Influence of Auxins and Wounding on Glucosinolate Biosynthesis in Hairy Root Cultures of Chinese Cabbage (Brassica rapa ssp. pekinensis)

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Cruciferous vegetables are well-known for their health benefits owing to the presence of the biologically active compounds glucosinolates. This study aimed to investigate the effect of auxins and wounding on GSL biosynthesis in the hairy root cultures of Chinese cabbage. Five different glucosinolates, i.e., glucoerucin, glucobrassicin, 4-methoxyglucobrassicin, gluconasturtiin, and neoglucobrassicin, were detected in the hairy root cultures of Chinese cabbage. The concentrations of neoglucobrassicin and 4-methoxyglucobrassicin were considerably higher than those of other glucosinolates in response to both Auxin and wounding. Glucosinolate accumulation was higher at low concentrations of auxins. Of the different auxins, 0.1 mg/L indole-3-acetic acid led to the highest accumulation of gluconasturtiin and neoglucobrassicin. The levels of glucosinolates in the hairy root cultures varied widely with time of wounding. The highest level of glucobrassicin was found 72 h after wounding, achieving a level 3.4-times greater than basal conditions. The level of gluconasturtiin was 1.5-times higher than that before wounding, and no gluconasturtiin was detected as time from wounding elapsed. Wound-induced phenolic metabolism in mature leaves appears to be induced by signals different than those functioning in young leaves. Therefore, hairy root cultures might be a valuable alternate approach for the production of glucosinolate compounds from Chinese cabbage through wounding and also with other phytohormones or elicitors.

Key words: Auxins, Chinese cabbage, Glucosinolates, Hairy root cultures, Wounding

Plant species of Brassicaceae have been recognized as important components of healthy diets based on their high contents of vitamin C, vitamin A, folic acid, dietary fiber, minerals, and bioactive phytochemicals, especially glucosinolates1,2. According to recent epidemiological studies, intake of cruciferous vegetables such as Chinese cabbage, cabbage, radish, kale, turnip, watercress, cauliflower, broccoli, and Brussels sprouts containing glucosinolates play an important role in antioxidation and prevention of various cancers, acting as potential chemopreventive agents3. Glucosinolates, secondary metabolites containing sulfur and nitrogen, are a diverse group of over 120 structures and vary by side chain (R
Glucosinolates have been assigned to three classes: aliphatic, aromatic, and indolic glucosinolates derived from the amino acids methionine, phenylalanine, and tryptophan. A number of glucosinolates have anti-carcinogenic activities, especially the aliphatic glucoraphanin (4-methylsulfinybutyl), the indolic glucobrassicin (3-indolylmethyl), and the aromatic gluconasturtiin (2-phenylethyl). Recently, studies have concentrated on improving health-promoting compounds such as glucosinolates, particularly glucoraphanin (the precursor of sulforaphane) and glucobrassicin (indole-3-carbinol). Glucosinolate accumulation can be induced by a variety of factors, such as insect attack, mechanical damage, and fungal infection. Mechanical wounding (i.e., abrading, crushing, and cutting) induces an increase in the production of wound repair and defense compounds.

Hairy roots are considered as a type of plant disease and a product of infection from Agrobacterium rhizogenes, a natural soil bacterium. These roots have important characteristic features, including potential for rapid growth in media without growth hormone. In addition, hairy root cultures of several important plant species produce similar or higher amounts of secondary metabolites than do the parent plants. Production of secondary metabolites in hairy root systems is influenced by a number of factors, including temperature, light, pH, composition of the medium and exogenous treatment with plant growth regulators.

In this study, we evaluated variations of glucosinolate contents in response to auxin and wounding in the hairy root cultures of Chinese cabbage using high-performance liquid chromatography (HPLC) and electrospray ionization-mass spectrometry (ESI-MS).

**MATERIALS AND METHODS**

**Chemicals**

Diethylaminoethyl (DEAE)-Sephadex A-25, sinigrin (2-propenyl GSL), and aryl sulfatase (Type H-1, EC 3.1.6.1) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). HPLC-grade acetonitrile (CH₃CN) and methanol (MeOH) were obtained from J. T. Baker (Phillipsburg, NJ, USA). Ultrapure water having a resistivity of 18.2 MΩ/cm was produced using PureLab Option from ELGA Labwater (Model LA 621; Marlow, UK).

**Establishment of hairy root cultures**

Excised hypocotyls of 10-day-old Brassica rapa ssp. pekinensis seedlings were used as the explant material for co-cultivation with A. rhizogenes R1000. The excised hypocotyls were dipped in the A. rhizogenes culture in liquid inoculation medium for 15 min, blotted dry on sterile filter paper, and incubated in the dark at 25°C on agar-solidified MS medium. After two days of co-cultivation, the explant tissues were transferred to a hormone-free medium containing MS salts and vitamins (0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine-HCl, 0.1 mg/L thiamine-HCl, and 2.0 mg/L glycine), 30 g/L sucrose, 500 mg/L cefotaxime, and 8 g/L agar. Numerous hairy roots emerged from the wound sites within 3 to 4 weeks. The hairy roots were separated from the explant tissues and sub-cultured in the dark at 25°C on agar-solidified MS medium. After repeated transfer to fresh medium, rapidly growing hairy root cultures were obtained. Isolated roots (200 mg) were transferred to 30 mL MS liquid medium containing 30 g/L sucrose in 100 mL flasks. Root cultures were maintained at 25°C on a gyratory shaker (100 rpm) in a growth chamber in the dark.

**Auxin treatments**

Optimal conditions of hairy root growth and glucosinolate biosynthesis were selected by determining the effects of 0.1, 0.5, and 1.0 mg/L auxins—indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), and 1-naphthaleneacetic acid (NAA). After 3 weeks of culture, hairy roots were harvested. Dry weight and glucosinolate contents were determined. Three flasks were used for each culture condition, and experiments were performed in duplicate.

**Wounding treatments**

For wounding experiments, hairy root cultures were collected in sterile Petri plates and most of the medium was removed with a pipette. The hairy roots were wounded by repeatedly cutting into the small piece with a sterile scalpel blade for 10 min. Wounded hairy roots were collected by vacuum filtration, frozen in liquid
N$_2$, and stored at –80°C for glucosinolate analysis.

**Extraction of desulfo-glucosinolates (DS-GSLs) and HPLC analysis**

DS-GSLs were extracted using a slight modification of the procedures reported in a previous study$^{18}$. Briefly, crude GSLs were extracted from 100 mg freeze-dried powder by boiling in 1.5 mL 70% (v/v) MeOH at 70°C for 5 min by using a water bath. After centrifugation at 12,000 rpm at 4°C for 10 min in a Hanil microcentrifuge (MICRO 17R; Incheon, Korea), the supernatant was collected in a 5-mL test tube, and the residue was re-extracted twice as described above. The combined supernatants were used as the crude GSL extract. Desulfation of the crude GSL extracts was performed using the DEAE Sephadex A-25 anion exchange column, which was prepared by adding the slurry of DEAE-Sephadex A-25 previously activated with 0.5 M sodium acetate. The crude GSL extracts were loaded onto a pre-equilibrated column. After the column was washed with 1 mL (×3 times) ultrapure water to remove cations and neutral ions, aryl sulfatase (E.C.3.1.6.1 type H-1 from *Helix pomatia*; 75 µL) was loaded onto each column. After the desulfation reaction overnight (16) at room temperature, the desulfated GSLs were eluted with 0.5 mL (×3 times) ultrapure water. The eluates were filtered through a 0.45-µm Teflon PTFE syringe filter and immediately analyzed using HPLC or stored at 4°C in a refrigerator until chemical analysis.

The DS-GSLs were separated on a reversed-phase Inertsil ODS-3 column (150 × 3.0 mm i.d.; particle size, 3 µm; GL Sciences, Tokyo, Japan) by using an E-type cartridge guard column (10 × 3.0 mm i.d., 5 µm) and an Agilent Technologies 1260 series HPLC system (Palo Alto, CA, USA). The column oven temperature and detection wavelength were set at 40°C and 227 nm, respectively, and the flow rate was 0.4 mL/min. The mobile phase consisted of ultrapure water (solvent A) and CH$_3$CN (solvent B). The gradient program used was as follows: 0 min, 0% B; 0–2 min, 0% B; 2–7 min, 10% B; 7–16 min, 31% B; 16–19 min, 31% B; 19–21 min, 0% B; 21–27 min, 0% B (total 27 min). The individual GSLs were quantified according to their HPLC area and response factor by comparing with those of an external standard, 5 mL of sinigrin solution (0.1 mg/mL) that was subjected to the same extraction process$^{19}$.

**LC/ESI-MS analysis for the quantitation of DS-GSLs**

The MS data were acquired using ESI-mass spectrometer with an API 4000 Q TRAP system (Applied Biosystems, Foster City, CA, USA) in the positive ion mode ([M+H]$^+$) that was equipped with an Agilent 1200 series HPLC. The analytical conditions for MS were as follows: ion spray voltage: 5.5 kV; curtain gas (20 psi), nebulizing gas (50 psi), and heating gas (50 psi): high purity nitrogen (N$_2$); heating gas temperature: 550°C; declustering potential: 100 V; entrance potential: 10 V; and spectra scanning range: m/z 100–800 (scan time, 4.8 s).

**RESULTS AND DISCUSSION**

The variation in glucosinolate content in response to different concentrations of auxins in the hairy root cultures of Chinese cabbage was investigated. Five different glucosinolates, i.e., glucoerucin, glucobrassicin, 4-methoxyglucobrassicin, gluconasturtiin, and neoglucobrassicin (Table 1), were detected in the hairy root cultures of Chinese cabbage by using HPLC and LC/ESI-MS analyses. The total and individual levels of some glucosinolates in the hairy root cultures did not vary among the auxin treatments (Table 1). Of the five glucosinolates, neoglucobrassicin and 4-methoxyglucobrassicin contents were considerably higher. The accumulation of neoglucobrassicin in the hairy root cultures was 128, 54, 10, and 3 times higher than those of glucoerucin, gluconasturtiin, glucobrassicin, and 4-methoxyglucobrassicin, respectively. Further, the level of 4-methoxyglucobrassicin was 49.3, 20.8, and 3.82 times higher than those of glucoerucin, gluconasturtiin, and glucobrassicin, respectively. Glucosinolate accumulation was found to be higher at low concentrations of auxins; with increasing concentration of auxins, the levels of glucosinolate decreased in most cases. Auxin treatments produced almost similar and even lower amount of glucosinolate in the hairy root cultures of Chinese cabbage. Among the auxins, IAA at 0.1 mg/L led to the highest accumulation
of gluconasturtiin and neoglucobrassicin. Treatment with 0.1 mg/L IBA produced the highest amount of glucoerucin. Similarly, 0.1 mg/mL NAA produced the highest amount of 4-methoxyglucobrassicin.

Accumulation of secondary metabolites in any parts of mother or transformed plants is largely dependent on their sources of origin; however, it can be affected by treatments with phytohormones and elicitors, as well as by environmental factors. Auxins are known to play important roles in plant growth and root development. However, the effects of auxins might vary across different plant species. In this study, auxins did not play a vital role in the accumulation of glucosinolates in the hairy root cultures of Chinese cabbage. However, low concentrations of auxins led to higher accumulation of glucosinolates. Previously, we showed that low concentrations of IAA led to the highest accumulation of glucosinolates in the hairy root cultures of broccoli.10

Using HPLC and LC/ESI-MS analysis, we detected five glucosinolates, i.e., progoitrin, glucobrassicin, 4-methoxyglucobrassicin, gluconasturtiin, and neoglucobrassicin in the hairy root cultures of Chinese cabbage (Table 2). The content of glucosinolates in the hairy root cultures varied markedly with time of wounding. Among the glucosinolates, the level of glucobrassicin increased rapidly with increasing wounding time. The level of increasing glucobrassicin was shown for all time points after wounding except at 3 h. The highest level of glucobrassicin was found at 72 h after wounding, achieving a 3.4-fold higher level compared to no wounding. In the case of gluconasturtiin, the level of this glucosinolate increased 1.5-times higher than that before wounding, and gluconasturtiin was undetectable with increasing wounding time in the hairy root cultures of Chinese cabbage. The concentrations of neoglucobrassicin and 4-

### Table 1. Effects of auxins on glucosinolate biosynthesis in hairy root cultures of Chinese cabbage

<table>
<thead>
<tr>
<th>Auxin</th>
<th>Glucosinolate (µmol/g dry wt.)</th>
<th>Glucoerucin</th>
<th>Glucobrassicin</th>
<th>4-methoxyglucobrassicin</th>
<th>Gluconasturtiin</th>
<th>Neoglucobrassicin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 0.0</td>
<td>0.11±0.00</td>
<td>1.42±0.25</td>
<td>5.42±0.09</td>
<td>0.26±0.06</td>
<td>14.08±1.86</td>
<td>21.31±2.04</td>
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<tr>
<td>IAA 0.1</td>
<td>0.10±0.01</td>
<td>1.31±0.45</td>
<td>4.79±0.68</td>
<td>0.28±0.12</td>
<td>13.57±2.42</td>
<td>20.06±3.57</td>
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<tr>
<td>IAA 0.5</td>
<td>0.11±0.01</td>
<td>1.06±0.04</td>
<td>4.54±0.80</td>
<td>0.13±0.04</td>
<td>10.91±1.51</td>
<td>16.74±1.62</td>
</tr>
<tr>
<td>IAA 1.0</td>
<td>0.11±0.02</td>
<td>1.13±0.11</td>
<td>4.93±0.63</td>
<td>0.12±0.09</td>
<td>10.25±1.04</td>
<td>16.53±0.37</td>
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<td>IBA 0.1</td>
<td>0.08±0.00</td>
<td>1.44±0.50</td>
<td>4.56±0.88</td>
<td>0.19±0.04</td>
<td>10.25±0.62</td>
<td>16.52±1.55</td>
</tr>
<tr>
<td>IBA 0.5</td>
<td>0.09±0.01</td>
<td>1.3±0.40</td>
<td>4.63±0.97</td>
<td>0.13±0.02</td>
<td>11.96±1.87</td>
<td>18.12±3.23</td>
</tr>
<tr>
<td>IBA 1.0</td>
<td>0.10±0.02</td>
<td>0.97±0.13</td>
<td>4.60±0.13</td>
<td>0.16±0.02</td>
<td>10.52±1.53</td>
<td>16.36±1.50</td>
</tr>
<tr>
<td>NAA 0.1</td>
<td>0.11±0.02</td>
<td>1.39±0.79</td>
<td>4.95±1.13</td>
<td>0.23±0.1</td>
<td>12.91±3.22</td>
<td>19.6±5.18</td>
</tr>
<tr>
<td>NAA 0.5</td>
<td>0.10±0.01</td>
<td>1.00±0.31</td>
<td>4.51±1.28</td>
<td>0.22±0.05</td>
<td>9.18±0.74</td>
<td>15.02±2.06</td>
</tr>
<tr>
<td>NAA 1.0</td>
<td>0.09±0.01</td>
<td>0.86±0.19</td>
<td>4.12±0.45</td>
<td>0.15±0.03</td>
<td>9.49±1.95</td>
<td>14.69±2.54</td>
</tr>
</tbody>
</table>

### Table 2. Effects of wounding on glucosinolate biosynthesis in hairy root cultures of Chinese cabbage

<table>
<thead>
<tr>
<th>Wounding (h)</th>
<th>Glucosinolate (µmol/g dry wt.)</th>
<th>Glucoerucin</th>
<th>Glucobrassicin</th>
<th>4-methoxyglucobrassicin</th>
<th>Gluconasturtiin</th>
<th>Neoglucobrassicin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.17 ± 0.00</td>
<td>1.50 ± 0.00</td>
<td>7.43 ± 0.01</td>
<td>0.17 ± 0.06</td>
<td>13.09 ± 0.01</td>
<td>13.41 ± 0.08</td>
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<td>3</td>
<td>0.21 ± 0.00</td>
<td>1.37 ± 0.02</td>
<td>5.28 ± 0.03</td>
<td>0.25 ± 0.12</td>
<td>13.41 ± 0.08</td>
<td>13.41 ± 0.08</td>
</tr>
<tr>
<td>6</td>
<td>0.20 ± 0.00</td>
<td>1.54 ± 0.01</td>
<td>4.82 ± 0.05</td>
<td>0.00 ± 0.00</td>
<td>12.05 ± 0.01</td>
<td>12.05 ± 0.01</td>
</tr>
<tr>
<td>12</td>
<td>0.23 ± 0.00</td>
<td>2.52 ± 0.03</td>
<td>4.34 ± 0.12</td>
<td>0.00 ± 0.00</td>
<td>12.06 ± 0.01</td>
<td>12.06 ± 0.01</td>
</tr>
<tr>
<td>24</td>
<td>0.22 ± 0.00</td>
<td>3.82 ± 0.03</td>
<td>4.08 ± 0.19</td>
<td>0.00 ± 0.00</td>
<td>10.14 ± 0.01</td>
<td>10.14 ± 0.01</td>
</tr>
<tr>
<td>48</td>
<td>0.21 ± 0.00</td>
<td>4.36 ± 0.02</td>
<td>3.60 ± 0.23</td>
<td>0.00 ± 0.00</td>
<td>8.18 ± 0.01</td>
<td>8.18 ± 0.01</td>
</tr>
</tbody>
</table>
methoxyglucobrassicin were much higher than other glucosinolates. The accumulation of neoglucobrassicin in the hairy root cultures of Chinese cabbage was 77-, 52-, and 9-times higher than that of gluconasturtiin, progoitrin, and glucobrassicin, respectively. The level of neoglucobrassicin increased slightly only after wounding at 3 h and then tended to decrease with the elapsing of wounding time. The accumulation level of 4-methoxyglucobrassicin was 44- and 30-times higher than that of gluconasturtiin and progoitrin, respectively. As wounding time increased, the levels of 4-methoxyglucobrassicin and progoitrin decreased.

The content of secondary metabolites in any part of a plant, either a mother plant or transformed plant, largely depends on their source of origin, but it can also be affected by treatment with phytohormones, elicitors, wounding, and even by environmental factors. Chlorogenic acid showed the highest accumulation after wounding in Romaine, Butter leaf, and iceberg lettuce cultivars, while there was no significant change induced by exposure of non-wounded leaves to different concentrations of methyl jasmonates. The accumulation of phenolic compounds is a significant part of the process triggered by wounding.

CONCLUSION

A hairy root culture could be a valuable alternate approach for the production of health-benefitting secondary metabolites, especially glucosinolate compounds from Chinese cabbage. Wounding at different time points influenced the accumulation of glucosinolates in the hairy roots of Chinese cabbage, whereas auxin treatments did not perform well for the accumulation of glucosinolate and our laboratory efforts are aimed at further improving glucosinolate compounds in hairy root cultures of Chinese cabbage using other treatment methods.

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