XL DNA Analyzer. PCR reactions were carried out using a Light cycler 480 (Roche, Mannheim,

Germany). The thermal cycling conditions were as follows: 95 ℃ for 5 min and 40 cycles of 95℃ for 15 s, 58℃ for 10 s, and 72℃ for 10 s. The primer sequences used to amplify each gene are summarized in Table II-1.

Gene expression levels were analyzed by reverse transcription quantitative PCR (RT-qPCR) using gene specific primer sets. Quantities of total RNA were normalized by comparison of band intensities for *Ubiquitin*. Thermal cycling conditions for RT-qPCR were as follows: 94℃ for 5 min and 28 cycles of 94℃ for 30 s, 58℃ for 30 s, and 72℃ for 5 min. The amplified PCR products were separated on 2% agarose gel.

Luciferase activity driven by *COX-2*, *AP-1* and *NF-κB*

The JB6 P+ mouse epidermal cell line which was kindly provided by Dr. Zigang Dong (University of Minnesota, Austin MN) (Lee *et al*., 2009) was cultured in monolayers on minimal essential medium (MEM) supplemented with 5% (v/v) fetal bovine serum (FBS) and 0.1% penicillin/streptomycin at 37℃ in a humidified atmosphere of 5% CO_2. The JB6 P+ cells were stably transfected with COX-2, AP-1, or NF-κB luciferase reporter plasmid containing the G418 resistance gene and maintained in 5% FBS-MEM supplemented with 200 mg/mL G418. The cells were seed to 96-well plate, and the plates were incubated in a 5% CO_2 incubator at 37℃.

When cultured cells reached approximately 80% to 90% confluency, the cells were starved with 0.1% FBS-MEM for 24 h. After that, cells were treated with various concentrations (0, 10, 20, 40, and 100 μg/mL) of *Aloe vera* adventitious root extracts (non-treated, 500 μM SA-treated, 1000 μM SA-treated, and 2000 μM SA-treated) for 1 h followed by exposure to UVB (0.05 J/cm^2) and incubation for 4 h. JB6 P+ cells treated with UVB were disrupted with 100 μL lysis buffer [0.1 M potassium phosphate buffer (pH 7.8), 1% Triton X-100, 1 mM DTT, and 2 mM EDTA]. The luciferase activity was measured using a luminometer (Luminoskan Ascent; Thermo Electron, Helsinki, Finland).

Table III-1 Primer sets used in this study

	Primer	Sequence
Gene cloning	OKS F	5'-ATGAGTTCACTCTCCAACGC-3'
	OKS R	5'-TCACATGAGAGGCAGGCTG-3'
	PKS4 F	5'-ATGAGTTCACTCTCCAACTCTC-3'
	PKS4 R	5'-TTACATGAGAGGCAGGCTGTGA-3'
Real-time PCR	OKS F	5'-GGCGGGTCTGATGTTCTAC-3'
	OKS R	5'-CGAAACTTCTCGGGACGA-3'
	OKSL-1 F	5'-AGATGGAACCGCAACAGT-3'
	OKSL-1 R	5'-TGGGAAACTTCTTGAGGAAT-3'
	Ubiquitin F	5'-GGTGGAGTCTTCGGATACCA-3'
	Ubiquitin R	5'-TGCTCTCCTTCTGGATGTTG-3'

RESULTS

Optimization for suspension culture condition of *Aloe vera* adventitious root

I tried to optimize the suspension culture condition to facilitate growth of *Aloe vera* adventitious root. Three-weeks-old adventitious roots cultured on solid media were transferred into MS liquid media including 30 g/L sucrose, together with 0.5 mg/L IBA, IAA or NAA, respectively. After 4 weeks culture, the adventitious roots were ordinarily grown in MS media supplemented with IAA and IBA, but not with NAA, resulting in induction of anomalous callus from adventitious roots. According to the result, I chose the media with IAA and IBA for following experiments to produce biomass and secondary metabolites.

Then I examined the influence of various growth media and IBA and IAA condition on production of biomass and aloe emodin and chrysophanol due to representative secondary metabolites in *Aloe vera.* Compared to them, MS media supplemented with 0.3 mg/L IBA was most suitable for biomass and aloe emodin and chrysophanol production (Table III-2). The adventitious roots grown in the MS media supplemented with 0.3 mg/L IBA reached the maximum production of biomass and aloe emodin and chrysophanol at 35 day (Figure III-2A and Figure III-2B).

Next, I also performed kinetic analysis in the adventitious roots-cultured

media (MS media supplemented with 0.3 mg/L IBA) to determine optimal timing for elicitor treatment. Sucrose, fructose, glucose, nitrate, and phosphate in the media were analyzed throughout the cultivation of adventitious roots (Figure III-2C). Sucrose began to sharply fall within 14 days and it was completely hydrolyzed into glucose and fructose as previous results (Sturm and Tang 1999). The fructose and glucose detected in the growth media at 28 days. Fructose content was gradually decreased at 35 day while glucose level remained subtle, indicating that it seemed to be not preferred as nutrient for growth. The phosphate and nitrate were gradually absorption, and then began to be exhausted at 35 days similar with glucose.

Table III-2 Effect of plant hormones and media on growth of *Aloe vera* adventitious roots and accumulation of aloe emodin and chrysophanol after 35 days

Plant growth condition (mg/L)	Growth ratio*	Aloe emodin (μg/g)*	Chrysophanol (μg/g)*
0.1 IAA	0.79±0.11^{b}	3.08±0.63^{ab}	34.93±14.02^{a}
0.3 IAA	1.32±0.11^{ab}	2.99±0.27^{ab}	46.63±0.35^{ab}
0.5 IAA	1.38±0.36^{ab}	2.36±0.39^{a}	33.36±0.74^{a}
0.1 IBA	1.12±0.34^{ab}	1.69±0.04^{a}	35.73±0.92^{a}
0.3 IBA	2.42±0.93^{a}	4.42±0.54^{b}	63.65±1.28^{b}
0.5 IBA	0.99±0.61^{b}	2.25±0.31^{a}	37.52±0.97^{a}
0.3 IBA+MS	2.44±0.52^{b}	3.59±1.92^{a}	45.07±3.51^{a}
0.3 IBA+1/2 MS	1.16±0.46^{a}	1.39±0.29^{a}	18.34±0.60^{b}
0.3 IBA+2 MS	0.71±0.28^{a}	3.05±0.17^{a}	42.12±7.97^{a}
0.3 IBA+B5	1.31±0.42^{ab}	2.39±1.10^{a}	38.99±5.78^{a}
0.3 IBA+SH	1.03±0.40^{a}	2.30±0.91^{a}	36.77±0.58^{a}

* The different characters in each column mean significantly differences ($P<0.05$) according to LSD test

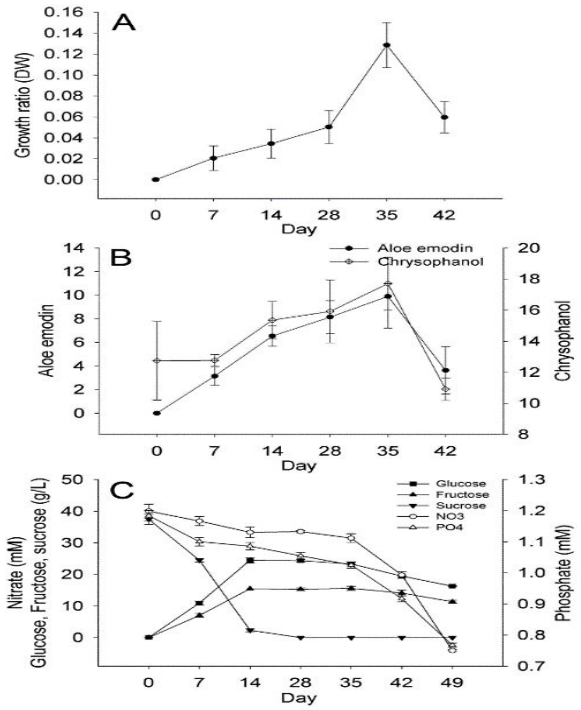

Figure III-2 Kinetic analysis of *Aloe vera* suspension culture. (A) Biomass accumulation of *Aloe vera* adventitious roots cultivated on MS liquid media supplemented with 0.3 mg/L IBA. (B) Accumulation patterns of aloe emodin and chrysophanol in *Aloe vera* adventitious root cultured on MS media including 0.3 mg/L IBA. (C) Nutrient consumption pattern in growth media during 42-day cultivation.

Optimization of elicitor effects on accumulation of aloe emodin and chrysophanol in *Aloe vera* adventitious roots

Elicitation conditions were investigated using using *Aloe vera* adventitious roots cultured under optimized suspension culture conditions. MJ, SA, and ethaphon were added in various concentrations to 35-d-old adventitious roots, and contents of aloe emodin and chrysophanol in the adventitious roots and in the media were measured, respectively. Maximum production of aloe emodin and chrysophanol occurred in adventitious roots treated with 1000-2000 μM SA (Figure III-3). Time course analysis revealed that when adventitious roots were treated with various concentrations of SA, MJ, and ethephon, accumulation of aloe emodin and chrysophanol in adventitious roots was increased by more than 10-11 and 5-13 folds at 24 h, respectively, and that in the growth medium rose at 24-72 h of 2000 μM SA treatment (Figure III-4A and III-4B). Treatment with 500 μM MJ and 500 μM ethephon also led to increases in aloe emodin and chrysophanol at 24 h (Figure III-3B and III-3C). With 500 μM MJ, endogenous levels of aloe emodin and chrysophanol steadily increased and spiked at 24 h with over 4-7-folds and 3-5 folds increases, respectively. Exogenous levels of these metabolites increased at 48-72 h following MJ treatment (Figure III-4C and III-4D). Treatment with 500 μM ethephon induced endogenous aloe emodin and chrysophanol at 12 h, with 5- and 4-folds increases, whereas it did not promote secretion of the these metabolites into the growth culture medium (Figure III-4E and III-4F). SA treatment showed the

most marked effect on production of aloe emodin and chrysophanol, and thus I investigated the responses of the tricyclic aromatic quinone biosynthesis pathway to SA elicitation.

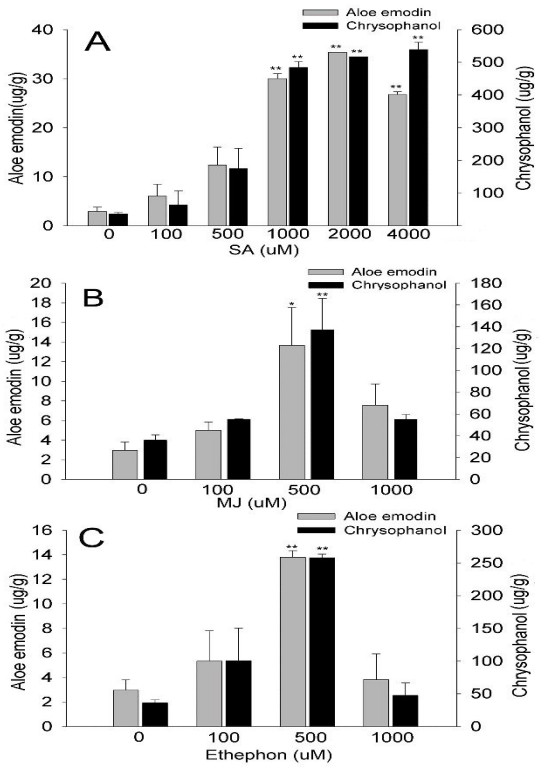

Figure III-3 Effect of elicitor treatments on aloe emodin and chrysophanol production in *Aloe vera* adventitious roots. Aloe emodin and chrysophanol production in *Aloe vera* adventitious roots treated with SA (A), MJ (B), and ethephon (C) for 24 h. Data are represented as means of replicate samples ± standard deviation. Statistical analysis was carried out using the Tukey test (* $p<0.05$, ** $p<0.01$). Asterisks indicate significant differences compared to aloe emodin and chrysophanol contents obtained from non-treated adventitious roots.

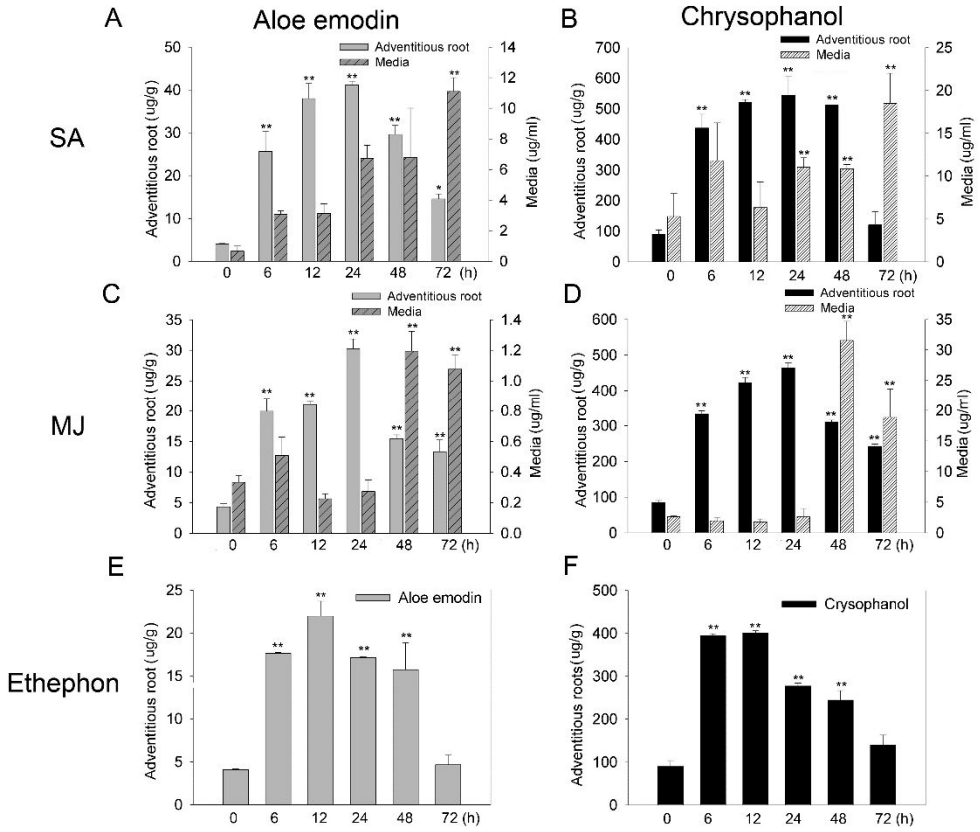

Figure III-4 Time-course analysis of aloe emodin and chrysophanol production following elicitation. Content of aloe emodin (A, C, and E) and chrysophanol (B, D, and F) in adventitious roots and culture medium following non and elicitation with 2000 μM SA (A and B), 500 μM MJ (C and D), or 500 μM ethephon (E and F). Data are represented as means of replicate samples ± standard deviation. Statistical analysis was carried out using the Tukey test (* $p<0.05$, ** $p<0.01$). Asterisks indicate significant differences compared to aloe emodin and chrysophanol contents obtained from adventitious roots before elicitation.

Primary metabolite analysis using GC-MS and LC-MS

I speculated that SA might affect primary metabolites that play roles as intermediates in the biosynthetic pathways for secondary metabolites as well as induce considerable changes in secondary metabolites including aloe emodin and chrysophanol. To monitor alterations in primary metabolites including malonyl-CoA, which is a precursor for the biosynthesis of tricyclic aromatic quinones, extracts from *Aloe vera* adventitious roots treated with 0, 500, 1000, and 2000 μM SA for 24 h were analyzed by GC-MS and LC-MS. Primary metabolites profiling analyzed by GC-MS did not reveal any difference upon treatment with different concentration of treated SA. The score plot of OPLS-DA showed that the SA-treatment replicates did not form separate clusters based on peak intensity (Figure III-5A). Similar patterns were observed in the PCA and PLS-DA score plots (data not shown). Peaks associated with glycolysis and the tricarboxylic acid cycle (TCA), which is the principal pathway to form malonyl-CoA, were not significantly changed (Figure III-5B and Table III-3) and were not segregated clearly in the same multivariate analyses (data not shown). Interestingly, LC-MS analysis revealed that malonyl-CoA was remarkably decreased in SA-treated adventitious roots in a concentration-dependent manner, suggesting that consumption of malonyl-CoA is affected by biosynthesis of tricyclic aromatic quinones including aloe emodin and chrysophanol (Figure III-5B and Table III-3). Succinyl-CoA and acetyl-CoA, which are involved in the TCA cycle, could not be detected in either non- elicited or

elicited samples elicited or elicited samples.

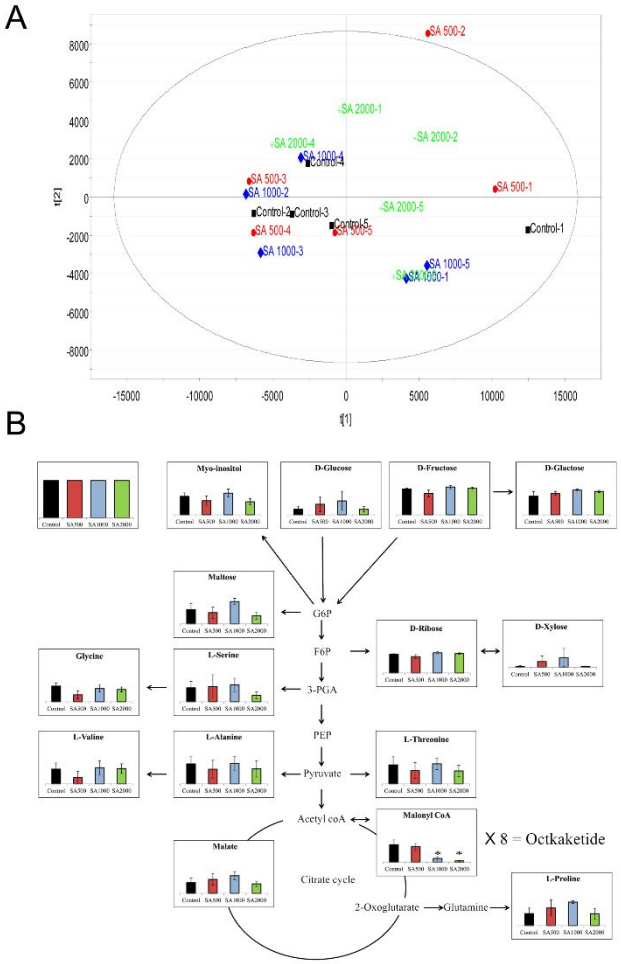

Figure III-5 Alteration of primary metabolites in *Aloe vera* adventitious roots in response to SA. (A) OPLS-DA of primary metabolites obtained from adventitious roots treated with 0, 500, 1000, or 2000 μM SA analyzed by GC-MS. (B) Alteration of primary metabolites associated with TCA and glycolysis in response to SA elicitation. The level of malonyl-CoA decreased in a SA dose-dependent manner. Levels of other metabolites did not change in response to SA elicitation. G6P: Glucose-6-phosphate, F6P: Fructose 6-phosphate, PEP: phosphoenolpyruvate.

Table III-3 Alteration of primary metabolites in *Aloe vera* adventitious roots following SA treatment.

Class	Retention time	Compounds	Fold change (Relative to control)		
			SA500	SA1000	SA2000
Amino acids	13.3	L-alanine[ab]	0.73	1.02	0.75
	17.0	L-valine[ab]	0.43	1.10	1.04
	19.4	glycine[ab]	0.45	0.84	0.79
	21.1	L-serine[ab]	1.08	1.20	0.46
	21.6	L-threonine[ab]	0.70	1.06	0.68
	25.1	L-proline[ab]	1.46	1.95	0.99
Carboxylic acids	24.3	malic acid[ab]	1.30	1.64	0.88
Sugar alcohols	25.5	threitol[ab]	0.63	1.14	0.57
	37.2	myo-inositol[ab]	0.77	1.18	0.71
Sugars	32.1	D-xylose[ab]	6.72	11.19	1.05
	33.2	D-ribose[ab]	0.87	1.07	1.03
	33.3	D-fructose[ab]	0.83	1.08	1.03
	33.4	D-glucose[ab]	1.87	2.47	0.97
	33.5	galactose oxime[ab]	1.14	1.33	1.26
	34.3	mannose[ab]	0.92	1.25	0.57
	34.5	mannoonic acid[ab]	ND	2.43	1.71
	36.2	D-ribofuranoside[ab]	0.74	0.85	1.04

	39.3	D-mannopyranose[ab]	0.84	1.13	0.39
	49.2	melibiose[ab]	0.68	1.49	0.80
	50.0	maltose[ab]	0.79	1.57	0.58
	44.3	unknown[b]	3.10	1.98	1.35
	46.4	unknown[b]	0.85	1.19	0.60
	47.2	unknown[b]	0.75	1.13	0.47
	47.4	unknown[b]	0.72	1.01	0.50
	50.2	unknown[b]	0.68	1.86	0.26
	56.0	unknown[b]	0.82	0.95	0.58
	58.2	unknown[b]	3.41	1.27	3.10
	59.1	unknown[b]	0.33	0.37	0.57
	60.0	unknown[b]	0.26	2.58	5.02
CoA derivatives	3.1	malonyl CoA[c]	0.88*	0.21**	0.07**
	3.1	acetyl CoA[c]	ND	ND	ND
	3.0	succinyl CoA[c]	ND	ND	ND

[a] Identified by NIST Mass Spectral Library;

[b] Analyzed by GC/MS;

[c] Analyzed by LC/MS in negative mode

ND indicates 'not detectable'

[*] Asterisks indicate significant differences compared to control intensity (Tukey test, $p<0.05$*, $p<0.01$**)

Isolation and gene expression of *Aloe vera* octaketide synthase genes

The decrease in malonyl-CoA might result from activation of the type III polyketide biosynthesis pathway. To investigate changes in expression levels of the *OKS*s following SA treatment, the full length cDNAs for *Aloe vera OKS* (*AvOKS*) and *OKS like-1* (*AvOKSL-1*) were isolated from adventitious roots based on the sequences of *Aloe arborescense OKS* (*AaOKS*, Accession number : AY567707) and *PKS4* (*AaPKS4*, Accession number : FJ536166.1). Each *AvOKS* candidate gene had a 1212-bp open reading frame (ORF). Compared with *AaOKS*, *AaPKS4*, and *AaPKS5* in *Aloe arborescense,* the deduced amino acid sequences of *AvOKS* and *AvOKSL-1* were 90-99% identical and included conserved active sites such as the chalcone synthase (CHS) active sites (Met 147, Gly 221, Gly 226, and Pro 388), the catalytic triad of CHS (Cys 174, His 316, and Asn 349), gatekeepers (Phe 225 and Phe 275), and Gly 207, Leu 266, and Val 351 (Figure III-6 and Table III-4) (Mizuuchi *et al*., 2009).

Real-time PCR and RT-qPCR analysis showed that the expression of *AvOKS* and *AvOKSL-1* was increased in proportion to the concentration of treated SA (Figure III-7 and Figure III-8). The transcripts for *AvOKS* and *AvOKSL-1* were up-regulated more than 6-fold at 6 h of 1000 μM SA elicitation. This suggests that SA induced the expression of *AvOKS* and *AvOKSL-1*, and in turn, the elevated enzyme activity accelerated the condensation of malonyl-CoA, resulting in increased production of tricyclic aromatic quinone derivatives including aloe

emodin and chrysophanol.

Figure III-6 Alignment of amino acid sequences of *Aloe vera* OKS and OKSL-1. OKS and OKSL-1 sequences from *Aloe vera* were compared with OKS from *Aloe arborescens* (Accession: AY567707.1), PKS4 from *Aloe arborescens* (Accession: FJ536166.1), and PKS5 from *Aloe arborescens* (Accession: FJ536167.1).

Table III-4 Comparison between amino acid sequences identities of OKS and OKSL-1 from *Aloe vera* and OKS, PKS4, and PKS5 from *Aloe arborescens.*

	AaOKS	AaPKS4	AaPKS5
AvOKS	401/403 (99%)	401/403 (99%)	366/403 (91%)
AvOKSL-1	388/401 (97%)	388/401 (97%)	363/403 (90%)

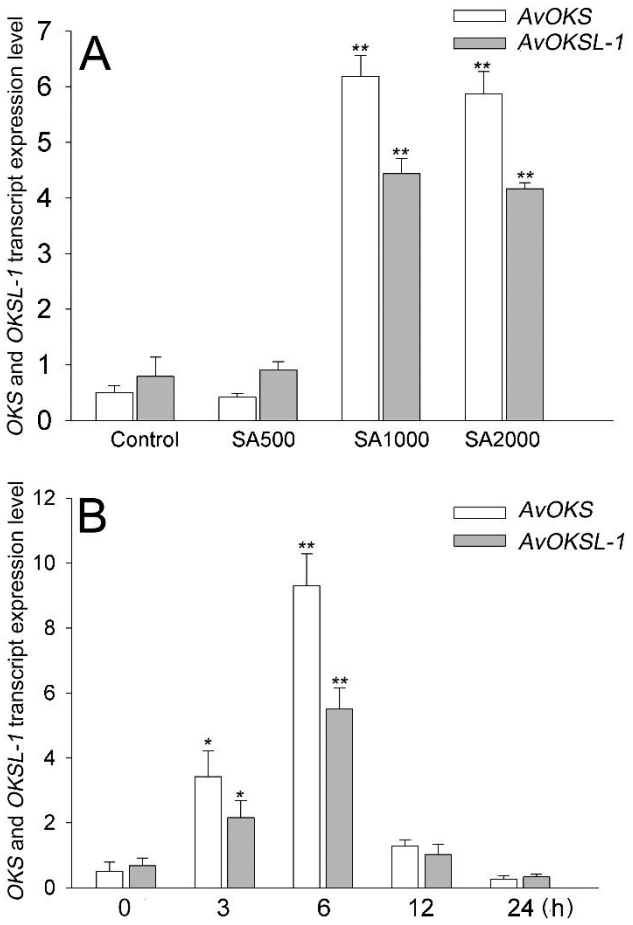

Figure III-7 Effect of SA elicitation on transcript accumulation of *OKS* and *OKSL-1*. (A) Transcript accumulation of *OKS* and *OKSL-1* at 6 h of 0, 500, 1000, and 2000 μM SA treatment relative to that of *Ubiquitin*. (B) Time course analysis of gene expression of *OKS* and *OKSL-1* in the presence of 1000 μM SA relative to that of *Ubiquitin*. Each value is the mean of replicates and error bars mean standard deviation. Statistical analysis was carried out using the Tukey test (* $p<0.05$, ** $p<0.01$). Asterisks indicate significant differences compared to control groups.

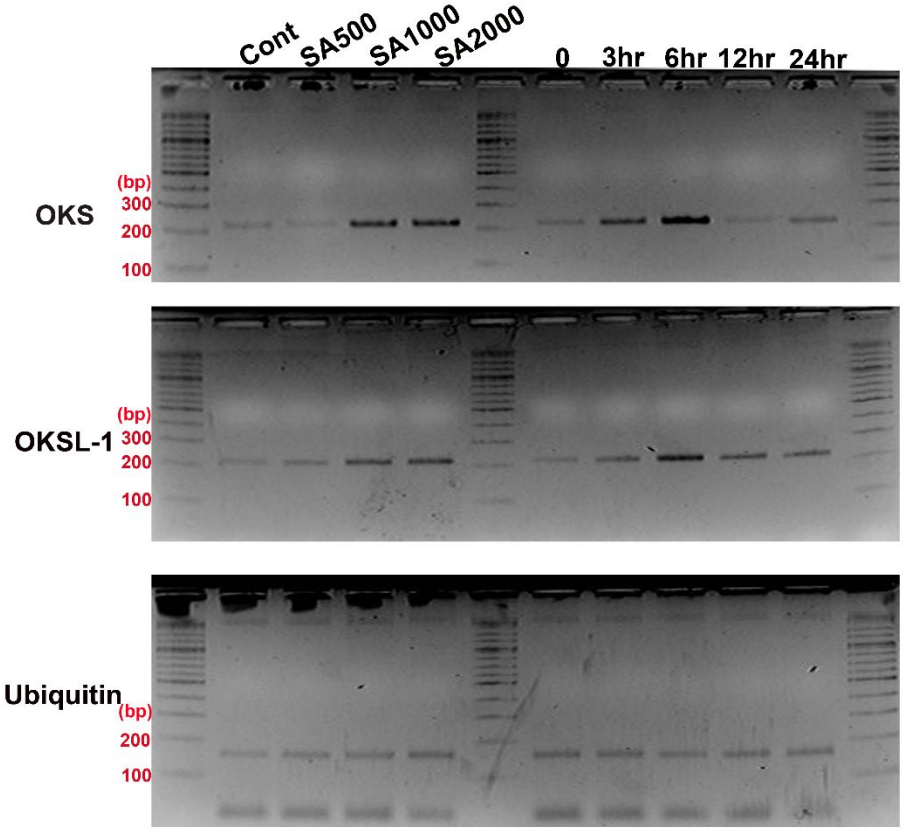

Figure III-8 Expression levels of *OKS* and *OKSL-1* in response to SA treatment. RT-qPCR expression profile of *OKS* and *OKSL-1* at 6 h of 0, 500, 1000, and 2000 μM SA treatment (Lane : Cont, SA500, SA1000, and SA2000) and time course analysis of gene expression of *OKS* and *OKSL-1* in the presence of 1000 μM SA (Lane : 0, 3hr, 6hr, 12hr, and 24hr). Quantities of total RNA were normalized by comparison with the band intensity for *Ubiquitin*, and the PCR products for *Ubiquitin*, *OKS,* and *OKSL-1* were separated on 2% agarose gels.

Global metabolite analysis

I carried out UPLC-ESI/MS analysis in adventitious roots treated with 0, 500, 1000, or 2000 μM SA for 24 h to identify which metabolites along with aloe emodin and chrysophanol were induced by SA. Based on RT and mass-to-charge ratio (m/z), 1850 and 634 peaks were obtained in positive and negative mode, respectively (Figure III-9). According to multivariate results of the UPLC-ESI-MS data sets, the PCA and PLS-DA score plots showed clear segregation between elicitor-treated groups and the untreated group in positive and negative modes (Figure III-10 and Table III-5). The OPLS-DA analysis also revealed obvious differences among different SA-treated groups in positive and negative modes, indicating that metabolite alterations were influenced by SA concentration (Figure III-11 and Table III-5). The compounds in the UPLC-ESI-MS data sets induced by SA treatment were classified by one way analysis of variance (ANOVA) ($p<0.05$) in comparison with the control to sort significantly increasing variables. I eliminated variables below 200 m/z because the basic structure of tricyclic aromatic quinones has a molecular weight above 200 m/z. Consequently, I obtained 370 and 130 variables, which were assigned using RT and m/z, in positive and negative modes, respectively (Table III-6).

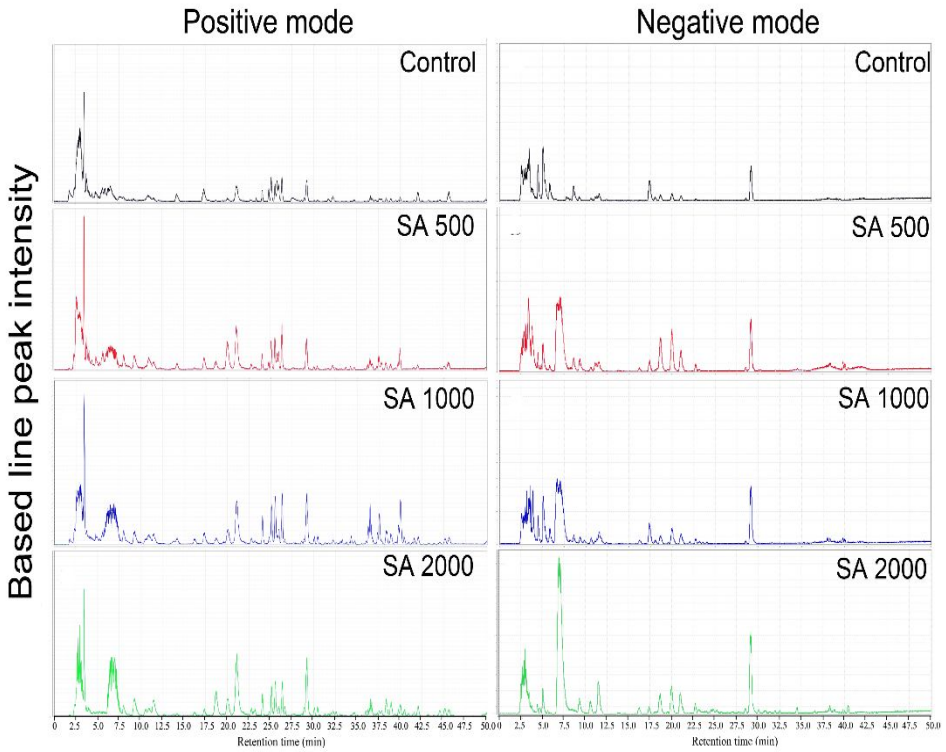

Figure III-9 Chromatograms of control, 500 (SA 500), 1000 (SA 1000), and 2000 (SA 2000) μM SA treated adventitious roots through UPLC-ESI-MS in positive and negative modes.

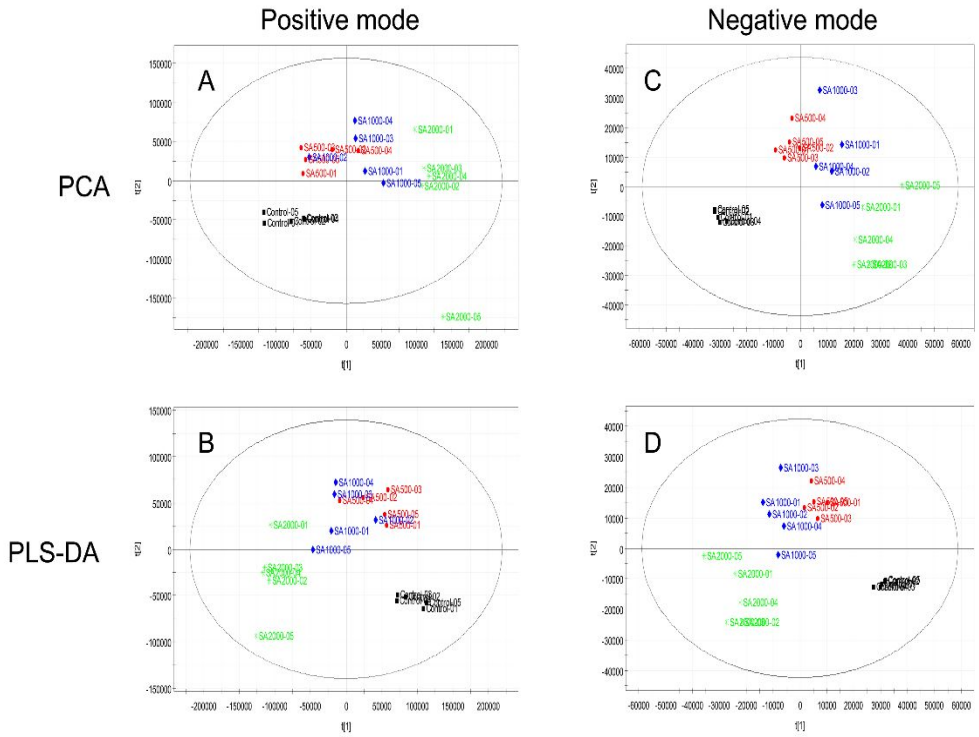

Figure III-10 PCA and PLS-DA score plots. The PCA (A, C) and PLS-DA (B, D) score plots of control (black), 500 (red), 1000 (blue), and 2000 (green) μM SA-treated adventitious roots analyzed by UPLC-ESI-MS in positive (A, B) and negative (C, D) mode.

Table III-5 Statistical parameters of PCA, PLS-DA, and OPLS-DA in positive and negative modes.

Treatment	Model	Positive mode		Negative mode	
		R^2X	Q^2	R^2X	Q^2
Control, 500 µM SA, 1000 µM SA, and 2000 µM SA	PCA	0.44	0.10	0.62	0.40
	PLS-DA	0.56	0.76	0.52	0.76
	OPLS-DA	0.68	0.51	0.80	0.68
Control, 500 µM SA, and 500 µM MJ	PCA	0.51	0.33	0.53	0.33
	PLS-DA	0.56	0.87	0.53	0.61
	OPLS-DA	0.62	0.86	0.53	0.60

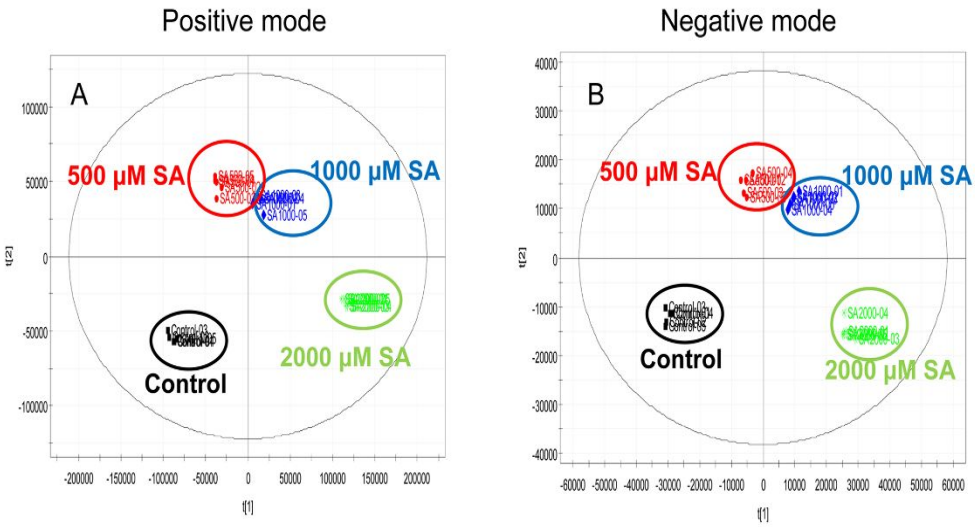

Figure III-11 OPLS-DA score plot. OPLS-DA score plots of control (black), 500 µM SA (red), 1000 µM SA (blue), and 2000 µM SA (green)-treated adventitious roots analyzed by UPLC-ESI-MS in positive (A) and in negative (B) mode.

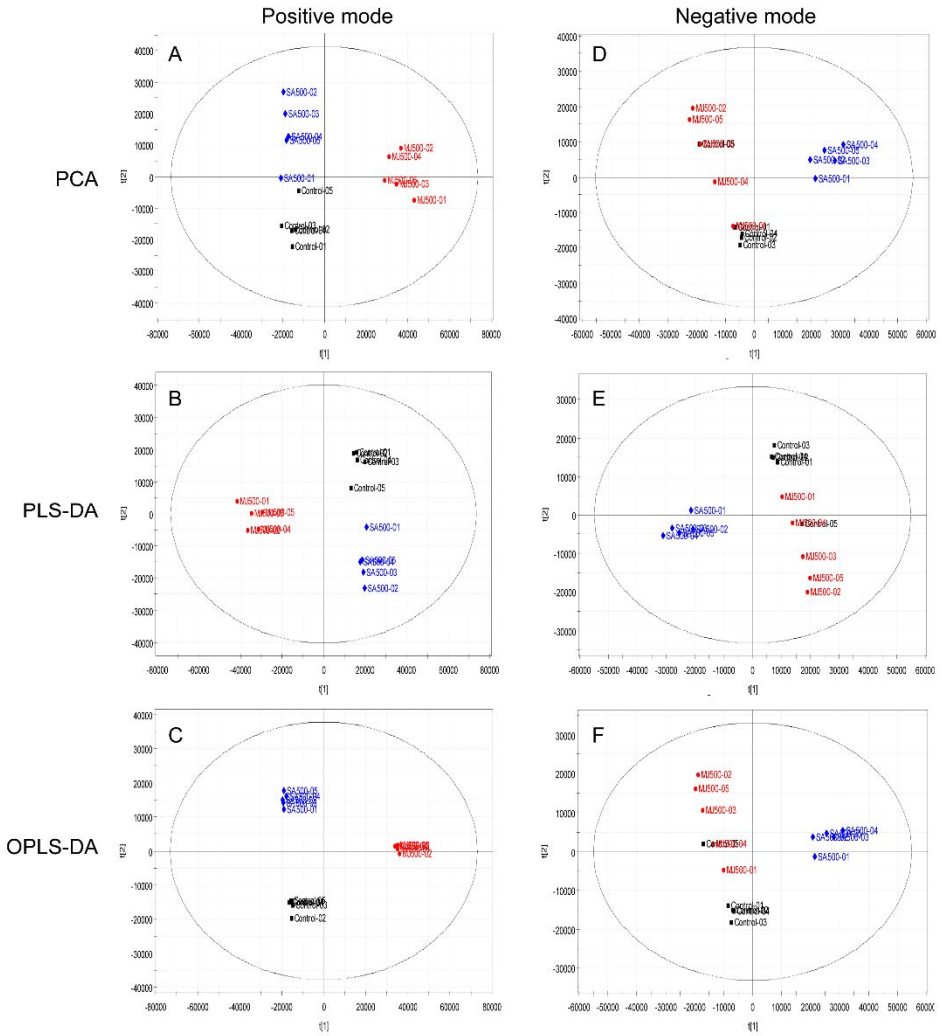

Figure III-12 PCA, PLS-DA, and OPLS-DA score plots. PCA (A and D), PLS-DA (B and E), and OPLS-DA (C and F) score plots of control (black), 500 μM SA (blue), and 500 μM MJ (red)-treated adventitious roots analyzed by UPLC-ESI-MS in positive (A, B, and C) and negative (D, E, and F) mode.

Table III-6 Statistically significant peak numbers

Mode	Peak detection	Induced peaks[a]			Reduced peaks[a]
		SA500	SA1000	SA2000	
Negative	634	40	20	70	114
Positive	1850	172	63	135	267

[a] Number of induced and reduced peaks is based on comparison with peaks of control groups

Identification of SA-induced metabolites using UPLC-ESI-MS/MS

Significantly increased variables in positive and negative modes were analyzed by UPLC-ESI/MS/MS to obtain structural information for unknown compounds induced by SA elicitation. Tentative structures of 37 variables including chrysophanol (SA 18) and aloe emodin (SA 19) were identified in positive and negative modes based on the Chemical Analysis Working Group (Table III-7) (Sumner *et al*., 2007). These compounds mainly displayed losses of H_2O (18 Da), CO (28 Da), CH_3 (15 Da), CH_3COOH (60 Da), and COOH (45 Da), and in rare cases, degradation of CH_3CN (41 Da) was detected (Table II-6) (Song *et al*., 2011; Kiehne *et al*., 1996). Among the identified compounds, variables numbered SA 9, 12, 14, 18, and 28 were designated as VIPs that were capable of discriminating between the samples in PLS-DA models.

Chrysophanol (SA 18) and aloe emodin (SA 19) were observed in adventitious roots treated with 1000~ 2000 μM SA. Chrysophanol (SA 18) was detected at 36.4 min, predominately due to the elimination of a CO residue to produce 225 m/z. A MS/MS spectra of deprotonated aloe emodin (SA 19) was monitored at 19.5 min and produced one fragment at 240 m/z by loss of CHO. These MS/MS fragment patterns of aloe emodin and chrysophanol were the same as those of the authentic compounds.

A total of 3 compounds (SA 11, 15, and SA 16) were highly likely to be

tricyclic aromatic quinone derivatives. The MS/MS fragment patterns of SA 11 and SA 15 were similar to that of emodin, and SA 16 showed a similar fragment pattern to that of aloe emodin. SA 11 appeared at 21.2 min and gave a deprotonated molecule at 269 m/z. Those MS/MS spectra produced daughter ions of 241 m/z [M-H-CO]$^-$, 213 m/z [M-H-2CO]$^-$, and 195 m/z [M-H-2CO-H_2O]$^-$. This fragment pattern was similar to that of emodin (Ye *et al*., 2007), but the RT was different from that of the pure standard compound, implying that the compound was not emodin per se but might have a similar structure. The SA 15 compound eluted at 8.6 min and produced a deprotonated daughter molecule at 313 m/z. The base peak was further cleaved into 269, 241, 225, and 197 m/z ions. The 269 m/z resulted from elimination of COOH adduct. The 241 m/z ion was produced by disassociation of CO, followed by degradation of a hydroxyl group to form 225 m/z and a CO adduct to give 197 m/z. I tentatively determined that the COOH substitution might exist in a structure resembling emodin (Ye *et al*., 2007). In addition, the spectrum of SA 16 showed a similar pattern to aloe emodin. The initial fragment arose from disassociation of CH_3COCH_3 to produce 269 m/z and cleavage of CHO to form 240 m/z as the base peak. The corresponding structure is considered to resemble aloe emodin with a CH_3COCH_3 substitution.

Table III-7 UPLC-ESI/MS/MS data of metabolites induced by SA treatment in *Aloe vera* adventitious roots

Mode	No.	Precursor $[M\pm H]^{\pm}$	RT[a]	Fold change[b]			MS^{2C}	Tentative compounds
				SA500	SA1000	SA2000		
negative mode	SA1	299	3.7	38.2**	15.4**	1.6	MS2-10ev[299] : 299(100), 211(20) MS2-20ev[299] : 240(81)	240 $[M-H-CH_3COOH]^-$
	SA2	287	20.0	4.4*	3.5	3.5	MS2-30ev[287] : 238(100), 209(33), 287(15), 239(9), 269(8)	269 $[M-H-H_2O]^-$ 238 $[M-H-H_2O-CH_2OH]^-$ 209 $[M-H-H_2O-CH_2OH-CHO]^-$
	SA3	239	23.2	3.0	4.9	8.9**	MS2-30ev[239] : 239(100), 224(15), 210(39), 238(21), 196(16)	224 $[M-H-CH_3]^-$ 210 $[M-H-CHO]^-$ 196 $[M-H-CH_3-CO]^-$
	SA4	239	25.2	2.7	4.0	50.0**	MS2-30ev[239] : 239(100), 238(44), 224(44)	224 $[M-H-CH_3]^-$
	SA5	239	27.8	5.2	4.4	20.4**	MS2-30ev[239] : 238(100), 239(42), 224(34), 210(23)	224 $[M-H-CH_3]^-$ 210 $[M-H-CHO]^-$
	SA6	239	31.4	1.4	2.6	26.3**	MS2-30ev[239] : 224(100), 196(73), 239(57)	224 $[M-H-CH_3]^-$ 196 $[M-H-CH_3-CO]^-$
	SA7	239	31.9	3.6	4.6	12.9**	MS2-30ev[239] : 210(100), 224(16), 238(81), 196(50), 239(19)	224 $[M-H-CH_3]^-$ 210 $[M-H-CO]^-$,196 $[M-H-CH_3-CO]^-$

SA8	257	10.6	2.6	3.2	5.0*	MS2-30ev[257] : 187(100), 38(73), 215(67), 257(49), 172(35), 239(29)	239 [M-H-H_2O]$^-$ 215 [M-H-C_2H_2O]$^-$ 187 [M-H-C_2H_2O-CO]$^-$
SA9	257	21.1	2.7**	2.8**	3.8**	MS2-30ev[257] : 239(100), 211(36), 257(23), 224(21), 172(22)	239 [M-H-H_2O]$^-$ 224 [M-H-H_2O-CH_3]$^-$ 211 [M-H-H_2O-CO]$^-$
SA10	269	9.7	1.3	1.2	4.0*	MS2-30ev[269] : 241(100), 195(61), 269(43), 213(46)	241 [M-H-CO]$^-$ 213 [M-H-2CO]$^-$ 195 [M-H-2CO-H_2O]$^-$
SA11	269	21.3	7.7**	7.8**	11.1**	MS2-30ev[269] : 197(100), 241(58), 225(44), 269(43)	241 [M-H-CO]$^-$ 225 [M-H-CO_2]$^-$ 197 [M-H-2CO]$^-$
SA12	273	9.4	6.5**	7.1**	7.8**	MS2-20ev[273] : 255(100), 237(56), 273(36), 226(35)	255 [M-H-H_2O]$^-$ 237 [M-H-2H_2O]$^-$ 226 [M-H-H_2O-CHO]$^-$
SA13	301	4.8	1.4	2	5.5**	MS2-25ev[301] : 257(100), 215(70), 239(54), 187(28), 301(17)	257 [M-H-COO]$^-$ 239 [M-H-COO-H_2O]$^-$
SA14	311	29.2	1.5	1.6*	2.0**	MS2-35ev[311] : 224(100), 253(20), 225(15), 311(2)	253 [M-H-CH_3COCH_3]$^-$ 224 [M-H-CH_3COCH_3-CHO]$^-$
SA15	313	8.7	8.9*	5.3	11.0**	MS2-30ev[313] : 269(100), 13(25) MS2-30ev[269] : 225(100), 241(75), 197(59), 269(25)	269 [M-H-COOH]$^-$ 241 [M-H-COOH-CO]$^-$ 225 [M-H-COOH-CO-O]$^-$

								197 [M-H-COOH-2CO-O]$^-$
	SA16	327	22.8	3.2**	2.9**	4.5**	MS2-35ev[327] : 240(100), 239(86), 211(58), 269(19)	269 [M-H-CH_3COCH_3]- 240 [M-H-CH_3COCH_3-CHO]$^-$
	SA17	329	16.3	9.7	18.4	24.2**	MS2-20ev[329] : 283(100), 268(36), 240(20)	283 [M-H-$COOH_2$]$^-$ 268 [M-H-$COOH_2$-CH_3]$^-$ 240 [M-H-$COOH_2$-CH_3-CO]$^-$
	SA18[d]	253	36.6	3.1	5.6**	6.9**	MS2-25ev[253] : 225(100), 253(11)	255 [M-H-CO]$^-$
	SA19[e]	269	19.6	3.8**	5.2**	4.9**	MS2-22ev[269] : 269(100), 40(92)	240 [M-H-CHO]$^-$
Positive mode	SA20	259	8.1	2.2*	2.0	1.6	MS2-20ev[259] : 241(100) MS2-20ev[241] : 213(100), 41(54), 185(26)	241 [M+H-H_2O]$^+$ 213 [M+H-CO]$^+$ 185 [M+H-2CO]$^+$
	SA21	275	8.5	40.8**	19.2	1.2	MS2-20ev[275] : 227(100), 75(12), 255(2)	255 [M+H-H_2O-H_2]$^+$ 227 [M+H-H_2O-H_2-CO]$^+$
	SA22	257	20.1	8.2*	7.1	6.8	MS2-20ev[257] : 211(100), 39(51), 257(22) MS2-20ev[239] : 211(100), 39(24), 185(27)	239 [M+H-H_2O]$^+$ 211 [M+H-CO]$^+$ 185 [M+H-2CO]$^+$
	SA23	358	39.6	1.9**	1.4	0.5	MS2-10ev[358] : 317(100) MS2-30ev[317] : 317(100), 235(85), 273(71), 258(19)	358 [M+H-CH_3CN]$^+$ 273 [M+H-CH_3CN-COO^-]$^+$ 258 [M+H-CH_3CN-COO^--CH_3]$^+$

SA24	255	29.4	3.7	6.4*	5.8*	MS2-30ev[255] : 181(100), 227(40), 209(20), 255(7)	227 $[M+H-CO]^+$ 209 $[M+H-CO-H_2O]^+$ 181 $[M+H-2CO-H_2O]+$
SA25	240	30.6	3.2	3.7*	3.0*	MS2-20ev[240] : 240(100), 94(18), 222(9), 212(2)	222 $[M+H-H_2O]^+$ 212 $[M+H-CO]^+$
SA26	240	38.5	3.1	4.6**	4.2**	MS2-30ev[240] : 194(57), 240(10), 222(4), 212(3)	194 $[M+H-CO-H_2O]^+$
SA27	256	38.5	3.2	5.0**	4.6**	MS2-30ev[256] : 181(100), 209(17), 227(8), 256(7)	227 $[M+H-CHO]^+$ 209 $[M+H-CHO-H_2O]^+$ 181 $[M+H-CHO-H_2O-CO]^+$
SA28	443	6.9	2.1	2.5	9.7**	MS2-20ev[433] : 443(100), 233(28), 353(18), 413(16)	413 $[M+H-CH_2O]^+$ 353 $[M+H-CH_2O-CH_3COOH]^+$ 233 $[M+H-CH_2O-CH_3COOH-Glc]^+$
SA29	351	8.3	2	6.4*	6.4*	MS2-20ev[315] : 297(100) MS2-30ev[297] : 213(100), 41(66), 185(38), 269(14), 297(5)	297 $[M+H-H_2O]^+$ 269 $[M+H-H_2O-CO]^+$ 241 $[M+H-H_2O-2CO]^+$ 213 $[M+H-H_2O-3CO]^+$
SA30	257	9.3	9.9*	11.6**	15.3**	MS2-20ev[257] : 229(100), 39(21), 257(12) MS2-20ev[229] : 229(100), 11(80), 201(32)	239 $[M+H-H_2O]^+$ 229 $[M+H-CO]^+$ 211 $[M+H-CO-H_2O]^+$ 201 $[M+H-2CO]^+$

SA31	317	11.5	1.3	1.3	2.8*	MS2-20ev[317] : 285(100) MS2-20ev[285] : 183(100), 211(47), 285(35), 239(29), 267(26), 229(5), 257(2)	285 $[M+H-CH_3OH]^+$ 267 $[M+H-CH_3OH-H_2O]^+$ 257 $[M+H-CH_3OH-CO]^+$ 239 $[M+H-CH_3OH-H_2O-CO]^+$ 229 $[M+H-CH_3OH-2CO]^+$ 211 $[M+H-CH_3OH-2CO-H_2O]^+$ 183 $[M+H-CH_3OH-3CO-H_2O]^+$
SA32	259	18.8	9.8	11.8	27.0**	MS2-20ev[259] : 241(100) MS2-20ev[241] : 241(100), 23(49), 195(44)	241 $[M+H-H_2O]^+$ 223 $[M+H-2H_2O]^+$ 213 $[M+H-H_2O-CO]^+$
SA33	259	21.2	4.5	3.8	5.9*	MS2-20ev[259] : 241(100), 23(84), 195(31), 213(9), 259(3)	195 $[M+H-2H_2O-CO]^+$
SA34	271	21.4	4.1	4.5	5.5*	MS2-30ev[271] : 173(100), 01(70), 229(50), 271(6)	229 $[M+H-C_2H_2O]^+$ 201 $[M+H-C_2H_2O-CO]^+$ 173 $[M+H-C_2H_2O-2CO]+$
SA35	297	22.9	2.9	2.7	3.2*	MS2-20ev[297] : 297(100), 69(90), 241(53), 213(31), 185(10)	269 $[M+H-CO]^+$ 241 $[M+H-2CO]^+$ 213 $[M+H-3CO]^+$ 185 $[M+H-4CO]^+$
SA36	256	36.7	3.2	2.6	9.5*	MS2-30ev[256] : 228(32), 210(21), 256(11)	228 $[M+H-CO]^+$ 210 $[M+H-CO-H_2O]^+$

	SA37	277	40.6	7.3	30.5	63.0**	MS2-20ev[277] : 277(56), 249(30), 241(21), 259(16), 226(10),	259 $[M+H-H_2O]^+$ 249 $[M+H-CO]^+$ 241 $[M+H-2H_2O]^+$ 226 $[M+H-2H_2O-CH_3]^+$

[a] RT, Retention time

[b] Average intensity relative to average control intensity

[c] Main fragments (Relative intensity)

[d] SA 18 was identified as chrysophanol confirmed using authentic standard

[e] SA 19 was identified as aloe emodin confirmed using authentic standard

[*] Asterisk indicates significantly difference relative to control according to Tukey's test ($p<0.05$ *, $p<0.01$ **)

The anti-inflammatory activity of SA-treated *Aloe vera* adventitious roots

Metabolite profiling analysis revealed that a number of metabolites including aloe emodin and chrysophanol were induced by SA elicitation. Tricyclic aromatic quinones such as aloe emodin and chrysophanol have been reported to possess anti-inflammatory activity (Yen *et al*., 2000). Based on these results, I investigated whether SA treatment of adventitious roots led to enhancement of anti-inflammatory activity in UVB-treated mouse skin cells, which are a well-established cell line for screening anti-inflammatory agents.

All extracts obtained from non-treated and SA-treated adventitious roots maintained mouse skin cell viability at 10-100 μg/mL concentrations (Figure III-13). Extracts obtained from 1000 μM SA-treated and 2000 μM SA-treated adventitious roots at 25-100 μg/mL suppressed the UVB-induced promoter activity of *COX-2* (Figure III-14). UVB-induced transactivation of *NF-κB* and *AP-1* was strongly repressed by extracts from 500 μM SA-treated adventitious roots at 100 μg/mL and 1000 μM SA-treated adventitious roots at 25 μg/mL, respectively (Figure III-14). Although *NF-κB* was slightly inhibited by the extract from non-treated adventitious roots at 100 μg/mL, the extract could not suppress promoter activity of *COX-2* and transactivation of *AP-1*.

These results indicated that anti-inflammatory activity was enhanced in the adventitious root extracts by SA elicitation, resulting from the induction

of a number of metabolites with anti-inflammatory activity. In addition, anti-inflammatory activity was dependent on the concentration of SA used to treat adventitious roots. Although I could identify the metabolites induced by SA treatment as described above, further detailed study is needed to confirm which metabolites account for the anti-inflammatory activity.

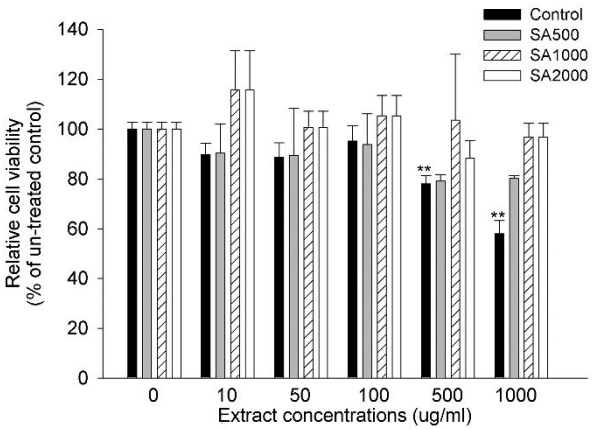

Figure III-13 Effect of extracts obtained from elicitor-treated adventitious roots on JB6 P+ cell viability. Extracts were obtained from *Aloe vera* adventitious roots untreated (Control) or treated with 500 μM SA, 1000 μM SA, or 2000 μM SA. JB6 P+ cells were treated with the indicated amounts of each extract for 4 h, and then 20 μL CellTiter 96 Aqueous One solution was added to the cells and they were incubated for an additional 4 h. Cell viability was subsequently measured at 492 and 690 nm. Data are represented as means of replicate samples ± standard deviation. Statistical analysis was carried out using the Tukey test (* $p<0.05$, ** $p<0.01$). Asterisks indicate significant differences compared to control groups.

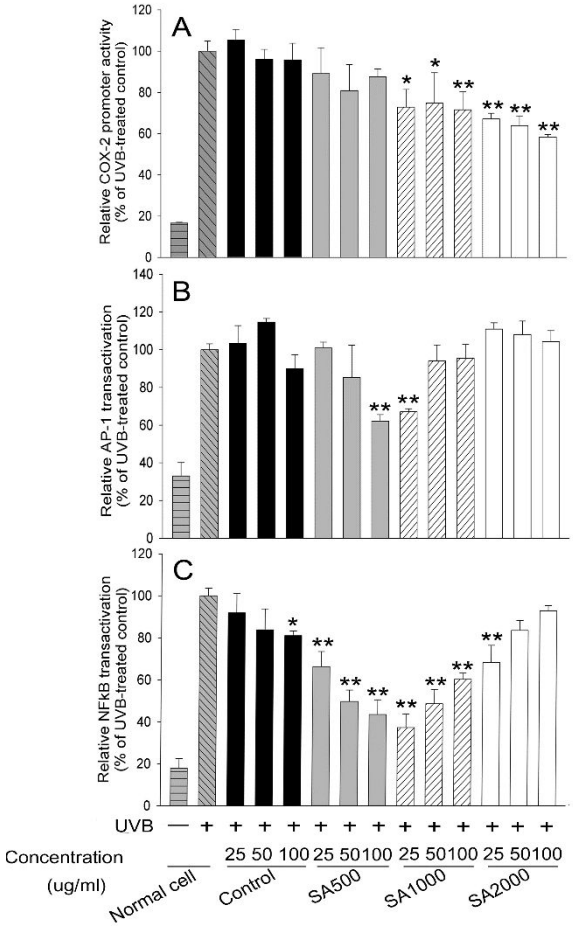

Figure III-14 Effect of extracts from SA-treated adventitious roots on UVB-induced expression in mouse skin cells. UVB-exposed JB6 P+ cells that were stably transfected with plasmids containing the luciferase reporter gene fused to the *COX-2* promoter (A), the *AP-1* gene (B), or the *NF- κB* gene (C) were incubated with extract from 0, 500, 1000, or 2000 μM SA-treated adventitious roots for 1 h and harvested after 4 h. Data are represented as means of replicate samples ± standard deviation. Statistical analysis was carried out with the Tukey test (* $p<0.05$, ** $p<0.01$). Asterisks indicate significant differences compared to groups treated with UVB alone. Control indicates the extract from SA-untreated adventitious roots.

DISCUSSION

In this study, I investigated metabolic changes and enhancement of anti-inflammatory activity in SA-treated *Aloe vera* adventitious roots. Prior to this series of experiments, there was a need for development of *in vitro* cell culture systems for *Aloe vera* in order to retain cell lines in a controlled environment. Although previous studies attempted to optimize callus induction on solid media in *Aloe* species (Yagi *et al*., 1998; Kawai *et al*., 1993), suspension culture had been limited, because phenolic compounds released from the cultured cells eventually led to cell death (Roy *et al*., 1991). In this work, I optimized suspension culture conditions for *Aloe vera* adventitious roots and overcame heavy browning (Figure III-2, Table III-1). Using the adventitious roots, I examined the elicitation effects of MJ, SA, and ethephon on the production of aloe emodin and chrysophanol.

MJ, SA, and ethephon are plant-derived elicitors that mediate the signal transduction involved in plant defense responses (Bulgakov *et al*., 2002). SA is associated with pathogen-related defense mechanisms and is required for establishment of plant systemic acquired resistance. Treatment with exogenous SA was previously reported to induce secondary metabolites, e.g. anthraquinones in *Rubia cordifolia* (Bulgakov *et al*., 2002), soluble phenolic compounds in *Matricaria chamomilla* and *Salvia miltiorrhiza* (Kováčik *et al*., 2009; Dong *et al*., 2010), podophyllotoxin in *Linum albums*

(Yousefzadi *et al*., 2010) and artemisinin in *Artemisia annua L* (Pu *et al*., 2009). In our study, the production of aloe emodin and chrysophanol was increased by all of these elicitors, but SA remarkably elevated the level of aloe emodin and chrysophanol by more than 10-11 and 5-13 folds at 24 h, implying defense-related function of the quinone compounds (Figure III-3 and III-4).

I performed UPLC-ESI/MS/MS analysis to identify additional SA-induced tricyclic aromatic quinones along with aloe emodin and chrysophanol. I identified 37 novel compounds induced by SA elicitation through MS/MS fragmentation, and three compounds were determined likely to have tricyclic aromatic quinone structures (Table II-5). Our analysis of overall primary metabolite profiles and transcription of *OKS* genes provided conclusive information about key components in the type III polyketide biosynthesis pathway that was previously uncertain in plants. In previous reports, it was predicted that tricyclic aromatic quinones such as aloe emodin and chrysophanol were synthesized from the precursor of malonyl-CoA in a process mediated by OKSs (Abe *et al*., 2005; Mizuuchi *et al*., 2009). However the activity of the plant OKS enzymes was tested in *E. coli*, and it remained unclear whether the condensation process could indeed produce the end products in aloe, as opposed to the shunt products SEK and SEK4b (Figure III-1). Our results showed that aloe emodin and chrysophanol biosynthesis correlated with *OKS* gene expression and malonyl-CoA content, supporting the hypothesis that the condensation of malonyl-CoA mediated by OKSs

results in production of tricyclic aromatic quinones in *Aloe vera* (Figure III-3,4,5,7,8 and Table III-3,7). On the other hand, some tricyclic aromatic quinones were not induced following SA elicitation. Rhein and emodin were decreased or not changed following SA elicitation, respectively (data not shown), and indicating that SA elicitation might also affect the downstream pathway of the condensation process.

Our metabolite analysis suggests that there is cross-talk in SA and MJ signaling in *Aloe vera*. UPLC-ESI-MS based-analysis showed clear differences between 500 μM SA-treated and 500 μM MJ-treated groups in positive mode (Figure III-12 and Table III-5). This reveals that SA and MJ can lead to different responses in overall metabolite accumulation in *Aloe vera* adventitious roots. In many cases, SA and MJ have been found to act antagonistically (Zhao *et al*., 2005), but some cooperative activity was observed in naphthodianthrone and phloroglucinol production in *Hypericum* species and ginsenoside accumulation in *Panax ginseng* (Coste *et al*., 2011; Ali *et al*., 2006). In *Aloe vera*, the biosynthesis of many secondary metabolites was SA- or MJ-dependently regulated, but some metabolites such as tricyclic aromatic quinones seemed to be cooperatively regulated.

Finally, I examined whether SA elicitation enhanced the anti-inflammatory activity of adventitious root extracts. I verified anti-inflammatory effects using JB6 P+ cells and the UVB model, which is suitable for examining chemopreventive effects of phytochemicals (Jung *et al*.,

2013; Jung *et al*., 2008; Kwon *et al*., 2009). Our results showed that anti-inflammatory activity was affected differently by extracts from adventitious roots treated with different concentrations of SA (Figure III-14). This indicates that SA-induced metabolites including aloe emodin and chrysophanol might regulate anti-inflammatory activity in UVB-exposed mice skin cells. Our metabolite profiling data revealed that the identified compounds mainly possessed hydroxy, hydroxymethyl, and carboxyl groups. There is accumulating evidence that the presence of these residues might have an effect on inflammation or angiogenesis associated with inflammation (He *et al*., 2009; Park *et al*., 2009; Tamarat *et al*., 2002). For example, the presence of 2 hydroxy groups located in aloe emodin might play an important role in its anti-inflammatory activity (Park *et al*., 2009). Emodin with hydroxyl group at C-6 position, rhein with carboxyl groups at C-6 position, and aloe emodin with hydroxymethyl group at C-3 position might contribute those anti-angiogenetic properties (He *et al*., 2009; Park *et al*., 2009). These types of evidence might partially account for the enhancement of anti-inflammatory activity in SA-treated adventitious roots.

Aloe vera is a well-known commercial crop; however, only a few metabolomic approaches have been applied to it. In this study, based on an optimized *in vitro* culture system system for *Aloe vera,* I showed that SA elicitation led to activation of the tricyclic aromatic quinone biosynthesis pathway and the accumulation of secondary metabolites. Moreover, the

extracts from SA-treated *Aloe vera* had enhanced anti-inflammatory activity in UVB-treated mouse skin cells. Taken together, these results provide a possible biological function of quinones related to SA-dependent defense responses and reveal the potential of SA-induced metabolites as chemopreventive agents.

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국문 초록

알로에 베라(*Aloe vera,* family: Asphodeaceae)는 약리 성분을 풍부하게 포함하고 있는 유용한 약용작물이다. 알로에 베라의 대표적인 이차대사 산물은 알로에 에모딘과 크리소파놀과 같은 안스라퀴논 계열의 물질로, 삼환계 방향족 퀴논 구조를 가지고 있으며 타입 III 폴리케타이드 생합성 경로를 통해 합성된다고 알려져 있다. 하지만 식물체 내에서는 타입 III 폴리케타이드 생합성 경로가 어떤 신호 분자에 의해 활성화되는지는 밝혀진 바가 없다. 본 실험은 알로에 베라 잎에서 부정근을 유기해 배양 조건을 확립하고, 메틸자스모닉산, 살리실산, 에틸렌을 유도자로 처리하여 타입 III 폴리케타이드 생합성 경로와 대사산물의 변화를 확인하였다. 그 결과 MS 배지에 0.3 mg/L IBA 을 첨가한 배지에서 35일간 배양하였을 때 부정근의 생체 중량과 알로에 에모딘, 크리소파올놀의 함량이 최대로 증가하였다. 또한 메틸자스모닉산, 살리실산, 에틸렌을 35일동안 키운 부정근에 처리하여 그 중 살리실산이 알로에 에모딘과 크리소파놀의 함량을 최대로 증가시키는 것을 확인하였다

또한 살리실 산에 타입 III 폴리케타이드 생합성 경로의 변화를 확인하기 위해 일차, 이차대사산물의 분석과, 관련 유전자의 분리 및 발현량 확인을 수행한 결과 트리싸이클릭퀴논 구조의 전구물질인 말로닐-코에이(Malonyl-CoA)의 함량이 유의성 있게 감소하였고 옥타케타이드 합성 유전자의 발현량은 증가하였다. 또한 살리실 산으로 다수의 물질이 증가하였으며 그 중 알로에 에모딘과 크리소파올을 포함하는 37개의 물질이 동정하였다. 살리실 산을 처리한 부정근의 추출액은 UVB을 처리한 쥐 피부 세포에서 염증관련 유전자의 발현을 억제를 확인하였다. 이는 살리실 산(Salicylic acid)이 식물체 유래 타입 III 폴리케타이드 생합성 경로를 조절하는데 중요한 역할을 한다고 볼 수 있으며 결과적으로 살리실 산에 의한 대사체의 변화는 알로에 베라의 약리효과를 강화시키는데 효과적임을 확인했다.

주요어: 살리실릭 산, 알로에 베라, 부정근, 대사체학, 염증 억제 작용

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