

# UV-B Irradiation Changes Specifically the Secondary Metabolite Profile in Broccoli Sprouts: Induced Signaling Overlaps with Defense Response to Biotic Stressors

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(Received December 9, 2011; Accepted June 20, 2012)

Only a few environmental factors have such a pronounced effect on plant growth and development as ultraviolet light (UV). Concerns have arisen due to increased UV-B radiation reaching the Earth's surface as a result of stratospheric ozone depletion. Ecologically relevant low to moderate UV-B doses ( $0.3\text{--}1\text{ kJ m}^{-2}\text{ d}^{-1}$ ) were applied to sprouts of the important vegetable crop *Brassica oleracea* var. *italica* (broccoli), and eco-physiological responses such as accumulation of non-volatile secondary metabolites were related to transcriptional responses with Agilent One-Color Gene Expression Microarray analysis using the  $2\times 204\text{ k}$  format *Brassica* microarray. UV-B radiation effects have usually been linked to increases in phenolic compounds. As expected, the flavonoids kaempferol and quercetin accumulated in broccoli sprouts (the aerial part of the seedlings) 24 h after UV-B treatment. A new finding is the specific UV-B-mediated induction of glucosinolates (GS), especially of 4-methylsulfinylbutyl GS and 4-methoxy-indol-3-ylmethyl GS, while carotenoids and Chl levels remained unaffected. Accumulation of defensive GS metabolites was accompanied by increased expression of genes associated with salicylate and jasmonic acid signaling defense pathways and up-regulation of genes responsive to fungal and bacterial pathogens. Concomitantly, plant pre-exposure to moderate UV-B doses had negative effects on the performance of the caterpillar *Pieris brassicae* (L.) and on the population growth of the aphid *Myzus persicae* (Sulzer). Moreover, insect-specific induction of GS in broccoli sprouts was affected by UV-B pre-treatment.

**Keywords:** *Brassica* array • Broccoli • Glucosinolates • Insect performance • Plant defense signaling • UV-B.

**Abbreviations:** ANOVA, analysis of variance; ESI, electrospray ionization; GS, glucosinolate; MS, mass spectrometry;

PAR, photosynthetically active irradiation; ROS, reactive oxygen species; UV, ultraviolet.

## Introduction

Plants are sedentary and, therefore, cannot escape environmental stresses such as insect herbivory, pathogen attack, ultraviolet-B (UV-B) radiation, ozone or drought. Although different stress factors may have different molecular targets, a common response to unfavorable environmental conditions is the occurrence of oxidative stress with increased levels of reactive oxygen species (ROS) (Greene 2002). However, a range of environmental factors, including temperature, radiation, air pollution, microorganisms, insects and nutrients, modifies the secondary metabolite composition of plants (Jahangir et al. 2009), thus altering plant stress tolerance (Mittler 2002) and the nutritional value of crop plants for the human diet (Jansen et al. 2008, Verkerk et al. 2009). Only a few environmental factors have such a pronounced effect on plant growth and development as UV-B radiation (280–315 nm). Concerns have arisen in the last decades because the stratospheric ozone layer has been depleted, leading to increased levels of solar radiation reaching the Earth's surface (McKenzie et al. 2007). The threat to ensuring productivity in global agriculture and horticulture due to ozone depletion and loss of plant species cannot be overstated nor should it be overlooked.

Elevated UV-B radiation affects plants directly by altering plant growth and development, biomass production, leaf characteristics and flowering time (Fagerberg and Bornman 2005, Hectors et al. 2007). UV-B may indirectly modify plant–herbivore/microbe interactions above and below ground (Caldwell et al. 2007). Studies with the model plant *Arabidopsis thaliana* (L.) Heyhn. revealed two different UV-B-specific signaling

*Plant Cell Physiol.* 53(9): 1546–1560 (2012) doi:10.1093/pcp/pcs096, available online at [www.pcp.oxfordjournals.org](http://www.pcp.oxfordjournals.org)

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pathways, one dependent on UV RESISTANCE LOCUS 8 (UVR8) (Brown et al. 2005, Cloix and Jenkins 2008, Jenkins 2009). At low UV-B conditions, expression of the ELONGATED HYPOCOTYL 5 (HY5) transcription factor is induced by UVR8 and CONSTITUTIVELY PHOTOMORPHOGENETIC 1 (COP1), which in turn regulates genes involved in photomorphogenetic UV-B response and metabolic accumulation (Favory 2009). Just recently, Rizzini and co-workers (2011) demonstrated that the perception of UV-B is facilitated by the UVR8 protein, probably by a tryptophan-based mechanism. Besides UV-B-specific signaling, high UV-B doses induce DNA damage and subsequent signaling components, ROS and wound/defense signaling molecules such as jasmonic acid, salicylic acid, nitric oxide, ethylene and ABA (Jenkins et al. 2009).

Some studies report negative plant-mediated effects of elevated UV-B levels on insects, such as decreased oviposition rates of the Lepidoptera *Plutella xylostella* L. (Caputo et al. 2006), poor caterpillar performance (Lindroth et al. 2000), less leaf tissue consumption by larvae of the moth *Manduca sexta* (L.) (Rousseaux et al. 2004) and a lower insect incidence under field conditions (Mazza et al. 1999). In contrast, Kuhlmann and Müller (2009) observed no UV-B-dependent effects on insect incidence on *Brassica oleracea* var. *italica* plants in the field cultivated under different UV-B transmittance coverings. Even less understood is the impact of enhanced UV-B on pathogen infections. Reports show that radiation may either decrease or increase plant susceptibility to pathogens (Raviv and Antignus 2004).

The production of flavonoids and related phenolic compounds as a response to UV-B in several plant species is well documented (Kakani et al. 2003, Caldwell et al. 2007). Most studies used acute enhanced UV-B levels and focused on the accumulation of flavonoids including anthocyanins, and hydroxycinnamic acids as UV-B protectants by serving as active oxygen scavengers (Mackerness 2000, Peng et al. 2003, Ruhland et al. 2005, Hectors et al. 2007, Jansen et al. 2008). However, less is known about the response of other non-volatile compounds such as glucosinolates (GS) under ambient moderate ( $1\text{--}3\text{ kJ m}^{-2}\text{ d}^{-1}$ ) or low UV-B supplementation ( $<1\text{ kJ m}^{-2}\text{ d}^{-1}$ ). Recent studies have highlighted that ecologically relevant low UV-B levels can trigger the induction of phenolic compounds and the accumulation of carotenoids and GS (e.g. Huyskens-Keil et al. 2007, Perez et al. 2009, Schreiner et al. 2009). GS and their hydrolysis products are known to have a function in plant resistance against insects and pathogens (Mewis et al. 2005, Halkier and Gershenzon 2006, Mewis et al. 2006, Kim and Jander 2007, Clay et al. 2009). The accumulation of flavonoids and GS is influenced by the same signaling pathways involving jasmonic acid (Mackerness et al. 1999, Textor and Gershenzon 2009).

GS are sulfonated thioglycosides sharing a common glycone moiety with a variable aglycone side chain. Based on the side chain structure, GS are divided into three classes: aliphatic GS derived principally from methionine, indolyl GS from tryptophan, and aromatic GS from phenylalanine. GS biosynthesis

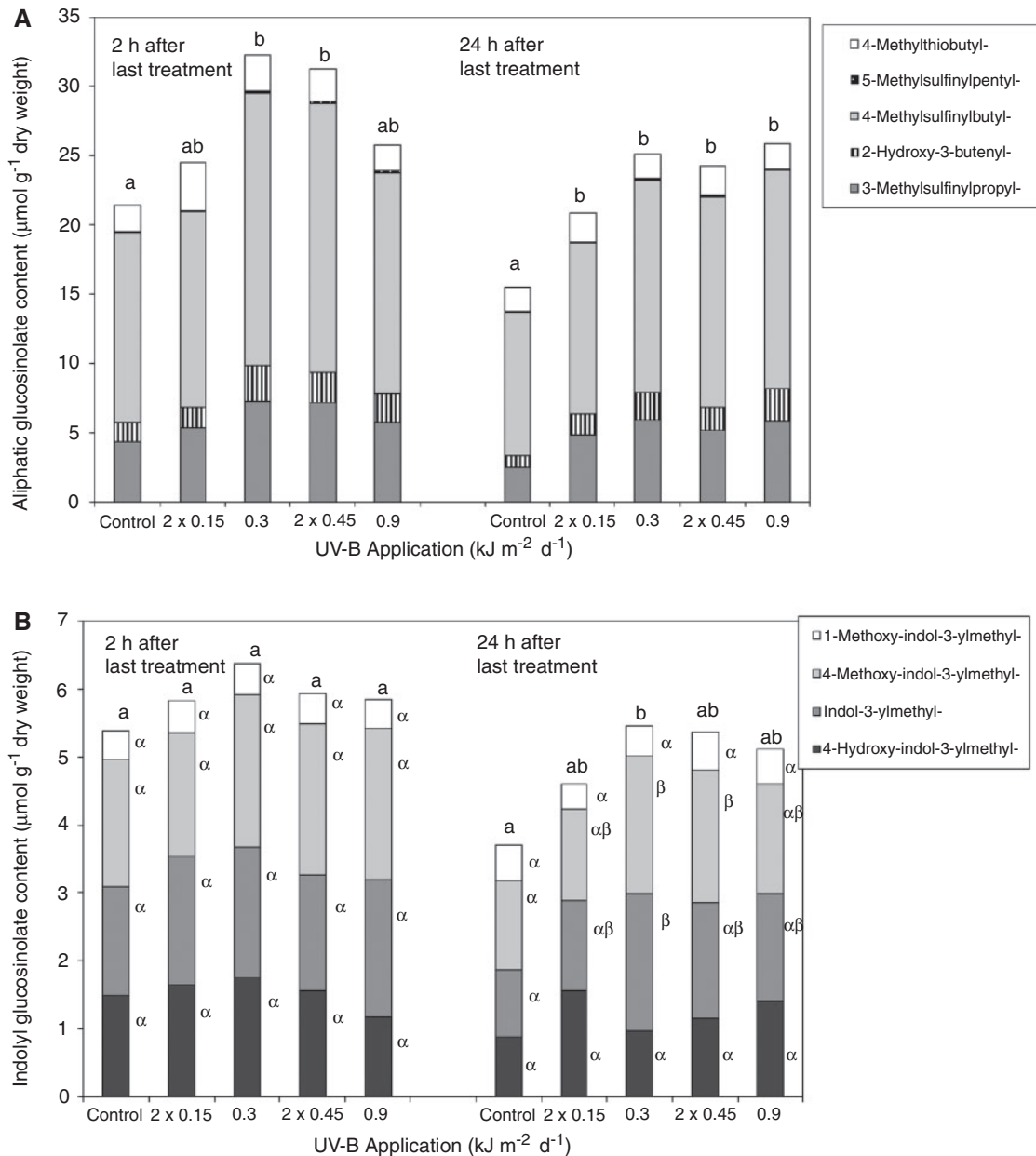
proceeds through three independent steps: (i) chain elongation of selected precursor amino acids; (ii) formation of the core GS structure; and (iii) side chain modifications. The GS biosynthetic pathway has been defined in *A. thaliana*, and most genes associated with GS biosynthesis have been isolated and characterized (Halkier and Gershenzon 2006, Sønderby et al. 2010). In contrast, little is known about GS-specific genes in *B. oleracea* crops and only a few quantitative trait loci (QTLs) are well characterized; *BoGSL-Elong* encodes a methylthioalkylmalate synthase (MAM) responsible for four-carbon side chain elongation (4 C), whereas *BoGSL-PRO* leads to formation of the three-carbon side chain structure of GS (3 C) (Li et al. 2001, Li and Quiros 2002). In *A. thaliana*, AOP2 catalyzes the conversion of S-oxygenated GS to alkenyl GS and AOP3 leads to the formation of hydroxyalkyl GS (Kliebenstein et al. 2001), whereby *BoGSL-ALK* represents the *B. oleracea* homolog (Li and Quiros 2003).

Previous studies gave only limited insights into UV-B-mediated effects on non-volatile secondary metabolites not belonging to the flavonoids and they often used unrealistically high UV-B doses ( $>15\text{ kJ m}^{-2}\text{ d}^{-1}$ ). The first approach of our study was to determine the elicitor effects of ecologically relevant low ( $<1\text{ kJ m}^{-2}\text{ d}^{-1}$ ) and moderate UV-B ( $1\text{ kJ m}^{-2}\text{ d}^{-1}$ ) doses on GS accumulation and on transcriptome changes in a brassicaceous crop plant, *B. oleracea* var. *italica* (broccoli). Due to the high GS levels in broccoli sprouts (the aerial part of the seedlings), 20–50 times of those found in broccoli inflorescences (Fahey et al. 1997), and especially the high stress responsiveness and tolerance of young plants (Jung 2004), this ontogenetic stage was chosen to study UV-B-mediated changes. Since GS also define plant resistance to herbivory (Halkier and Gershenzon 2006), the second objective of our study was to test if the UV-B-provoked changes in accumulation of secondary metabolites influence the plant's resistance against insect herbivores. In a third approach, the combined effect of UV-B on insect-elicited GS accumulation was studied. The model insects *Myzus persicae* (Sulzer) (Hemiptera), *Pieris brassicae* (L.) (Lepidoptera) and *Phaedon cochleariae* (F.) (Coleoptera) were selected to represent three different insect orders that potentially could elicit different plant responses.

## Results

### UV-B dose effect on glucosinolate accumulation in broccoli sprouts

To investigate elicitor effects of UV-B on GS accumulation in broccoli, sprouts were exposed both once and twice to different equivalent low UV-B dosages. Already 2 h after UV-B application, changes in GS levels of sprouts (the aerial part of the plant) were observed compared with the control (Fig. 1A). Aliphatic GS increased significantly at dosages of once 0.3 and twice  $0.45\text{ kJ m}^{-2}\text{ d}^{-1}$ , whereas indolyl GS remained unchanged 2 h after UV-B exposure. Aliphatic GS levels were significantly higher by up to roughly 2-fold in all UV-B treatments compared



**Fig. 1** Aliphatic (A) and indolyl (B) glucosinolate contents of broccoli sprouts after single and double exposure to different UV-B doses. In the double treatments with two UV-B applications, the first application was done 24 h before harvest, followed by a second application 2 h before harvest, resulting in equivalent levels to single treatments. Sprouts were 13 days old. Different letters indicate significant differences among treatments at the two different harvest times, Tukey's HSD test  $P \leq 0.05$ . Statistical differences are given in Greek letters on the right of the bar for single compounds;  $n = 6$  biological replicates with sprouts cultivated from 0.5 g of seeds.

with control sprouts 24 h after the last exposure. Also, at this time point the indolyl GS levels were significantly higher compared with non-treated sprouts, but only at a single dose of  $0.3 \text{ kJ m}^{-2} \text{d}^{-1}$ ; however, there was no difference within UV-B treatments (Fig. 1B). When comparing the results of single and double (24 h interval) UV-B applications of an equivalent dose, no distinct effects on GS accumulation were observed. However, the highest GS levels were found in the sprouts treated once with  $0.3 \text{ kJ m}^{-2} \text{d}^{-1}$  UV-B, but this was not significantly different from the contents of other treated sprouts. No further

increase in GS contents of sprouts was achieved with the higher dosages of  $2 \times 0.45$  and  $0.9 \text{ kJ m}^{-2} \text{d}^{-1}$  after 2 and 24 h, respectively. It is worth noting that the GS contents per plant dry weight were generally lower 24 h post-treatment compared with 2 h. The total GS contents in control samples were about 25% lower after 24 h compared with the samples taken after 2 h. Also, the GS levels of sprouts exposed to  $0.15$ ,  $2 \times 0.15$ ,  $0.3$  and  $2 \times 0.45 \text{ kJ m}^{-2} \text{d}^{-1}$  UV-B were about 20% lower 24 h post-harvest compared with 2 h; the exception was for the dose of  $0.9 \text{ kJ m}^{-2} \text{d}^{-1}$  UV-B, with no changes in aliphatic GS.

Altogether, exposure of sprouts to UV-B led to an increase particularly in methylsulfinylalkyl GS such as 3-methylsulfinylpropyl GS and 4-methylsulfinylbutyl GS at 2 and 24 h after application (Fig. 1A). Indolyl GS responded differently to UV-B, in that 4-methoxy-indol-3-ylmethyl GS in particular increased 24 h post-treatment at UV-B doses of 0.3,  $2 \times 0.45$ , and  $0.9 \text{ kJ m}^{-2} \text{ d}^{-1}$  (Fig. 1B).

### UV-B-induced changes within secondary metabolite levels and corresponding gene expression in broccoli sprouts

In this experiment, the UV-B-induced overall secondary metabolite biosynthesis and corresponding gene expression in broccoli sprouts was investigated. The low UV-B doses did not influence the dry weight gain within 24 h. Furthermore, the water content of the UV-B-treated sprouts was  $87.97 \pm 1.71\%$  of the content of the control sprouts. However, a significant increase in all aliphatic GS, except 5-methylsulfinylpentyl GS, was observed in sprouts 24 h after application of  $0.6 \text{ kJ m}^{-2} \text{ d}^{-1}$  UV-B compared with control sprouts (Table 1), which was similar to the UV-B-mediated induction observed in the first experiment in the greenhouse (Fig. 1A). The effect of UV-B on indolyl GS depended on the compound. As shown in the previous experiment, 4-methoxy-indol-3-ylmethyl GS increased significantly compared with the control, whereas

1-methoxy-indol-3-ylmethyl GS decreased. UV-B exposure also led to elevated flavonoid levels in sprouts, whereby the accumulation of kaempferol was more pronounced than that of quercetin. In addition, profiles of  $\beta$ -carotene (the only carotenoid detected in sprouts) and Chl *a* and *b* were analyzed, but no differences were found upon treatment.

A DNA microarray analysis of the gene transcripts in RNA extracted from 12-day-old broccoli sprouts showed that UV-B induced the expression of genes homologous to those in *A. thaliana* that are related to specific stress responses stimulated by fungal and bacterial pathogens (Table 2; Supplementary Table S1). The pathogen- and salicylic acid-responsive genes *PR-1* (JCVI\_40837) and *PR-2* were increased 4- to 5-fold among the five most up-regulated genes. Also the expression of *PR-4* (HEL, JCVI\_16583) and *BG3* (JCVI\_19327), genes associated with salicylate and jasmonic acid signaling and also induced by pathogen attack and/or wounding, increased 3- to 4-fold. Furthermore, genes homologous to xenobiotic stress-responsive glutathione *S*-transferases, *ATGSTF3* (JCVI\_30), *ATGSTU25* (JCVI\_13432) and *ATGSTU8* (JCVI\_35521), and one linked to chitin binding and chitinase activity (*CHI*) had a 3-fold increase among the most up-regulated genes (Table 2; Supplementary Table S1).

When possible, elements on the *Brassica* array were identified by their homology to known genes of *A. thaliana* and were classified into their respective bins using MapMan (Thimm et al. 2004). The most responsive genes are listed according to their potential function in secondary metabolite biosynthesis in Table 2. The *Brassica* microarray data revealed that UV-B increased the expression of genes presumably coding for CYP71A (JCVI\_19911) and CYP71B (DY019565) families of Cyt P450 monooxygenases (Supplementary Table S1) and especially CYP71A12 and CYP71B14, the potential orthologs of those involved in phytoalexin biosynthesis in *A. thaliana*, whose transcripts increased about 2-fold in response to UV-B (Table 2). UV-B also induced changes in the expression of GS biosynthesis genes; the most responsive, with about a 2-fold increase, was *FMO GS-OX5* (JCVI\_13189), involved in the conversion of methylthioalkyl GS into methylsulfinylalkyl GS. In addition, transcript levels of the transcription factor *MYB51* (EX121009) were elevated relative to the non-treated control, as were gene transcripts homologous to those involved in indolyl GS biosynthesis, e.g. *CYP81F2* (AM388472), which catalyzes the hydroxylation of indol-3-yl-methyl GS to 4-hydroxy-indol-3-yl-methyl GS. As well as the induction of *FMO GS-OX5* and *MAM1*, expression levels of many genes homologous to those of aliphatic GS biosynthesis decreased about 2-fold, such as, for example, *CYP79F1*, *MAM3* and *BCAT4* (EX056879, JCVI\_30455, JCVI\_34763, Table 2; Supplementary Table S1). Also transcripts for the epithiospecifier protein *ESP* (JCVI\_2436) involved in degradation processes were about 2-fold down-regulated by UV-B. The transcript abundance of genes homologous to *TGG1* and *TGG2*, coding for myrosinase enzymes, either did not change significantly (data not shown) or were changed <2-fold (JCVI\_18277, JCVI\_20363, JCVI\_29722).

**Table 1** Glucosinolate, flavonoid, carotenoid and Chl contents of broccoli sprouts treated with  $0.6 \text{ kJ m}^{-2} \text{ d}^{-1}$  UV-B compared with the control, harvested 24 h after application

Group	Specific compound	Content ( $\mu\text{mol g}^{-1}$ DW)	
		Control	UV-B
Glucosinolates	3-Methylsulfinylpropyl-	23.77 $\pm$ 1.84	34.27 $\pm$ 1.37*
	( <i>R</i> )-2-Hydroxy-3-butenyl-	0.95 $\pm$ 0.10	1.48 $\pm$ 0.04*
	4-Methylsulfinylbutyl-	47.69 $\pm$ 4.11	70.95 $\pm$ 2.89*
	5-Methylsulfinylpentyl-	0.21 $\pm$ 0.08	0.34 $\pm$ 0.02
	4-Methylthiobutyl-	9.90 $\pm$ 0.74	13.40 $\pm$ 1.35*
	4-Hydroxy-indol-3ylmethyl-	4.09 $\pm$ 0.06	4.21 $\pm$ 0.20
	Indol-3ylmethyl-	2.92 $\pm$ 0.09	3.10 $\pm$ 0.19
	4-Methoxy-indol-3ylmethyl-	1.08 $\pm$ 0.04	1.79 $\pm$ 0.19*
	1-Methoxy-indol-3ylmethyl-	0.53 $\pm$ 0.04	0.33 $\pm$ 0.01*
	Total	91.23 $\pm$ 4.97	129.98 $\pm$ 5.69*
Flavonoids	Kaempferol	0.54 $\pm$ 0.01	1.11 $\pm$ 0.02*
	Quercetin	0.20 $\pm$ 0.00	0.31 $\pm$ 0.00*
	Total	0.74 $\pm$ 0.01	1.42 $\pm$ 0.02*
Carotenoids	$\beta$ -Carotene	3.1 $\pm$ 0.48	2.9 $\pm$ 0.25
	Total	3.1 $\pm$ 0.48	2.9 $\pm$ 0.25
Chls	Chl <i>a</i>	6.5 $\pm$ 1.03	5.9 $\pm$ 0.39
	Chl <i>b</i>	2.9 $\pm$ 0.53	2.5 $\pm$ 0.18
	Total	9.4 $\pm$ 1.40	8.4 $\pm$ 0.70

Plants were 13 days old.

An asterisk indicates a significant difference among metabolite contents of treatments, ANOVA \* $P \leq 0.05$ ,  $\pm$ SD,  $n = 3$  biological replicates with sprouts from 1 g of seeds.



**Table 2** UV-B-induced changes of transcript levels of genes related to secondary metabolite biosynthesis pathways or stress response in broccoli sprouts 24 h after exposure

Putative function	Identifier	Transcript changes <sup>a</sup>	Description
Phytoalexins	JCVI_19911	2.12	Moderately similar to AT2G30750, CYP71A12, CYTOCHROME P450 FAMILY 71 SUBFAMILY A POLYPEPTIDE 12, oxygen binding
	DY019565	1.86	Weakly similar to AT5G25180, CYP71B14, CYTOCHROME P450 FAMILY 71 SUBFAMILY B POLYPEPTIDE 14, oxygen binding
Glucosinolates	JCVI_13189	1.85	Moderately similar to ATG12140, FMO GS-OX5, FLAVIN-MONOXYGENASE GLUCOSINOLATE S-OXYGENASE
	AM388472	1.68	Weakly similar to AT5G57220, CYP81F2, CYTOCHROME P450 81F2
	EX121009	1.39	Moderately similar to AT1G18570, HIG1, MYB51, MYBDOMAIN PROTEIN 51, HIGH INDOLIC GLUCOSINOLATE 1
	EV175932	0.88	Weakly similar to AT4G37410, CYP81F4, CYTOCHROME P450 81F4
	JCVI_8389	0.78	Moderately similar to AT5G23010, IMS3, MAM1, 2-ISOPROPYL-MALATE SYNTHASE 3
	JCVI_33391	0.63	Highly similar to AT4G39950, CYP79B2, CYTOCHROME P450 79B2
	JCVI_6771	-1.22	Highly similar to AT2G31790, UGT74C, UDP-GLUCOSYL TRANSFERASE
	JCVI_2436	-1.85	Moderately similar to AT1G54040, ESR, TASTY, ESP, EPITHIOSPECIFIER PROTEIN
	EX056879	-1.87	Very weakly similar to AT1G16410, BUS1, SPS1, BUSHY 1, CYP79F1, SUPERSHOOT 1, CYTOCHROME P450 79F1
	JCVI_30455	-1.88	Highly similar to AT5G23020, IMS2, MAM3, MAML, 2-ISOPROPYL-MALATE SYNTHASE 2
	JCVI_34763	-2.3	Weakly similar to AT3G19710, BCAT4, BRANCHED-CHAIN AMINOTRANSFERASE4, catalytic/methionine-oxo-acid transaminase
Flavonoids	JCVI_35049	3.45	Weakly similar to AT4G34135, UGT73B2, UDP-glycosyltransferase, flavonol 3-O-glucosyltransferase activity
	EX131200	1.80	Moderately similar to AT5G49690, UDP-glycosyltransferase family protein
	JCVI_2934	-1.63	Highly similar to AT5G08640, FLS, FLAVONOL SYNTHASE
	JCVI_3974	-1.77	Moderately similar to AT5G13930, CHS, TT4, CHALCONE SYNTHASE, naringenin-chalcone synthase
	JCVI_2577	-1.90	Moderately similar to AT5G05270, chalcone-flavanone isomerase family
Isoprenoids	JCVI_20479	4.73	Moderately similar to AT1G64970, TMT1, VTE4, G-TMT, GAMMA-TOCOPHEROL METHYLTRANSFERASE
	JCVI_2005	1.41	Moderately similar to AT1G74470, geranylgeranyl reductase
Stress response	JCVI_40837	4.85	Very weakly similar to AT2G14610, PR-1, PR1, PATHOGENESIS-RELATED GENE 1
	JCVI_33787	4.63	Moderately similar to AT3G11340, UDP-glucuronosyl/UDP-glucosyl transferase family protein
	JCVI_24991	4.31	Moderately similar to AT3G57260, PR2, BG2, PR-2, BGL2, glucan 1,3-beta-glucosidase, hydrolyzing O-glycosyl compounds
	JCVI_16762	3.99	Moderately similar to AT3G13080, EST2, MRP3, ATM RP3, Arabidopsis thaliana multidrug resistance-associated protein 3
	JCVI_19327	3.81	Moderately similar to AT3G57240, BG3, BETA-1,3-GLUCANASE 3, hydrolyzing O-glycosyl compounds
	JCVI_291	3.77	Moderately similar to AT4G11650, ATOSM34, OSMOTIN 34
	EV116790	3.64	Moderately similar to AT5G13320, GDG1, WIN3, PBS3, AVRPPHB SUSCEPTIBLE 3
	JCVI_18994	3.30	Moderately similar to AT2G43570, CHI, putative chitinase
JCVI_16583	2.93	Weakly similar to AT3G04720, HEL, PR-4, PR4, PATHOGENESIS-RELATED 4	

<sup>a</sup> Log 2-fold change of expression; only transcript changes with a *P*-value < 0.05 are shown.

Among the genes associated with flavonoid biosynthesis, UV-B strongly induced by 3-fold the expression of a homolog of UGT73B2 (JCVI\_35049), involved in glycosylation of flavonoids, and a gene encoding a UDP-glycosyltransferase family protein. On the other hand, the levels of many transcripts of genes of flavonoid biosynthesis decreased, such as, for example,

homologs of FLS and CHS (JCVI\_2934, JCVI\_3974, **Table 2; Supplementary Table S1**). Furthermore, UV-B had only marginal effects on the transcript levels of genes involved in isoprenoid biosynthesis, and only a homolog of TMT1 (JCVI\_20479), a  $\gamma$ -tocopherol methyltransferase, increased 5-fold.

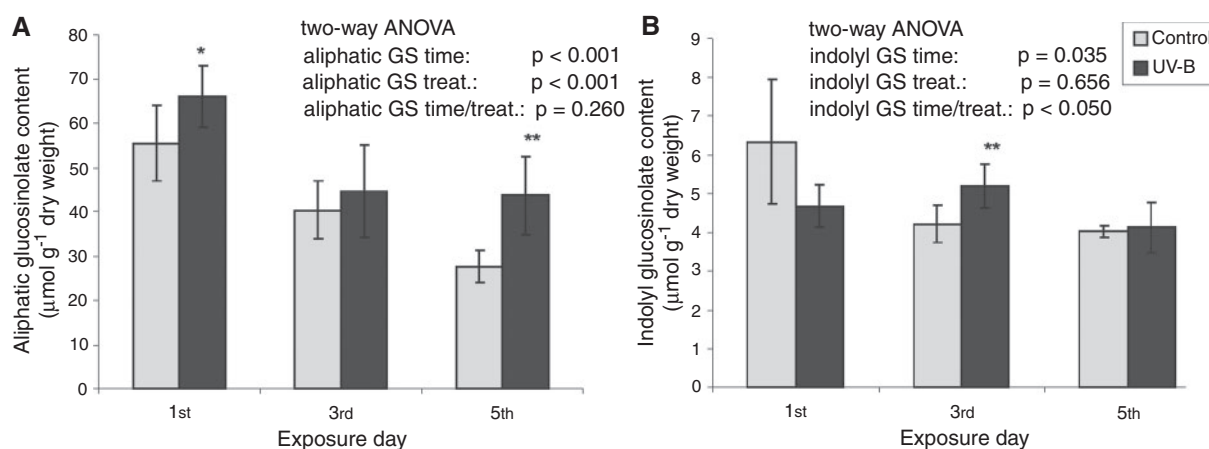
### Long-term effects of UV-B on glucosinolate accumulation in broccoli sprouts and plant resistance against insect herbivory

The experiments demonstrate that UV-B elevates GS levels in broccoli sprouts followed by an up-regulation of genes involved in the biotic stress response and the final step of GS biosynthesis. Therefore, an experiment was conducted to investigate the UV-B-mediated effects on insect–plant interactions. Sprouts which received a daily dose of  $1 \text{ kJ m}^{-2}$  UV-B for 5 d were chosen for the insect treatment. The UV-B doses and application time used corresponded to the mean ambient UV-B levels measured in Großbeeren, Germany during the summer. After 5 d of UV-B exposure, sprouts contained significantly higher aliphatic GS levels when compared with untreated sprouts (Fig. 2A). The aliphatic and indolyl GS content of control and treated broccoli sprouts (the aerial part of the seedling) decreased during the 5 d of treatment. UV-B influenced only aliphatic GS levels significantly [two-way analysis of variance (ANOVA), Fig. 2A, B], whereas the interaction time and treatment were only significant for levels of indolyl GS. Sprouts pre-exposed for 5 d to UV-B and non-irradiated sprouts (GS levels are displayed in Fig. 2) were used for the subsequent bioassays with insects for 3 d.

**Table 3** Performance of the aphid *M. persicae* and the caterpillar *P. brassicae* after 3 d on UV-B-treated (five times  $1 \text{ kJ m}^{-2} \text{ d}^{-1}$ ) and untreated broccoli sprouts

Species	Control	UV-B
	Aphid number	
<i>Myzus persicae</i>	$7.3 \pm 0.7$	$2.3 \pm 0.9^{**}$
	Weight gain (mg)	
<i>Pieris brassicae</i>	$44.0 \pm 4.9$	$27.1 \pm 3.3^*$

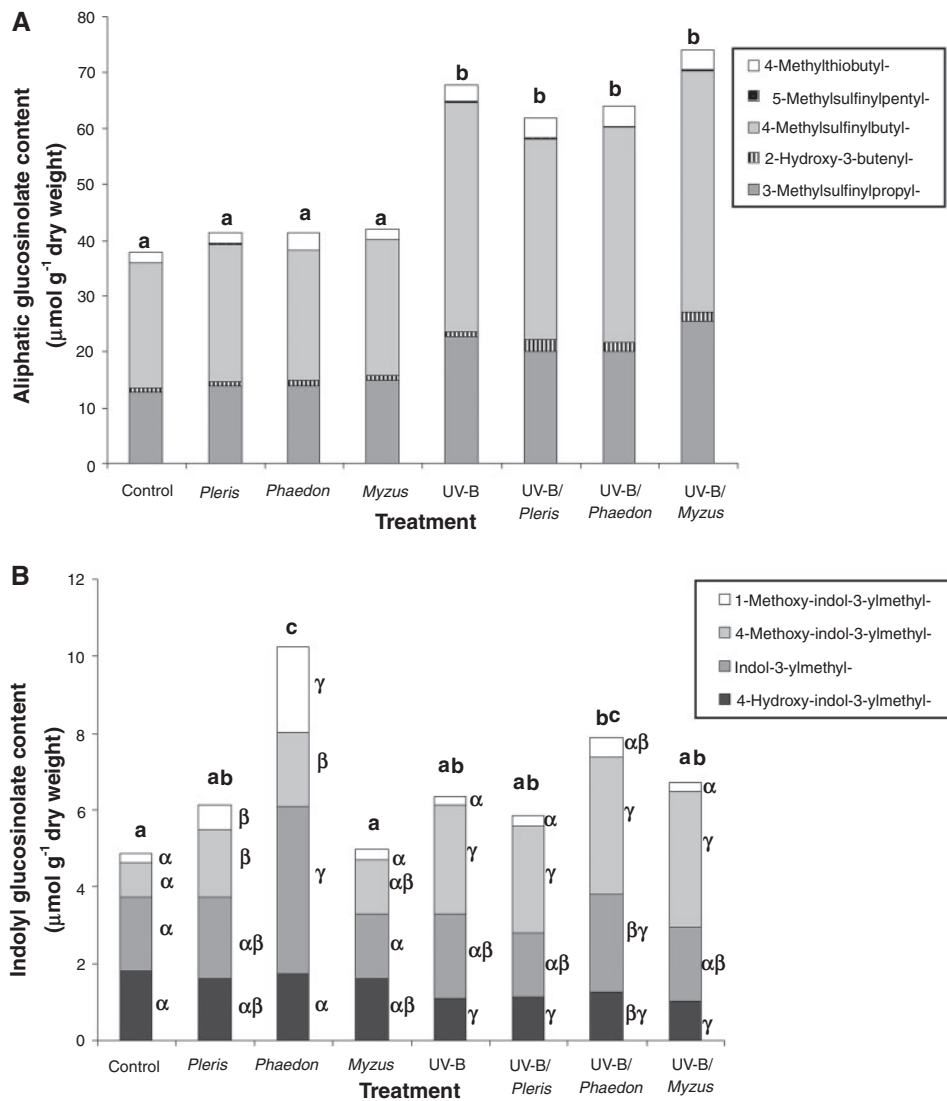
An asterisk indicates a significant difference among insect performance on treatments, ANOVA  $*P < 0.05$ ,  $**P < 0.01$ ,  $\pm$ SD,  $n = 12$ .



**Fig. 2** Aliphatic (A) and indolyl (B) glucosinolate contents of broccoli sprouts on days 1, 3 and 5, after exposure to  $1 \text{ kJ m}^{-2} \text{ d}^{-1}$  UV-B for 5 d. Sprouts were 13 days old. treat. = treatment. Asterisks indicate significant differences among treatments at the different harvest times, RMANOVA  $*P \leq 0.05$ ,  $**P \leq 0.001$ ; data are means  $\pm$ SD,  $n = 6$  biological replicates with sprouts cultivated from 0.5 g of seeds.

After 3 d without receiving UV-B and without insect feeding, sprouts that had been pre-exposed to UV-B had significantly (nearly 2-fold) higher aliphatic GS levels and had a distinct profile of indolyl GS compared with non-irradiated sprouts (Fig. 3A, B). The most significant difference in the indolyl GS profile in response to UV-B was the approximately 4-fold increased abundance of 4-methoxy-indol-3-ylmethyl GS (Fig. 3B). Levels of the other indolyl GS in the UV-B-pre-exposed sprouts were not different from the control or, in the case of 4-hydroxy-indol-3-ylmethyl GS, the levels decreased significantly (Fig. 3B).

Because insects can also induce the accumulation of GS, the insect-mediated defense responses were compared with those induced by UV-B or a combination of both stressors. GS accumulation in response to 3 d of insect feeding was different from the response elicited by UV-B only. There was no effect of insect feeding on aliphatic GS levels (Fig. 3A) but for the indolyl GS, there were increases in the abundance and changes in the relative proportions of individual indolyl GS after feeding by the two chewing insects (Fig. 3B). The GS induction by the insects of the three orders differed. After feeding by the specialist caterpillar *P. brassicae*, 1-methoxy-indol-3-ylmethyl GS levels were 3-fold higher than in the control. Although 4-methoxy-indol-3-ylmethyl and 1-methoxy-indol-3-ylmethyl GS accumulated significantly after caterpillar feeding, the total indolyl GS level after herbivory of *P. brassicae* was not significantly different from that of the control (Fig. 3B). Herbivory by the specialist beetle *P. cochleariae* in comparison with control sprouts resulted in significantly higher indolyl GS contents. The levels of 1-methoxy-indol-3-ylmethyl GS increased significantly and were about 10-fold higher compared with control sprouts, while the abundance of 4-hydroxy-indol-3-ylmethyl GS was not significantly affected (Fig. 3B). GS levels in sprouts remained unchanged when compared with the control after phloem sucking for 3 d by the generalist aphid *M. persicae*.



**Fig. 3** Aliphatic (A) and indolyl (B) glucosinolate contents of 3-week-old broccoli sprouts after exposure for 3 d to insect species compared with unchallenged plants. Sprouts were treated with  $1 \text{ kJ m}^{-2} \text{ d}^{-1}$  UV-B five times before exposure to insects for 3 d or remained unchallenged. Different Arabic letters indicate significant differences among treatments, ANOVA, UV-B covariate, followed Tukey's HSD test  $P \leq 0.05$ . Statistical differences are given in Greek letters to the right of bar for single compounds;  $n = 6$  biological replicates with sprouts cultivated from 0.5 g of seeds.

In all treatments of plants exposed to the combination of UV-B and insects, the aliphatic GS content of sprouts was significantly higher than in the non-irradiated and insect-free control (ANOVA covariate UV-B  $P < 0.05$ ), mainly due to the marked increase in 4-methylsulfinylbutyl GS. Interestingly, 4-methoxy-indol-3-ylmethyl GS levels were about 3- to 4-fold higher when compared with the control in all combination treatments and also significantly higher when compared with sprouts after exposure to insects only (Tukey's HSD test  $P < 0.001$ ). It has to be emphasized that the *P. cochleariae*-mediated strong plant response in terms of indolyl GS was not observed in UV-B-pre-exposed sprouts and no increase in 1-methoxy-indol-3-ylmethyl GS was observed when compared with the control (Tukey's HSD test  $P = 0.998$ ).

UV-B influenced not only the GS profile of broccoli sprouts but also the plant resistance against insect feeding (Table 3). Significantly lower numbers of *M. persicae* were found on UV-B-treated sprouts compared with aphids found on the control (Table 3). Even the aphid population size decreased on sprouts pre-exposed to UV-B. Also, the weight gain of *P. brassicae* was significantly lower after 3 d of feeding on sprouts cultivated with supplemental UV-B than on the control sprouts (Table 3).

## Discussion

The analysis of UV-B-induced changes in metabolite levels has focused largely on the flavonoids, and the effects have

usually been associated with increases in these compounds (Frohnmeyer and Staiger 2003, Ruhland et al. 2005). We focused here on another group of secondary metabolites, the GS, which are not involved in UV shielding but are important in the plant defense against herbivores and pathogens. Even a single and low dose of 0.3 or 0.6 kJ m<sup>-2</sup> d<sup>-1</sup> UV-B applied to non-UV-B-acclimated broccoli sprouts increased GS levels specifically within 2 h after treatment. Interestingly, greater doses of up to 1 kJ m<sup>-2</sup> d<sup>-1</sup> UV-B and multiple exposure times did not elicit a stronger response of aliphatic GS accumulation. Our study reveals that ecologically relevant UV-B levels can trigger in the sprout part of the plant the accumulation of 4-methylsulfinylbutyl GS, the precursor of presumably health-promoting sulforaphane (4-methylsulfinylbutyl isothiocyanate; Haack et al. 2010). Two-fold increases in aliphatic GS levels have been found in sprouts after exposure to UV-B compared with non-irradiated sprouts. It cannot be clearly concluded whether the GS are synthesized de novo in the sprouts or if they are translocated from the roots to the aerial part of the plant. However, the GS profile is different in roots compared with leaves and siliques, with a higher proportion of indolyl GS in the roots as determined in *A. thaliana* (Brown et al. 2003). Another possibility is that UV-B might slow down the rate of GS degradation in sprouts and that the rate of degradation might be inversely correlated with the UV-B dose. The reduction of GS to amines and hydrolysis to yield the corresponding amino acids has been demonstrated (for a review, see Bones and Rossiter 2006); these substrates could be used for other metabolic plant processes, e.g. protein biosynthesis. Elevated GS levels after UV-B treatment were reported in other plant species (Schreiner et al. 2012). A low UV-B dose treatment of *Tropaeolum majus* L. induced a 6-fold increase in benzyl GS levels of young, immature green seeds, whereas the GS response in mature leaves and inflorescences was not as pronounced (Schreiner et al. 2009). Initially, enhanced UV-B radiation (1.55 W m<sup>-2</sup>) induced production of several GS such as 4-methylsulfinylbutyl GS in *A. thaliana*. However, after continued exposure to UV-B, the expression of GS metabolism-related genes was inhibited, and the GS content, especially that of indolyl GS, declined (Wang et al. 2011).

Linkage of UV-B-mediated changes in secondary metabolite content to the corresponding transcriptome has not been performed with broccoli so far and was, therefore, one aspect of our study. Gene expression of GS biosynthetic genes determined 24 h after exposure to UV-B only partially corresponded to UV-B-mediated accumulation of GS. It is believed that elicitor signal perception initiates a signal transduction network that leads to activation of transcription factors as an early response and subsequent regulation of expression of biosynthetic genes involved in secondary metabolite production (Zhao et al. 2005). In agreement with this, genes homologous to those of *A. thaliana* involved in later steps of GS biosynthesis were markedly induced in broccoli sprouts. The most responsive gene was a gene homolog of *FMO GS-OX5*, involved in the conversion of methylthioalkyl GS into methylsulfinylalkyl

GS (Li et al. 2008), thus confirming the observed accumulation of 4-methylsulfinylbutyl GS in sprouts. Also the expression of a homolog of *CYP81F2* was up-regulated by UV-B. *CYP81F2* catalyzes the hydroxylation of indol-3-ylmethyl GS to 4-hydroxy-indol-3-ylmethyl GS (Pfalz et al. 2009), the precursor of 4-methoxy-indol-3-ylmethyl, which accumulated after UV-B exposure in sprouts.

As shown in previous studies and as also described here, the levels of the UV-B protectants kaempferol and quercetin increased in UV-B-treated broccoli sprouts. The greatest increase, especially of kaempferol, was achieved with a single low dose application. Previous studies with brassicaceous plants showed a relatively higher increase in quercetin compared with kaempferol (Olsson et al. 1998, Reifenrath and Müller 2007). Quercetin flavonols have been speculated to be a more efficient free radical scavenger than kaempferol flavonols (Harborne and Williams 2000). However, these previous studies focused on mature plants, and a different metabolic response of the abundant kaempferol in broccoli sprouts seems likely. Ontogenetic effects are further supported by Krumben and co-workers (2007), who determined changes in quercetin and kaempferol concentrations during head ontogeny of three broccoli cultivars, with the highest contents in the mature inflorescence. Accumulation of flavonoids by UV-B is triggered by induction of the phenylpropanoid biosynthesis pathway (Jenkins et al. 2009), and genes coding for enzymes of the phenylpropanoid pathway are up-regulated in *A. thaliana*, including PAL and various flavonol synthases (Ulm et al. 2004). In the present study with broccoli sprouts pre-exposed to low UV-B levels, we have described a decrease in the transcript levels of many broccoli genes homologous to those of *A. thaliana* phenylpropanoid biosynthesis. Similar results were obtained by Hectors and co-workers (2007) with *A. thaliana* plants acclimated to low UV-B levels. An explanation for the observed decrease in expression of genes involved in phenylpropanoid biosynthesis would be that stable flavonoids had already accumulated in sufficient amounts to provide protection from low UV-B doses within the 24 h adaptation time. Correspondingly, two genes with homology to the UDP-glycosyltransferase family protein, *UGT73B2*, involved in the final step of glycosylation (Willits et al. 2004) were induced 3.5-fold following UV-B treatment. We did not observe effects of low supplemental UV-B on the isoprenoid and Chl content of broccoli sprouts, and also the corresponding gene expression was not changed.

Plant responses to UV-B are believed to be partly similar to the responses induced by insects, in which the jasmonic acid pathway has a central role (Izaguirre et al. 2007, Demukra et al. 2010). However, gene expression changes in the present study revealed that salicylic acid pathway signaling is induced by a low non-damaging UV-B dose. In broccoli sprouts, *PR-1* and *PR-2* homologs were induced, while those genes in *A. thaliana* are salicylic acid and pathogen responsive (Gu et al. 2002). Recent studies have shown that PR genes are also regulated by environmental factors including



radiation and development processes (Seo et al. 2008). Furthermore, UV-B induced the expression of several genes homologous to those of glutathione-S-transferases. Although plant glutathione-S-transferase has been mainly studied with regard to its role in herbicide detoxification, little is known about its other biological functions in plants (Frova 2003). Among others, the up-regulation of *ATGSTF3* (*AtGSTF6*) by UV-B was determined, whereby induction of this gene by avirulent *Pseudomonas syringae* was mediated by salicylic acid and ethylene signaling (Lieberherr et al. 2003). The UV-B treatment-induced expression of gene homologs that are also induced by fungal and bacterial pathogens suggests that pre-conditioned sprouts may be altered in their tolerance to pathogen infections. UV-B also increased the transcript levels of *PR-4* (HEL) and *BG3*, which are genes associated with salicylate/jasmonic acid signaling and responsiveness to pathogens (Li et al. 2004, Seo et al. 2008). HEL is reported to be up-regulated by insect infestation but not by mechanical wounding (Reymond et al. 2000). The present study supports previous findings that the plant responses to UV-B partly overlap those of defense signaling induced by insects and pathogens (Kunz et al. 2008, Demukra et al. 2010). Furthermore, the microarray data revealed UV-B-mediated induction of homologs of *CYP71A12* and *CYP71B14*, potential orthologs of those genes involved in the biosynthesis of camalexin (3-thiazolyindole) in *A. thaliana*. Derived from tryptophan, *CYP71A13* and *CYP71A12* are capable of catalyzing indole-3-acetaldoxime which is further decarboxylated by *CYP71B15* to camalexin (Rauhut and Glawischnig 2009). It can be speculated that a phytoalexin analogous to camalexin which is present in broccoli such as brassinin (indolyl-3-methanamine; Pedras et al. 2007) will accumulate in broccoli sprouts after UV-B exposure, changing the plant tolerance against pathogens.

Because our studies revealed that UV-B elevates GS levels in sprouts and induced defense signaling, the influence of UV-B treatment on the plant resistance and response was tested. In agreement with UV-B-induced defenses, weight gain of the caterpillar *P. brassicae* was lower on broccoli sprouts pre-exposed to moderate ecologically relevant UV-B levels when compared with the non-treated control. Also the population growth of *M. persicae* was reduced on UV-B-treated sprouts. The induced defense response to environmental stress such as UV-B has been repeatedly associated with increased accumulation of secondary metabolites, cell wall-reinforcing enzymes and defensive proteins with toxic, anti-digestive and anti-nutritive activity, which reduce the palatability of plants to the subsequent attackers (Kessler and Baldwin 2002, Caldwell et al. 2007).

Low to moderate ambient UV-B doses induced accumulation of mainly aliphatic methylsulfinylalkyl GS and 4-methoxy-indol-3-ylmethyl GS in the sprout part of broccoli, which was distinct from the GS response elicited by the insects. After 3 d of exposure to *M. persicae*, the GS profile in the broccoli sprouts did not change, but feeding by the

caterpillar *P. brassicae* and the beetle *P. cochleariae* elicited a plant response and levels of methoxylated indolyl GS increased. In *A. thaliana* and broccoli sprouts, 1-methoxy-indol-3-ylmethyl GS in particular has been shown to be responsive to jasmonate application (Mewis et al. 2005, Pérez-Balibrea et al. 2011) and increased about 10-fold after feeding by *P. cochleariae* in the present study. In a recent study, salicylic acid application to broccoli sprouts resulted in an increase of short-chain methylsulfinylalkyl GS, whereas levels of indolyl GS were unchanged (Pérez-Balibrea et al. 2011). Interestingly in this study, chitosan, a natural polypeptide and deacetylated derivative of chitin, increased levels of 4-methylsulfinylbutyl GS and 4-methoxy-indol-3-ylmethyl GS in sprouts, which is similar to the UV-B-mediated induction of GS in our study. *Pieris brassicae* and *P. cochleariae* elicited, like UV-B and chitosan, 4-methoxy-indol-3-ylmethyl GS in broccoli sprouts, but pre-conditioning of sprouts to moderate UV-B levels suppressed the induction of 1-methoxy-indol-3-ylmethyl GS mediated by the chewing insects. Although the plant GS response to insects in UV-B-pre-exposed sprouts was diminished, the increased levels of 4-methoxy-indol-3-ylmethyl GS and aliphatic GS in UV-B-pre-treated sprouts could contribute to the reduced performance of *P. brassicae* and *M. persicae*. A negative relationship between population growth and increasing aliphatic GS contents was determined for the generalist aphid *M. persicae* feeding on *A. thaliana* (Mewis et al. 2005). Increased concentrations of 4-methoxy-indol-3-ylmethyl GS in *A. thaliana* affected *M. persicae* growth (Kim and Janders 2007) as well as plant resistance against pathogens (Clay et al. 2009). Although *P. brassicae* is oligophagous and feeds on brassicaceous plants, Rohr and co-workers (2006) found a weak negative correlation between caterpillar weight gain and higher methylsulfinylalkyl GS content of *A. thaliana* ecotypes. However, the UV-B-mediated induction of other secondary metabolites with anti-feedant activity such as flavonoids (Simmonds et al. 2003) could have influenced the insects negatively.

Low levels of UV-B have been shown to increase defense metabolite levels and lead to the elevated expression of genes associated with the salicylate and jasmonic acid signaling pathways in broccoli sprouts. The data suggest that these photomorphogenetic UV-B levels have a signaling role for UV-B-biotic interactions. Therefore, application of low and moderate ambient UV-B at an early growth stage may also provoke plant immunity against attack by insects and pathogens. Based on the induction of pathogenesis-related proteins, studies are currently in progress to identify broccoli phytoalexins and confirm the effects of supplemental low UV-B on broccoli sprout resistance against pathogens. Beside UV-B effects on the plant tolerance to enemies, the elicitation of flavonoids and aliphatic GS in sprouts has potentially important benefits for the human consumer, due to their anti-inflammatory and anti-carcinogenic properties (Jeffery and Araya 2009).

## Materials and Methods

### Experimental set-up for different UV-B dose applications to broccoli sprouts (the aerial part of seedlings): first experiment

Seeds of broccoli, *B. oleracea* var. *italica* (Chrestensen, Erfurt Germany, 0.5 g per replication, produces about 150 sprouts), were sown on water-soaked fleece (synthetic fabric made from polyethylene, 5 cm × 5 cm) in aluminum trays filled with perlite and water as displayed in Fig. 4. Seeds were atomized daily until germination by using a water sprayer. Water was given as needed for optimal sprout growth. Trays were kept in a greenhouse (no UV-B transmission) at about 24°C during the day and 20°C during the night at a relative humidity of about 75%. Supplemental photosynthetically active irradiation (PAR) of 150  $\mu\text{mol m}^{-2} \text{s}^{-1}$  was provided to give a 16 h photoperiod.

UV-B treatment was carried out in special UV-B chambers with 12-day-old sprouts. Chambers were equipped with a single UV-B lamp (Philips 20 W ultraviolet B TL20W/12RS, Philips) and two daylight bulbs (Osram L18W/954 Lumilux de luxe Daylight, Osram). Broccoli sprouts were placed 40 cm below the irradiation source, with 0.042  $\text{Wh m}^{-2}$  UV-B and about 50  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PAR. The UV-B irradiation was determined prior to the experiment with a UV-B sensor (type DK-UVB 1.3-051, deka Sensor+Technologie GmbH) measuring in the spectral range from 265 to 315 nm. Different exposure times and intervals were used to investigate the elicitor effects of UV-B on GS accumulation. Treatments were: (1 a) two exposure episodes for 60 min; (1 b) a single exposure for 120 min; (2 a) two exposure episodes for 180 min; and (2 b) a single exposure for 360 min. The resulting UV-B doses per day used for sprout treatment were 0  $\text{kJ m}^{-2} \text{d}^{-1}$  in controls, 2 × 0.15  $\text{kJ m}^{-2} \text{d}^{-1}$  in treatment 1 a; 1 × 0.3  $\text{kJ m}^{-2} \text{d}^{-1}$  in treatment 1 b; 2 × 0.45  $\text{kJ m}^{-2} \text{d}^{-1}$  in treatment 2 a; and 1 × 0.9  $\text{kJ m}^{-2} \text{d}^{-1}$  in treatment 2 b. A 24 h interval separated treatments involving two episodes of UV-B exposure. In single treatments, sprouts were treated only once on the second application day. After treatments, the sprouts were returned to the greenhouse. Sprouts were harvested 2 and 24 h after the last treatment by cutting at the root base (Fig. 4). Plant material was flash-frozen in liquid nitrogen and stored at −40°C until freeze drying of samples and GS analysis. The experiment was conducted with about 150 sprouts per replication and six biological replications per treatment.

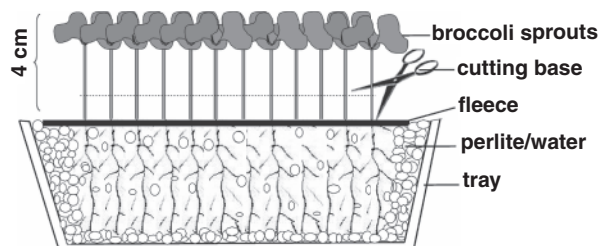


Fig. 4 Schema for broccoli sprout cultivation and harvest.

### Experimental set-up for single UV-B treatment of broccoli sprouts and chemical/molecular analysis: second experiment

For broccoli sprout cultivation, 1 g of seed (which produces about 300 sprouts) per replication was sown on water-soaked fleeces (5 cm × 10 cm) on perlite in water as described previously. Trays with sprouts were kept in a climate chamber at 21 ± 1°C, a relative humidity of 70 ± 5%, and with 200  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PAR for 12 h  $\text{d}^{-1}$ .

The UV-B treatment of 12-day-old sprouts was done in the same chambers as described in the experimental set-up for applications of different UV-B doses. Sprouts were exposed to UV-B irradiation for 240 min (0.6  $\text{kJ m}^{-2} \text{d}^{-1}$ ) and returned to the climate chamber until harvest 24 h later. Sprouts used as controls were exposed to 50  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PAR in similar chambers but without UV-B treatment. Sprouts were harvested by cutting at the base and were flash-frozen in liquid nitrogen. Samples for chemical analysis were stored at −60°C until freeze drying, and samples used for RNA extraction were kept at −80°C. The experiment was done in three replications per treatment with about 300 sprouts each. Additionally, sprout samples were used to determine the water content of UV-B-treated and control sprouts. For this purpose, 0.5 mg of sprouts were weighed in aluminum cups in three replications and dried for 24 h at 105°C. The dry weight of sprouts was determined and the water content calculated.

### Experimental set-up for long-term exposure of broccoli sprouts to UV-B and design of bioassays with insects: third experiment

Seeds of broccoli (0.5 g, which produces about 150 plants) were sown on fleece (5 cm × 5 cm) placed on top of water-soaked stonewool cubes (10 cm × 10 cm; Grodan). Water was applied daily to sprouts by using a dispenser. After sprout emergence, water was provided as needed for optimal sprout growth. Cubes with sprouts were kept on floats in a greenhouse under long-day conditions (about 16 h) at about 28°C during the day and 22°C during the night at 78% relative humidity.

UV-B treatment was performed on 14-day-old sprouts in a chamber that was larger than, but of similar design to, that used in the first experiments and it was equipped with one UV-B source (Philips UV-B L40 W/12RS) and two daylight bulbs (Osram Cool White L36 W/840). On five consecutive days, sprouts were exposed for 5 h  $\text{d}^{-1}$  to UV-B at a distance of 40 cm from the irradiation source (0.056  $\text{W m}^{-2}$  UV-B and 60  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PAR) for a total UV-B dose of 1  $\text{kJ m}^{-2} \text{d}^{-1}$  (ambient UV-B level). Non-UV-B-treated sprouts served as controls. An aliquot of sprouts was harvested into liquid nitrogen after the daily UV-B application on days 1, 3 and 5 for GS analysis. Chemical analysis of UV-B-treated and non-treated sprouts (the aerial part of seedlings) was done in six replications (about 150 sprouts per replication) per sampling day. These 18-day-old UV-B-treated and non-treated sprouts were used for the subsequent 3 d bioassays with insects.

The feeding bioassays with insects were done in cages of transparent Mylar cylinders (5 cm diameter, 9 cm high) with a top of fine mesh gauze (mesh wide < 0.1 mm) with the lower cage edge in stonewool. These cages contained the sprouts and insects, but maintained air exchange and allowed the insects to choose their feeding sites. The first bioassay was conducted with the aphid *M. persicae*. Five apterous aphids (adults and fourth nymph stage) were transferred to sprouts of the two treatments, and the population size increase was determined after 3 d. The second bioassay was conducted with the Lepidoptera *P. brassicae*. After determining the initial caterpillar weight, a single *P. brassicae* larva (third instar) was transferred to each replication of non-treated and UV-B-treated sprouts, and the final weight was determined after 3 d. In the third bioassay with *P. cochleariae*, five beetles were released to each replication of sprout treatments and were allowed to feed for 3 d. All assays were done in 12 replications. Sprouts with insects and insect-free control plants were caged and kept in the greenhouse. At the time of harvest, insects were removed and plants were cut directly above the root and immediately flash-frozen in liquid nitrogen. All material was stored at  $-80^{\circ}\text{C}$ . Plants for GS analysis were harvested in pairs (six replicate pairs).

### Insect rearing

Eggs of *P. brassicae* L. (Lepidoptera: Pieridae) were obtained originally from Insect Service GmbH and reared with the other insects in an insect acclimated room ( $24 \pm 2^{\circ}\text{C}$ , 14 h photoperiod) at the Leibniz-Institute of Vegetable and Ornamental Crops Großbeeren/Erfurt e.V. Mated butterfly females, fed with a 10% honey solution, laid eggs on *B. oleracea* var. *acephala* (kohlrabi) plants. After 2 weeks the first instars hatched and were reared on kohlrabi in insect cages until they reached the second instar. Then larvae were transferred to *B. oleracea* convar. *capitata* var. *sabauda* (savoy cabbage) leaves until pupation. Larvae of the third instar, previously fed with savoy cabbage, were used for the bioassays.

The initial population of *P. cochleariae* (F.) (Coleoptera: Chrysomilidae) was a gift from Bayer Crop Science. *Brassica rapa* var. *chinensis* (pak-choi) plants were provided as food for adult beetles in insect cages. For egg laying, a stem of savoy cabbage was given to the beetles. After the beetles laid eggs on savoy cabbage, leaves with eggs were transferred to a new cage for larval hatching. Larvae were fed savoy cabbage leaves, replaced on alternate days until larval pupation. Pupae were transferred to another cage for beetle hatching. Beetles, which were fed with pak-choi, were used for the bioassays with sprouts.

*Myzus persicae* (Sulzer) were collected from crucifer plants in the greenhouse of Katz Biotech AG and reared on pak-choi plants in an insect cage with about a 14 h photoperiod. New plants were added roughly every 2 weeks and old plants were removed after the aphids had settled on the new plants.

### Glucosinolate analysis

Lyophilized broccoli sprout material (20 mg) was extracted with 750  $\mu\text{l}$  of 70% methanol at  $80^{\circ}\text{C}$  in a heat block for 5 min and

centrifuged for 5 min at  $4,000 \times g$ . The pellet was re-extracted at  $80^{\circ}\text{C}$  twice with 500  $\mu\text{l}$  each, and the supernatants were combined. To quantify the GS content, 60  $\mu\text{l}$  of 1 mM *p*-hydroxybenzyl GS (isolated from *Sinapis alba* seeds) was added initially to the first methanol extract as internal standard. To convert this to desulfo GS, extracts were loaded on mini columns filled with 500  $\mu\text{l}$  of DEAE Sephadex A-25 (Sephadex A-25 was rehydrated in 2 M acetic acid). Columns were pre-treated with  $2 \times 1$  ml of 6 M imidazole-formate solution in 30% formic acid and washed twice with  $2 \times 1$  ml of ultrapure water before loading the GS extracts. The columns were then washed with  $2 \times 1$  ml of 0.02 M sodium acetate buffer (pH 4.0). A 75  $\mu\text{l}$  aliquot of aryl sulfatase solution (Sigma-Aldrich; H-1 from *Helix pomatia*, prepared according to Graser et al. 2001) was added to each column. Desulfation of GS was done overnight at room temperature. Desulfo GS samples were eluted with 1 ml of ultrapure water. A volume of 40  $\mu\text{l}$  of desulfo GS extracts was injected in a Dionex P680A HPLC-DAD system equipped with a narrow bore column (Acclaim™ 120, 250 mm  $\times$  2.1 mm, 5  $\mu\text{m}$ , RP18, Dionex). A 43 min gradient program was set for separation of GS with the eluents: (A) ultrapure water and (B) 40% acetonitrile (HPLC grade). The gradient was as follows: 0.5% B for 1 min, from 0.5% to 20% B for 7 min, 20% B for 2 min, from 20% to 50% B for 9 min, 50% B for 3 min, from 50% to 99% B for 6 min, a 5 min hold at 99% B, from 99% to 0.5% B for 3 min, and a 7 min final hold at 0.5% B. GS were monitored at 229 nm. Only the amounts of the nine major GS were calculated from HPLC peak areas related to those of the internal standard *p*-hydroxybenzyl GS by using response factors (RFs) computed to allyl GS [RFs: 3-methylsulfinylpropyl GS = 1.2; (R)-2-hydroxy-3-butenyl GS = 1; 4-methylsulfinylbutyl GS = 0.9; 5-methylsulfinylpentyl GS = 0.9; 4-methylthiobutyl GS = 0.9; 4-hydroxy-indol-3-ylmethyl GS = 0.2; indol-3-ylmethyl GS = 0.33; 4-methoxy-indol-3-ylmethyl GS = 0.35; 1-methoxy-indol-3-ylmethyl GS = 0.25; and *p*-hydroxybenzyl GS = 0.61]. 3-Butenyl GS and 2-phenylethyl GS were identified as minor GS.

Peak identity of the 11 detected GS in extracts was confirmed with liquid chromatography–mass spectrometry (LC-MS) analysis on an HPLC-1100 series chromatograph (Agilent Technologies) coupled to an Esquire 6000 electron spray ionization (ESI)-ion trap mass spectrometer (Bruker Daltonics) operated in negative ion mode in the range *m/z* 50–700. MS conditions were: a capillary voltage of  $-4,000$  V, a nebulizer pressure of 35 p.s.i., a drying gas flow of  $10\text{ l min}^{-1}$ , and a drying gas temperature of  $330^{\circ}\text{C}$ . Before reaching the ESI, the flow coming from the column was diverted in a ratio of 4:1. The mass spectrometer detected in full scan mode as well as AutoMS mode in order to produce  $\text{MS}^2$  and  $\text{MS}^3$  spectra. The following mass spectra of protonated molecular ions  $[\text{M}+\text{H}]^+$  were obtained at the indicated retention times: (i) 3-methylsulfinylpropyl GS, *m/z* 344 and 183, 7 min; (ii) (R)-2-hydroxy-3-butenyl GS, *m/z* 310 and 149, 8 min; (iii) 4-methylsulfinylbutyl GS, *m/z* 358 and 197, 10 min; (iv) 5-methylsulfinylpentyl GS, *m/z* 372 and 211, 11 min; (v) 3-butenyl GS, *m/z* 294 and 133, 13 min; (vi) 4-hydroxy-indol-



3-ylmethyl GS,  $m/z$  385 and 224, 14 min; (vii) 4-methylthiobutyl GS,  $m/z$  342 and 181; (viii) indol-3-ylmethyl GS,  $m/z$  369 and 207, 21 min; (ix) 2-phenylethyl GS,  $m/z$  344 and 183, 22 min; (x) 4-methoxy-indol-3-ylmethyl GS,  $m/z$  399 and 237, 23 min; and (xii) 1-methoxy-indol-3-ylmethyl GS,  $m/z$  399 and 237, 27 min.

### Flavonoid analysis (second experiment)

Flavonoids were determined as aglycones after acidic hydrolysis, using HPLC as described by Krumbein et al. (2007). For analysis, 0.5 g of the lyophilized sample of broccoli sprouts was extracted and hydrolyzed with 50% methanol (aqueous) containing 1.6 M HCl. After refluxing at 90°C for 2 h, the extract was cooled to room temperature, adjusted to 100 ml with 50% methanol and sonicated for 5 min. The extract was then filtered through a 0.45  $\mu\text{m}$  PTFE filter for HPLC analysis. The flavonoid composition and concentration were determined using a series 1100 HPLC (Agilent) equipped with a diode array detection system. A Prodigy column ODS(3) (250 mm  $\times$  4.6 mm, 5  $\mu\text{m}$ , Phenomenex) was used to separate flavonoid aglycones at a 1 ml  $\text{min}^{-1}$  flow and a temperature of 25°C. Eluent A consisted of water with 0.1% trifluoroacetic acid and 2% tetrahydrofuran, whereas eluent B was 100% acetonitrile. The following gradient was used: from 30% to 35% B in 5 min, from 35% to 39% B in 12 min, from 39% to 90% B in 5 min, 2 min at 90% B, from 90% to 30% B in 5 min, and 30% B for 5 min. The eluent was monitored at 270 nm. Contents were quantitated by calibration curves of the related pure standards (dihydroquercetin and kaempferol; Carl Roth GmbH). The identity of flavonoid aglycones was verified by HPLC–ESI–MS<sup>2</sup> by using an Agilent 1100 series (ion trap) in the negative ionization mode. Quercetin and kaempferol were identified from the deprotonated molecular ions  $[M-H]^-$  with  $m/z$  301 and 285, respectively.

### Carotenoid and Chl analysis (second experiment)

Carotenoid and Chl pigment contents of broccoli sprouts were determined photometrically. Freeze-dried plant material (0.5 g) was homogenized with 15 ml of acetone:hexane (4:5 v/v) using an Ultra-Turrax (T25, IKA-Technik Staufen). The extract was centrifuged at 4,000  $\times g$  for 10 min. The supernatant was applied to a folded filter and the flow-through was transferred to a volumetric flask and adjusted to 25 ml with the acetone:hexane solvent. An aliquot of each extract was transferred to quartz glass cuvettes and absorbances were measured at different wavelengths: 450 nm for total carotenoids, 453 nm for  $\beta$ -carotene, 505 nm for lycopene, 663 nm for Chl *a*, and 645 nm for Chl *b*. The quantitative determination of Chls and carotenoids in whole-pigment extract is complicated because of overlapping spectral regions (for more details, see Lichtenthaler and Buschmann 2001). When Chl *a* and *b* were in a pigment extract, the specific contributions of Chl *b* to the Chl *a* maximum and of Chl *a* absorbance to the Chl *b* absorbance maximum were subtracted (Chl *a*  $\mu\text{g ml}^{-1} = 10.1 \times E_{663} - 1.01 \times E_{645}$ ; Chl *b*  $\mu\text{g ml}^{-1} = 16.1 \times E_{645} - 2.57 \times E_{663}$ ). Total

carotenoid concentrations were determined at 450 nm and  $\beta$ -carotene at 453 nm by subtracting the Chl contributions ( $\beta$ -carotene  $\mu\text{g ml}^{-1} = 0.216 \times A_{663} \times - 1.22 \times A_{645} - 0.304 \times A_{505} + 0.452 \times A_{453}$ ).

### Microarray analysis (second experiment)

Frozen broccoli sprout material was ground in centrifuge tubes in liquid nitrogen in an orbital ball mill for 2 min at a frequency of 30 Hz (MM400 Retsch GmbH). Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen GmbH) according to the manufacturer's protocol, including the on-column DNase digestion step with the RNase-free DNase Set (Qiagen). RNA quality and quantity were determined by using a NanoDrop 1000 spectrometer (NanoDrop Biotechnology GmbH) with the software version ND-1000 3.2.1. Only RNA samples with sufficient yield  $>50 \text{ ng } \mu\text{l}^{-1}$  and a 260 nm/280 nm absorbance ratio  $>1.6$  were used for microarray analysis. The microarray analysis was done in three biological replications with RNA isolated from UV-B-treated and non-treated sprouts. At least 1 mg of total RNA from each replicate was sent on dry ice to Beckman Coulter Genomics (Morrisville, NC, USA, [http://www.beckmangenomics.com/genomic\\_services/gene\\_expression.html](http://www.beckmangenomics.com/genomic_services/gene_expression.html)) who performed the microarray analysis using the  $2 \times 104 \text{ k}$  format *Brassica* Array (Trick et al. 2009). This newly developed community microarray resource for transcriptome profiling in *Brassica* species contains 60-mer oligonucleotides specific for the 94,558 sequences defined as the *Brassica* unigene set (<http://brassica.bbsrc.ac.uk/>). Agilent One-Color Gene Expression Microarray analysis was performed by Beckman Coulter Genomics using quality control of purified RNA and target labeling reaction products, followed by multiple quality control steps during array hybridization, scanning and image analysis. The Open Source Microarray Processing Software Robin (<http://mapman.gabipd.org/web/guest/home>) was used to normalize generic single channel array data from text formatted raw data files (Lohse et al. 2010). Background correction, normalization of all arrays together and gene expression analysis giving results of the log fold change of expression in UV-treated sprouts in relation to the control were performed using the default settings of Robin with robust multiarray averaging (RMA). Assignment of the different genes represented by identifiers to respective bins and visualization of data sets was realized using MapMan 3.5.1 (<http://mapman.gabipd.org/web/guest/mapman-download>) with the automatically generated *Brassica* 95 k mapping. The *Brassica* mapping was done using the 95 k *Brassica* unigene set, and its translated protein sequences were automatically compared with the Arabidopsis TAIR9 database using the Mercator pipeline for automated sequence annotation (<http://mapman.gabipd.org/web/guest/app/mercator>). For each identifier, the gene with the highest homology was provided with an identifier and description. The respective bitscores were classified as follows: very weakly



similar (bitscore <100); weakly similar (bitscore 101–200); moderately similar (bitscore 201–500); highly similar (bitscore >500). Microarray analysis followed the recommendation of MIAME (<http://www.mged.org>), and raw data files will be made available in a public archive (e.g. ArrayExpress Archive; <http://www.ebi.ac.uk/arrayexpress/>).

### Statistical analysis

For the respective experiments, statistical differences within secondary metabolite contents among treatments were detected by ANOVA (when indicated with UV-B as covariate) and Tukey's HSD test in SYSTAT 11.0. A two-way ANOVA was used to determine significant effects of the factors time and UV-B treatment on secondary metabolite accumulation. The mean insect performance data were compared with one-way ANOVA.

### Supplementary data

Supplementary data are available at PCP online.

### Funding

This work was funded by the Bundesministerium für Bildung und Forschung [BMBF; AZ 31P5627].

### Acknowledgments

We thank Dr. Peter Meisner and Gerd Trautmann (Bayer Crop Science, Mohnheim, Germany) for providing the starting population of *P. cochleariae*, and Dr. James Burleigh for editing the manuscript. The authors also thank Dr. Michael Reichelt and Dr. Jonathan Gershenzon (Max Planck Institute for Chemical Ecology Jena, Germany) for their collaborative help.

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