Phylloquinone (vitamin K\textsubscript{1}) and glucosinolate contents in transgenic \textit{Brassica rapa} transformed with a bacterial isochorismate synthase gene

Sanimah Simoh\textsuperscript{1,2}, Ibrahim Abdel-Farid\textsuperscript{1}, Huub JM Linthorst\textsuperscript{3}, Robert Verpoorte\textsuperscript{1}

\textsuperscript{1} Section Metabolomics, Institute of Biology, Leiden University, Leiden, The Netherlands
\textsuperscript{2} Biotechnology Research Centre, Malaysian Agricultural Research & Development Institute (MARDI), Kuala Lumpur, Malaysia
\textsuperscript{3} Section Plant Cell Physiology, Institute of Biology, Leiden University, Leiden, The Netherlands

Abstract

The phylloquinone and glucosinolate contents of transgenic \textit{Brassica rapa} ssp. \textit{oleifera} transformed with a bacterial isochorismate synthase (ICS) gene were investigated. There was no significant difference in the phylloquinone accumulation in the transgenic plants in comparison to the controls whereas the glucosinolate profile was significantly altered particularly for indole and aliphatic glucosinolates. Although the total glucosinolate levels were not significantly changed, an increase of total indole glucosinolates was observed in the transgenic plants whereas total aliphatic glucosinolate levels were decreased. This indicates that the introduction of the ICS gene in \textit{B. rapa} ssp. \textit{oleifera}, which was expected to have an effect on the isochorismate production along the chorismate pathway did not have an important effect on the phylloquinone contents, whereas unexpected effects on glucosinolate profiles were observed. This might be due to an increase in the level of SA produced via isochorismate and its activation of plant defense.

Keywords: phylloquinone, glucosinolates, isochorismate synthase, transgenic \textit{Brassica rapa}. 
5.1 Introduction

The two naturally occurring vitamin Ks are phylloquinone (vitamin K\textsubscript{1}) and menaquinone (vitamin K\textsubscript{2}). Phylloquinone is synthesized in plants whereas menaquinone is synthesized by microorganisms. This fat-soluble vitamin functions as a cofactor in the processes of blood clotting and bone formation. In higher plants, phylloquinone plays an essential role as a cofactor for photosystem I in the electron transfer chains in all photosynthetic tissues. Phylloquinone is located in the chloroplast and is present in small amounts in plants. Dark-green, leafy vegetables and vegetable oils are regarded as the best sources of phylloquinone (Tikkanen, 2001; Damon \textit{et al}., 2005).

Chorismate, a central intermediate of the shikimate pathway, is a precursor for the aromatic amino acids, phenylalanine, tyrosine and tryptophan. Besides these routes that also lead to the biosynthesis of different types of secondary metabolites, there is also a minor route, the isochorismate route leading to the synthesis of compounds like ubiquinones, anthraquinones and phylloquinones. Isochorismate, is thought to play an essential role in the synthesis of phylloquinone because it is a precursor of \textit{o}-succinylbenzoic acid (OSB), an intermediate in phylloquinone biosynthesis (Hutson and Threlfall, 1980). In plants, isochorismate is also thought to be involved in the biosynthesis of salicylic acid (SA) (Moreno \textit{et al}., 1994; Verberne \textit{et al}., 1999), a signal compound that plays a role in inducing both local and systemic acquired resistant (SAR) in plant defense. Thus, providing isochorismate as a substrate was thought to have a profound effect on the accumulation of metabolites along the isochorismate utilizing pathway. Transgenic tobacco plants overexpressing two bacterial genes encoding the enzymes isochorismate synthase (ICS) and isochorismate pyruvate lyase (IPL) that convert chorismic acid to SA in a two step process, produced higher levels of phylloquinone (two-fold) as well as SA in comparison to the wild type (Verberne \textit{et al}., 2007; Verberne \textit{et al}., 2000). The products of both the bacterial ICS and IPL gene were targeted to the chloroplast. Transgenic tobacco plants with only the ICS gene accumulated a three to four times higher level of phylloquinone than control tobacco plants. In contrast to this, transgenic plants with only the IPL gene accumulated lower levels of phylloquinone as compared to the control plants. It was speculated that the introduction of IPL in the chloroplast may have had a negative effect on the phylloquinone accumulation as isochorismate was
channelled away to SA production (Verberne et al., 2007). These plants were also unable to accumulate normal levels of phylloquinone.

Glucosinolates (GLS) consist of a β-thioglucose moiety, a sulphonated oxime moiety and a side chain (R) derived from an amino acid. They are exclusively found in the Brassicaceae family, including *B. rapa* (Halkier and Gershenzon, 2006), where they act as defense compounds. The source of the side chain determines the class of GLS; aliphatic GLS are derived mostly from methionine, aromatic GLS are derived from phenylalanine or tyrosine, and indole GLS are derived from tryptophan. The initial step of GLS biosynthesis involves the conversion of amino acids to aldoximes catalyzed by cytochromes P450 monooxygenases encoded by the CYP79 gene family (Wittstock and Halkier, 2000). The defense function of GLS is attributed to the compounds that are formed by the action of the hydrolytic enzyme myrosinase, upon tissue damage (Burow et al., 2006). GLS and their breakdown products isothiocyanate, thiocyanate and nitriles are known to have different biological effects, such as antimicrobial, cancer prevention, allelopathic as well as goitrogenic activities (Wittstock and Halkier, 2002). Recent interests in these compounds mostly concern their cancer chemopreventive effect and disease resistance in plants. *Arabidopsis* plants transformed with CYP79 genes responsible for GLS biosynthesis produced high level of GLS as well as exhibited increased resistance against pathogenic bacteria (Brader et al., 2006). Furthermore, increased GLS levels paralleled the induction of salicylic acid (SA) mediated defense. Previous research by Kiddle et al. (1994) has shown that by applying salicylic acid (SA) to *Brassica* species, total GLS levels in the treated plants increased, whereas a study by van Dam et al. (2003) on the application of SA on roots of wild *Brassica* reported the opposite. Our previous experiments (Chapter 4) showed an increased level of salicylic acid in individual transgenic plants after introduction of the bacterial isochorismate synthase (ICS) gene. This suggests that overexpression of isochorismate synthase in *Brassica* leads to increased salicylic acid accumulation like in transgenic tobacco.

In the present study we investigated the effects of introducing the bacterial ICS gene into *B. rapa* on the accumulation of phylloquinone. We were also interested to know whether the introduction of this gene resulted in specific changes in aromatic and indole GLS, as these are derived from the shikimate pathway.
5.2 Materials and methods

5.2.1 Plant materials
Ten and 22 months old primary transgenic plants of *B. rapa* ssp. *oleifera* encoding bacterial isochorismate synthase gene (ICS), tissue culture-generated plants which served as controls and wild type plants which were grown in the greenhouse at 22 °C with 16:8 photoperiod per day. The shoot and mature leaves of transgenic and control plants were harvested at the same time and ground in liquid nitrogen prior to extraction.

5.2.2 Chemicals
Phylloquinone (vitamin K$_1$) and menaquinone-4 (vitamin K$_2$) used as internal standard were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Methanol, isopropanol, ethanol, n-hexane and dichloromethane were of HPLC grade (Biosolve, BV, Netherlands). Zink powder, particle size <60 µm (Merck, Darmstadt, Germany), zink chloride, sodium acetate (anhydrous) and glacial acetic acid were analytical grade.

5.2.3 Extraction and HPLC analysis of phylloquinone
Extraction of phylloquinone was according to the modified method of Piironen *et al.* (2000). Approximately one gram of ground leaf materials was placed into a 50 ml centrifuge tube containing 10 ml of isopropanol in which 2.5 ug of menaquinone-4 (vitamin K$_2$, served as internal standard) was added. The mixture was then incubated in a 80 °C water bath for 5 minutes. After cooling down, 30 ml of isopropanol:hexane (1:2, v/v) was added to the mixture, followed by sonication in the sonicator water bath for 10 minutes. Ten milliliter of purified Millipore water was added to the samples before vortexing for at least 2 minutes and then centrifuging at 1500 g for 10 minutes. Ten milliliter of the upper hexane layer was transferred into a 25 ml round bottom flask and evaporated to dryness by rotary evaporator at 40 °C. The residue was then dissolved in 1 ml ethanol before injecting into HPLC. Due to light sensitivity of vitamin K, all the extraction steps were carried out under subdued light or in the glassware covered with aluminium foil. All the stock solutions for standard vitamin K$_1$ and internal standard menaquinone-4 were prepared by dissolving the compounds in ethanol. All the samples were extracted in triplicate. The HPLC equipment for the analysis consisted of a pump module (LKB 2150, Bromma, Sweden), auto-injector
(Gilson 234, Villiers Le Bel, France) and Shimadzu RF-10AxL spectrofluorometric
detector (Shimadzu Corporation, Kyoto Japan). A reversed phase C18 Vydac column
(250 x 4.6 mm i.d, 5µm) coupled with a small guard column, dry-packed with
LiChrosorb RP-18 (Merck, Darmstadt, Germany) were used. A short column (20 x 2
mm i.d.) filled with zinc powder which served as post column reducer was connected
between the analytical column and fluorescence detector.

Analysis was performed using an isocratic eluent system which consisted of
dichloromethane: methanol (10:90, v/v) and 5 ml of a methanolic solution containing
1.37 g of zinc chloride, 0.41 g of sodium acetate and 0.30 g of glacial acetic acid
(Jacob and Elmadfa, 1996). Detection of phylloquinone was carried out at an
excitation wavelength of 243 and emission wavelength of 430 nm (Jacob and
Elmadfa, 1996). The flow rate used was 0.8 ml/min and injection volume was 20 µl.
All samples were analyzed in triplicate. The levels of phylloquinone were determined
based on the direct comparison of peak area ratios.

5.2.4 Extraction and HPLC analysis of glucosinolates (GLS)
GLS extracts were analysed using the modified method of Font et al. (2005). Hundred
milligram of dry weight sample was put in a 15 ml tube into which 2 ml of 70% methanol was added. The mixtures were heated in a 90 ºC water bath for 6 minutes, sonicated for 15 minutes and centrifuged for 10 minutes at 3500 rpm. This step was repeated and the supernatants obtained were pooled and loaded onto an ion-exchange column containing 0.5 ml Sephadex DEAE-A25. The column was then rinsed with 1 ml 70% methanol two times followed by 1 ml MilliQ water and 1 ml 20 mM sodium acetate buffer. Desulphation of glucosinolates was carried out by adding 20 µl of purified sulphatase (E.C. 3.1.6.1, type H-1 from Helix pomatia). The sulfatase was flushed down by adding 50 µl sodium acetate buffer onto the column which was then covered with aluminium foil and left to stand overnight. The next day the desulfoglucosinolates were eluted with 1 ml MilliQ water two times and placed in a -80 ºC freezer and finally freeze dried for at least two days. For analysis with HPLC, the residues were redissolved in an exact volume of MilliQ water, filtered with 0.2 µm nylon syringe and put in the HPLC vial.

The HPLC system used consisted of a Waters system equipped with a 626 pump and 600 S pump controller, a 717 plus auto sampler, and a 2996 photodiode array detector type 2996. The procedure employed a C-18, reverse phased column
(LiChrosper RP-18, 250x4.6 mm i.d., 5 µm particle size, Chrompack) with a gradient of water and acetonitrile. The results are given in ug/g dry weight of leaf materials calculated from the peak areas at 229 nm relative to the peak area of the sinigrin as a standard and using the relative response factors 1.0 for aliphatic and 0.3 for indole glucosinolates.

5.2.5 Statistical analysis
Where appropriate, one–way analysis of variance (ANOVA; p ≤ 0.05) was used to determine the significant difference of the observed phylloquinone and glucosinolate levels between the transgenic and control plants. The Minitab (12.1.2) was used for the analysis.

5.3 Results and discussion
5.3.1 Profile of phylloquinone accumulation
To determine a possible effect of introducing a bacterial ICS into B. rapa ssp. oleifera on the phylloquinone contents, the shoot and mature leaves of transgenic (entC) plants at 10 and 22 months old were analyzed by HPLC. The accumulation of phylloquinone in the transgenic plants, the control and wild type plants is summarized in Figure 5.1a-b.

The results from the shoot and mature leaves of 10 (Figure 5.1a) and 22 months old (Figure 5.1b) transgenic plants of the B. rapa show that the ICS gene did not have any significant effect on accumulation of phylloquinone in comparison with the control plants. Moreover, the transgenic B. rapa plants developed a normal phenotype similar to wild type plants. This is in contrast to the situation in tobacco (Verberne et al., 2007). These authors observed that transgenic tobacco transformed with an entC gene encoding ICS accumulated high levels of phylloquinone. In contrast, transgenic plants transformed with pmsB gene encoding IPL produced only low levels of phylloquinone and showed severely retarded growth. As ICS was targeted to the chloroplast where isochorismate is produced, these results (Verberne et al., 2007) showed that ICS was involved in the biosynthesis of phylloquinone.

Piironen et al. (1996) suggested that large variation in phylloquinone levels found in margarine is due to poor reproducibility of the extraction method. To reduce this problem, we combined all our results into three sets of replicates; transgenic plants,
tissue culture generated plants and wild types. However, as shown in Table 5.1, still no significant differences can be found.

Figure 5.1 Phylloquinone contents in shoot and mature leaves of 10 (a) and 22 months old (b) transgenic (L2, L4, L5, L9, L10, L12), tissue culture generated control (CTRL 1, CTRL2) and wild type (W) plants of Brassica rapa ssp. oleifera.

The results of Figure 5.1 show that the phylloquinone content at the two time points was approximately the same except it was decreased significantly in the shoots of 22 months old plants. As phylloquinone is primarily found in photosynthetic tissues, its accumulation may also vary (Ferland and Sadowski, 1992). It was found that the outer
leaves of cabbage and Brussel sprouts contain higher amount of phylloquinone in comparison with the inner leaves (Bolton-Smith et al., 2000).

**Table 5.1** Phylloquinone contents in shoots and mature leaves of 10 and 22 months old plants of *Brassica rapa* ssp. *oleifera*

<table>
<thead>
<tr>
<th></th>
<th>10 months old</th>
<th>22 months old</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Shoot</td>
<td>Mature leaves</td>
</tr>
<tr>
<td>Transgenic</td>
<td>1.72 ± 0.36</td>
<td>1.68 ± 0.24</td>
</tr>
<tr>
<td>Control</td>
<td>1.53 ± 0.18</td>
<td>1.76 ± 0.06</td>
</tr>
<tr>
<td>Wild type</td>
<td>1.40 ± 0.00</td>
<td>1.43 ± 0.00</td>
</tr>
</tbody>
</table>

*Combined data from 5 transgenic plants, 2 tissue culture - generated plants which served as controls, and wild type*

Previous research also showed that green leafy and flower vegetable of Brassicaceae were among the vegetables that are the richest source of phylloquinone, i.e. more than 1.0 µg/g vegetable (Damon et al., 2005). In our study, the highest contents of phylloquinone was detected in L9 (2.2 µg/g FW) and L12 (2.4 µg/g FW) for 10 months old shoots (Figure 5.1a) and 22 months old mature leaves (Figure 5.1b) respectively. Generally, the phylloquinone contents in all the tested plants in this present study were within the ranges as previously reported (Koivu et al., 1997; Bolton-Smith, 2000; Dismore et al., 2003).

### 5.3.2 Profile of glucosinolate accumulation

Four individual transgenic (L2, L4, L5 and L10) and two control (CTRL1, CTRL2) plants of *B. rapa* ssp. *oleifera* were determined for their GLS content. Eight GLS belonging to the classes of aliphatic and indole GLS were detected in the transgenic and control plants (Figure 5.2). However, none of the aromatic GLS were detected in this variety of *B. rapa*. The aliphatic GLS detected were progoitrin (PRO), gluconapoleiferin (GNL), gluconapin (GNA), and glucobrassicanapin (GBN), the indole GLS were glucobrassicin (GBC), 4-hydroxyglucobrassicin (4-OH), 4-methoxyglucobrassicin (4-MeO) and neoglucobrassicin (NEO).
Figure 5.2 Chromatograms of glucosinolate profiles from the control (a) and transgenic (b) plants of *Brassica rapa* ssp. *oleifera*. 1. Progoitrin 2. Gluconapoleiferin 3. Gluconapin 4. Glucobrassicanapin 5. Glucobrassicin 6. 4-hydroxyglucobrassicin 7. 4-methoxyglucobrassicin 8. Neoglucobrassicin

Figure 5.3a shows the profile of individual GLS accumulation in the transgenic and control plants of *B. rapa* ssp. *oleifera*. The data from transgenics were taken from the combination of 4 individual plants whereas for the controls from 2 plants (CTRL 1, CTRL 2). It is evident that the accumulation of all aliphatic GLS was decreased while all indole GLS were increased in the transgenic plants as compared to the controls. The accumulation of progoitrin gluconapin, glucobrassicanapin, glucobrassicin and neoglucobrassicin in the transgenic plants was significantly different ($p \leq 0.05$) in comparison to the control plants. A pronounced induction was observed particularly for indole GLS, glucobrassicin and neoglucobrassicin. The accumulation of glucobrassicin in transgenic plants was increased more than 10 fold ($P=0.025$) over the controls while neoglucobrassicin was increased almost three fold ($P=0.013$). The accumulation of the other two indole GLS; 4-hydroxyglucobrassicin and 4-methoxyglucobrassicin was slightly increased, however this was not significantly different from the controls ($P=0.182$ and 0.337, respectively). The most abundant GLS found was progoitrin which represents around 50% and 30% from the total GLS followed by glucobrassicatin which was around 33% and 20%, respectively for both controls and transgenic plants. The total GLS, aliphatic and indole GLS are shown in Figure 5.3b. It is evident that there was a significant change in the profile of total indole and aliphatic GLS in transgenic plants as compared to the controls, even though the total accumulation of GLS was not significantly different. The most
significant change was found in the total indole GLS, which was increased five fold (p=0.017) over the controls. This effect was mostly due to the increase of glucobrassicin and neoglucobrassicin (Figure 5.3a). Lower accumulation (10 µg/g dry weight, p=0.016) of total aliphatic GLS was observed in transgenics if compared to the controls, whereas in the control plants it was around 16 µg/g dry weight of leaf material.

**Figure 5.3** Individual (a) and total (b) glucosinolates including aliphatic and indole glucosinolates contents from tissue culture-generated control and transgenic plants of *Brassica rapa* ssp. *oleifera*. PRO: progoitrin GNL: gluconapoleiferin GNA: gluconapin GBN: glucobrassicanapin GBC: glucobrassicin 4-OH: 4-hydroxyglucobrassicin 4-MeO: 4-methoxyglucobrassicin NEO: neoglucobrassicin. Significant difference at p ≤0.05 from the data set of control plants is indicated by asterisk (*). Data for controls are taken from 2 individual tissue culture-generated plants and for transgenics from 4 individual plants (L2, L4, L5 and L10).
Previous work has shown that aliphatic and indole GLS productions are readily modulated in Brassicaceae by genetic and environmental stressors (Kliebenstein et al., 2001, Kim et al., 2003; Padilla et al., 2007). Induced accumulation of indole GLS, glucobrassicin and neoglucobrassicin was observed upon herbivory and fungal infection of Chinese cabbage (Rostas et al., 2002). A similar effect was observed in *B. napus* after herbivory by chrysomelid beetles (Koritsas et al., 1991) and treatment with methyl jasmonate (Doughty et al., 1995). Kiddle et al. (1994) observed increased accumulation of GLS including aromatic and indole GLS on the leaves of *B. napus* following soil-drench treatment with salicylic acid (SA) solution. Brader et al. (2006) reported that the increased level of GLS upon introduction of the CYP79 gene responsible for GLS biosynthesis, paralleled with the induction of the SA mediated response. In contrast to this, a study by van Dam et al. (2003) on two wild Brassica species showed that application of salicylic acid decreased accumulation of total GLS. Mikkelsen et al. (2003) reported that mutant Arabidopsis plants overexpressing SA had lower level of GLS than the wild type plant. Our previous results (Chapter 4) showed increased accumulation of SA in *B. rapa* plants transformed with the ICS gene. Like in tobacco the increased accumulation may have led to an increased synthesis of SA by endogenous *B. rapa* pyruvate lyase or by an increased spontaneous conversion to SA. SA is one of the signaling compounds for plant defense responses and it is conceivable that the increased levels of SA in the transgenic plants have resulted in enhanced production of the anti-insect indole GLS species. Apparently, the increased flux towards isochorismate did not directly affect the flux towards tryptophan or the indole glucosinolate.

5.4 Conclusion

It was shown that the introduction of the bacterial ICS gene in *B. rapa ssp. oleifera* has no significant effect on the phyloquinone contents. However, the profiles of GLS in the transgenic plants was changed, particularly the ratio of aliphatic to indole GLS. This might be due to increased level of SA that activates the plant defense system.

5.5 Acknowledgements

We thank Malaysian Agricultural Research and Development Institute (MARDI), Malaysia for the Ph.D grant to Sanimah Simoh.