Stress-Induced Biosynthesis of Dicaffeoylquinic Acids in Globe Artichoke

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Leaf extracts from globe artichoke (*Cynara cardunculus* L. var. *scolymus*) have been widely used in medicine as hepatoprotectant and choleretic agents. Globe artichoke leaves represent a natural source of phenolic acids with dicaffeoylquinic acids, such as cynarin (1,3-dicaffeoylquinic acid), along with its biosynthetic precursor chlorogenic acid (5-caffeoylquinic acid) as the most abundant molecules. This paper reports the development of an experimental system to induce caffeoylquinic acids. This system may serve to study the regulation of the biosynthesis of (poly)phenolic compounds in globe artichoke and the genetic basis of this metabolic regulation. By means of HPLC-PDA and accurate mass LC-QTOF MS and MS/MS analyses, the major phenolic compounds in globe artichoke leaves were identified: four isomers of dicaffeoylquinic acid, three isomers of caffeoylquinic acid, and the flavone luteolin 7-glucoside. Next, plant material was identified in which the concentration of phenolic compounds was comparable in the absence of particular treatments, with the aim to use this material to test the effect of stress application on the regulation of biosynthesis of caffeoylquinic acids. Using this material, the effect of UV-C, methyl jasmonate, and salicylic acid treatments on (poly)phenolic compounds was tested in different globe artichoke genotypes. UV-C exposure consistently increased the levels of dicaffeoylquinic acids in all genotypes, whereas the effect on compounds from the same biosynthetic pathway, for example, chlorogenic acid and luteolin-7-glucoside, was much less pronounced and was not statistically significant. No effect of methyl jasmonate or salicylic acid was found. Time-response experiments indicated that the level of dicaffeoylquinic acids reached a maximum at 24 h after UV radiation. On the basis of these results a role of dicaffeoylquinic acids in UV protection in globe artichoke is hypothesized.

KEYWORDS: Globe artichoke; UV-C radiation; abiotic stresses; phenolic compounds; dicaffeoylquinic acids

INTRODUCTION

In southern Europe, production of globe artichoke (*Cynara cardunculus* L. var. *scolymus*) is an important component of the regional economy, and Italy is the leading world producer (about 470 Mt per year). Globe artichoke ($2n = 2x = 34$) is cross-pollinated and highly heterozygous. Commercial production (at least in Europe) is mainly based on the cultivation of vegetatively propagated clones, which guarantee high yields of marketable product. The clonally propagated varieties are highly heterogeneous and, as evidenced by DNA marker analysis, show a significant element of within-cultivar variation (*1, 2*). In recent years a considerable number of new seed-propagated cultivars have been developed and commercialized as F1 hybrids, although they lack uniformity due to the considerable inbreeding depression globe artichoke suffers following selfing, which hampers the possibility to obtain true inbred lines.

The major use of globe artichoke and cardoon is for human food. However, various studies have demonstrated health-promoting properties of their extracts. Leaf extracts are reported to decrease blood cholesterol levels by reducing its synthesis in the liver and to reduce fat accumulation in other tissues (*3−5*). Clinical studies have indicated that globe artichoke can act as a hepatoprotective and choleretic agent, for the treatment of hepatobiliary dysfunction and digestion complaints (*6*). In vitro studies have shown globe artichoke leaf extracts to be active in protection of proteins, lipids, and DNA from free radical-mediated oxidative damage, and their antibacterial and antifungal activities have been well established (*7−14*).

Globe artichoke leaves contain a very high content of phenolic compounds, such as caffeoylquinic acids and luteolin glucosides (*8*). Caffeoylquinic acids are a group of esters of quinic acid and caffeic acid (*15, 16*) that are present in many plant species. Globe artichoke is very rich in this type of compound and

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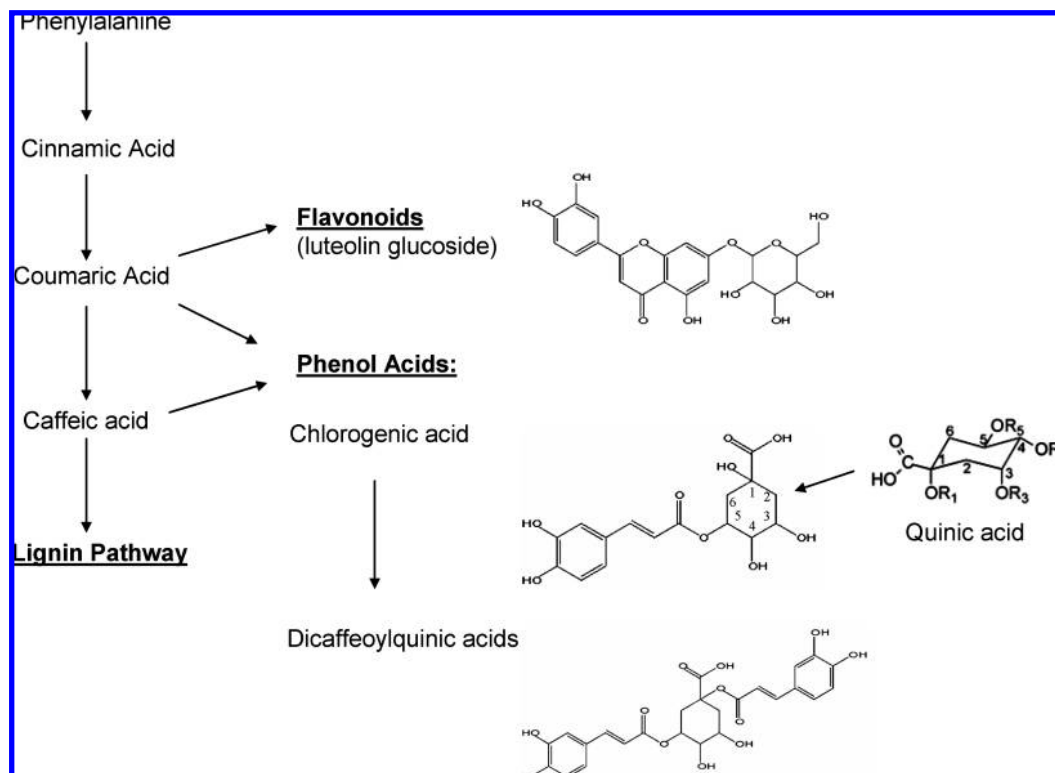§ Plant Research International.

Figure 1. Phenylpropanoid pathway in plants and structures of the major phenolic compounds in globe artichoke. Quinic acid is represented in chair form.

accumulates up to 50 g/kg of dry weight mainly as 5-caffeoylquinic acid (chlorogenic acid; **Figure 1**), whereas in mature flower heads its content is 1−2 g/kg (*17*). Other well-known sources of chlorogenic acids include coffee (60−100 g/kg of DW in green coffee beans), Solanaceous species such as tomato, potato (1 g/kg of DW) and eggplant, and Rosaceae fruits such as apple and pear (*18*). The 3,5, 4,5, and 3,4 isomers of dicaffeoylquinic acid are found in many plant species, such as coffee, tomato, and lettuce (*18, 19*). In globe artichoke extracts 1,3-dicaffeoylquinic acid (cynarin) is the main isomer (*20*) (**Figure 1**). Dicaffeoylquinic acids are of particular pharmaceutical interest, because they may function as an inhibitor of the activity of HIV integrase, which catalyzes the insertion of viral DNA into the genome of host cells (*21−23*).

Chlorogenic acid and dicaffeoylquinic acids derive from the phenylpropanoid pathway. This pathway starts from phenylalanine and leads to secondary metabolites such as flavonoids (e.g., luteolin) and phenolic acids (such as chlorogenic acid), but also to lignin (**Figure 1**). Generally, this pathway is induced upon biotic and abiotic stress such as wounding, UV irradiation, or pathogen attack (*24, 25*). Several authors have reported the specific involvement of chlorogenic acid in stress responses, as increases in its concentration were reported in lettuce upon wounding (*26*) and in tobacco upon UV-B irradiation and after insect attacks (*27*). Tomato plants with elevated levels of chlorogenic acid showed reduced levels of infection of *Pseudomonas syringae* and improved antioxidant activity (*28*). In contrast, much less is known about the accumulation of dicaffeoylquinic acids in response to these stress conditions.

The aim of this work was to develop a suitable system to test the effect of environmental factors on the biosynthesis of phenolic compounds in globe artichoke. The system is designed to deal with the highly diverse genetic background and outbreeding properties of the species. In later stages, the system will be applied to isolate genes involved in the biosynthesis of dicaffeoylquinic acids.

MATERIALS AND METHODS

Plant Material. Seeds of two varieties of globe artichoke, F1 hybrid 'Orlando' (Numhems) and multiclonal varietal type 'Violetto di Sicilia', were grown in an environmentally controlled climatic chamber located in Wageningen (The Netherlands) under a 16 h light/8 h dark regimen (light intensity = 120 μmol m^{-2} s^{-1} photons) at 20 °C in soil.

Experiments were performed on plants with five fully expanded leaves. Young leaves were collected from the plants at different ages (8, 10, 12, and 14 weeks). Foliar disks (10−12 weeks) were obtained from leaf cuttings using a cork borer with a diameter of 2 cm and immediately used for experiments. After treatment with different abiotic stresses, all plant material was immediately frozen in liquid nitrogen and stored at −80 °C until further analysis.

Abiotic Stress Application. *UV-C Treatment.* Detached globe artichoke foliar disks were exposed to UV-C (254 nm) light for 20 min, through a 16 W germicidal lamp at a distance of 20 cm. Control treatments were performed in another compartment with the same experimental approach but with the lamp off. Irradiated disks were placed at 21 °C in a Petri dish containing distilled water and maintained under a 16 h light/8 h dark regimen before harvesting. The harvesting was performed after 24 h in most experiments or after 0, 12, 24, and 36 h in the time exposure experiment.

Methyl Jasmonate and Salicylic Acid Application. Globe artichoke foliar disks were sprayed with solutions of 100 μM methyl jasmonate dissolved in ethanol or 50 μM salicylic acid dissolved in water. Treated disks were incubated for 20 min and placed at 21 °C in a Petri dish containing distilled water and maintained for 24 h (16 h light/8 h dark) before harvesting.

Extraction. Globe artichoke materials (foliar disks and leaves) were ground to a fine powder in liquid nitrogen. Then 100 mg of material was weighed and extracted with 3 volumes of pure methanol (0.1% formic acid). Compounds were extracted by sonicating the mixture in a water bath for 20 min, and after that, the samples were centrifuged for 10 min at 16000g. The supernatant was filtered through a 0.45 μm Anotop 10 filter (Whatman) before injection into HPLC.

HPLC-PDA Analysis. The HPLC system comprised a Water 600 controller, a Water 996 photodiode array detector (PDA), and a column oven at 40 °C. For the chromatographic separation a Luna C18(2) precolumn (2 × 4 mm) and analytical column (2 × 150 mm, 100 Å,

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Table 1. Properties of Phenolic Compounds in Globe Artichoke Leaf Extract after HPLC, Detection by Absorbance, and MS/MS[a]

peak	RT	$(M - H)^-$ (*m/z*)	formula	calcd mass	Δppm	postulated structure	MS/MS fragm	absorb. max
1	14.5	353.0891	$C_{16}H_{18}O_9$	353.0873	5.2	chlorogenic acid (5-caffeoylquinic acid)	191	324
2	15.0	353.0979	$C_{16}H_{18}O_9$	353.0873	4.0	caffeoylquinic acid II	191	324
3	17.3	353.0879	$C_{16}H_{18}O_9$	353.0873	1.8	caffeoylquinic acid III	191	324
4	25.8	447.0942	$C_{25}H_{24}O_{12}$	447.0927	3.3	luteolin 7-glucoside	285	343
5	27.9	515.1194	$C_{25}H_{24}O_{12}$	515.1190	0.4	dicaffeoylquinic acid I	179, 191, 353	326
6	28.2	515.1190	$C_{25}H_{24}O_{12}$	515.1190	0.1	dicaffeoylquinic acid II	191, 353	326
7	29	515.1193	$C_{25}H_{24}O_{12}$	515.1190	0.7	dicaffeoylquinic acid III	191, 353	326
8	30	515.1207	$C_{25}H_{24}O_{12}$	515.1190	3.4	dicaffeoylquinic acid IV	179, 191, 353	326
standard	19.2	515.1100	$C_{25}H_{24}O_1$	515.1190	−0.3	cynarin (1,3-dicaffeoylquinic acid)	191, 353	326

[a] Peak numbers refer to **Figure 1**. All metabolites are presented according to the recommended IUPAC numbering system (*31*).

particle size = 3 μm) were used. The mobile phases include degassed solutions of trifluoroacetic acid/ultrapure water (1:1000, v/v, eluent A) and trifluoroacetic acid/acetonitrile (1:1000, v/v, eluent B) in the following gradient system: initial, 5% B; linear gradient to 35% B in 45 min. Then the column was washed with 75% B for 10 min and equilibrated in 0% B before the next injection. The column temperature was kept at 40 °C, and the samples were kept at 20 °C. The flow rate was 1 mL/min, the injection volume was 10 μL, and the detection wavelength was recorded at 240−600 nm.

HPLC QTOF-MS. To identify the polyphenolic metabolites in the extracts, we used accurate mass LC-MS and MS/MS on a high-resolution time-of-flight (TOF) mass spectrometer with lockmass correction, in combination with spectral analysis using a PDA detector. HPLC was performed using a Waters Alliance 2795 HT HPLC system providing a linear gradient from 5 to 35% acetonitrile (acidified with 0.5% FA) at a flow rate of 1 mL/min. For the chromatographic separation a Luna C18(2) precolumn (2 × 4 mm) and analytical column (2 × 150 mm, 100 Å, particle size = 3 μm) were used. Eluting compounds were first detected online at 240−600 nm using a Waters2996 PDA, before entering a QTOF Ultima API mass spectrometer equipped with an electrospray ionization (ESI) source and a separate LockSpray. The eluent flow was split after PDA detection to obtain a flow of 0.2 mL/min into the MS. Before each series of analyses the mass spectrometer was calibrated using phosphoric acid/acetonitrile/water (1:1000:1000, v/v/v) solution. During sample analysis the capillary voltage was set at 2.75 kV and the cone at 35 V. Source and desolvation temperatures were set at 120 and 250 °C, respectively.

During mass spectrometry analysis, compounds may preferentially ionize in either positive or negative mode; polyphenolic acids ionize better in negative mode, and for this reason we chose that type of detection. In the negative ion mode the collision energy was 10 eV. Ions in the *m/z* range of 100−3000 were detected using a scan time of 0.9 s and an interscan delay of 0.1 s. Tandem MS was performed online using three different collision energies (5, 10, and 50 eV) on up to eight masses detected per survey scan. Leucine enkaphalin (Sigma), dissolved in 50% acetonitrile with 20 μM ammonium acetate (ESI⁻), was used as a lock mass and was measured every 10 s. Masslynx software version 4.0 was used for visualization and processing of data.

To identify the metabolites in globe artichoke leaf extracts, we combined HPLC-PDA and LC-MS data, using the same chromatographic conditions. The identification of compounds was based on the following parameters: (1) accurate mass, (2) retention time, (3) UV−vis spectral information, and (4) MS/MS fragmentation (*29*). Caffeoylquinic acids and luteolin 7-glucoside were identified according to UV−vis spectra, comparison with reference standard, accurate mass, and MS/MS fragmentation data. Quantification was performed by comparison to standard compounds chlorogenic acid and luteolin 7-glucoside from Apin (Abingdon) and cynarin (1,3-dicaffeoylquinic acid) from Carl Roth (Karlsruhe).

HPLC Antioxidant Detection System. The antioxidant compounds in the globe artichoke extract were determined by HPLC coupled to an online postcolumn antioxidant system, according to reference *30*. Compounds eluting from the analytical column passed first through a PDA detector (set at an absorbance range of 240−600 nm) and were then allowed to react for 30 s with a buffered solution of 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid, ABTS•⁺; Roche) cation radicals online, before passing through a second detector, which monitors

ABTS•⁺ radicals. The ABTS•⁺ radical solution was prepared by dissolving 55 g of ABTS in 50 mL of water, followed by the addition of potassium permanganate. After 16 h of incubation in the dark, the radical solution was diluted further in 3 volumes of 0.2 M sodium phosphate buffer, pH 8.0. The postcolumn reaction loop was a 3 m stainless steel tube (internal diameter = 0.508 mm) at 40 °C, and column eluate resided for exactly 30 s in this tube before detection. The decreased absorption of ABTS•⁺ by reaction with antioxidants was monitored as it passed through a dual-wavelength UV−vis detector (Waters) at 412 and 650 nm. The absorption at 412 nm is the maximum absorption of the ABTS•⁺ cation.

Statistical Analysis. Univariate analysis of variance (ANOVA) has been performed using SPSS version 15.0. Variance homogeneity has been evaluated with Levene's test, whereas multiple comparison has been realized following an LSD test.

RESULTS

Identification of Main Phenolic Compounds in Globe Artichoke Leaves. The qualitative and quantitative analysis of (poly)phenolic composition in globe artichoke leaf extracts was performed using reversed phase HPLC with photodiode array (PDA) and a high-resolution mass spectrometer (QTOF MS) operating in negative electrospray ionization mode. The compounds separated in the chromatographic system were identified on the basis of their absorption spectrum, the exact mass of the precursor ion, MS/MS fragments, and comparison to authentic standards (**Table 1**). The chromatographic profile of an aqueous−methanol extract from globe artichoke leaf, recorded at 312 nm, is illustrated in **Figure 2**. All data for metabolites presented in this paper are presented according to the recommended IUPAC numbering system for caffeoylquinic acids (*31*).

Identification of chromatographic peak 1 (at 14.5 min) was based on the comparison of its retention time (RT), absorbance spectrum, and MS/MS fragments with a chlorogenic acid (5-caffeoylquinic acid) standard. In the mass spectrometer, an *m/z* 353.0891 [M − H]⁻, corresponding to chlorogenic acid, and its fragment of *m/z* 191.05 (quinic acid) were observed. The λ_max of this compound was 324 nm.

Peaks 2 (at 15.0 min) and 3 (at 17.3 min) also represent caffeoylquinic acids, having similar λ_max (324 nm), precursor mass (*m/z* 353.0979 and 353.0879, respectively), and its fragment (*m/z* 191.05) as chlorogenic acid. Indeed, their precursor masses corresponded within 5 ppm to that of the chlorogenic acid standard ($C_{16}H_{18}O_9$). Thus, their different retention times indicate that these are isomers of chlorogenic acid. In further descriptions, we refer to these isomers as caffeoylquinic acids II and III, respectively.

Peak 4 (RT = 25.8) was identified as luteolin 7-glucoside, by comparison of retention time, λ_max (343 nm) and mass values to those of the authentic standard (−3.3 ppm). Mass spectrometry yielded a precursor ion of *m/z* 447.0942 and the luteolin fragment of *m/z* 285.
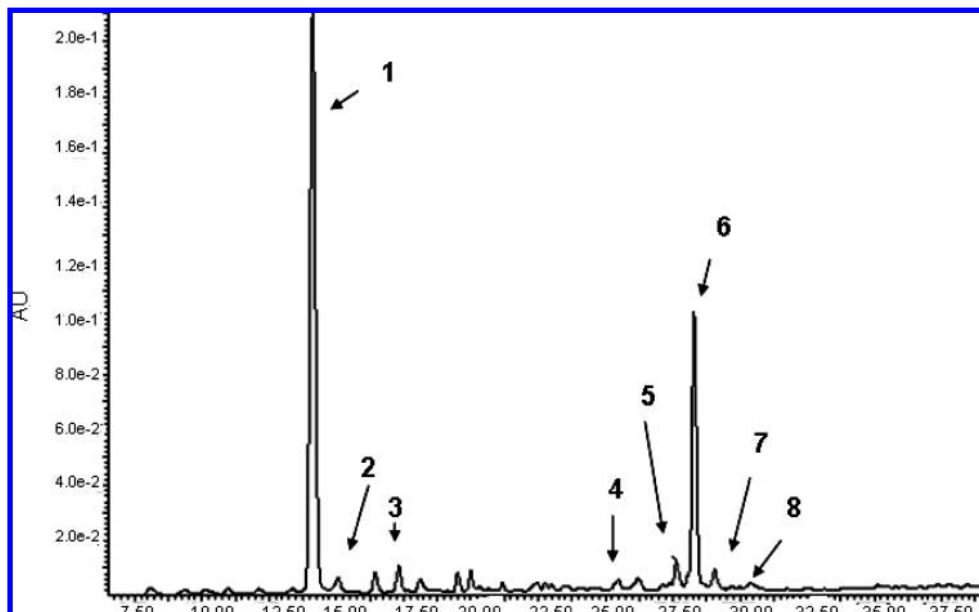
Figure 2. HPLC chromatogram at 312 nm of globe artichoke leaf extract. Identified compounds: (1) chlorogenic acid (5-caffeoylquinic acid); (2) caffeoylquinic acid isomer II; (3) caffeoylquinic acid isomer III; (4) luteolin 7-glucoside; (5) dicaffeoylquinic acid I; (6) dicaffeoylquinic acid II; (7) dicaffeoylquinic acid III; (8) dicaffeoylquinic acid IV.

Table 2. Comparison between the Total Contents of Chlorogenic Acid, Dicaffeoylquinic Acid Isomer II, and Luteolin 7-Glucoside in Different Types of Plant Material[a]

	chlorogenic acid (mg/g)	min/ max values	CV (%)	dicaffeoyl quinic acid isomer II (mg/g)	min/max values	CV (%)	luteolin 7-glucoside (mg/g)	min /max values	CV (%)
technical reproducibility[1]	0.043	0.042–0.044	2	0.10	0.095–0.103	5	NA[b]	NA	NA
variation between leaves[2c]	0.20	0.19–0.22	6	0.17	0.1–0.22	36	NA	NA	NA
variation within leaves (foliar disks)[3c]	0.15	0.14–0.19	22	0.20	0.16–0.28	32	NA	NA	NA
variation between plants of 8 weeks[4]	0.11	0–0.25	83	0.09	0.04–2.21	137	0.16	0–0.50	143
variation between plants of 12 weeks[4]	3.70	0.05–11.01	169	0.86	0.01–2.23	138	0.76	0–1.96	139
variation between plants of 14 weeks[4]	3.70	0.07–7.01	110	3.65	0.07–7.74	108	2.67	0–5.64	116

[a] The data are expressed in mg/g of fresh weight of material and are the mean of three determinations. The minimum and maximum values, as well as the coefficients of variation (CV; the standard deviation expressed as percentage of the mean value) are indicated. The data refer to (1) the same globe artichoke extract analyzed in triplicate, (2) three leaves from a single 'Orlando' plant, (3) three foliar disks from three leaves of the same 'Orlando' plant, (4) different leaves collected from different age plants (three genotypes per age, for a total of nine different genotypes of 'Orlando'). [b] Not analyzed. [c] Biological variation has been evaluated by analyzing 10-week-old plant.

Peaks 5, 6, 7, and 8 (RT = 27.9, 28.2, 29.0, and 30.5 min, respectively) were all identified as different dicaffeoylquinic acids. Their precursor masses (m/z 515.11 and 515.12, respectively) all corresponded within 5 ppm to that of the cynarin (1,3-dicaffeoylquinic acid) standard. In addition, their observed mass fragments of m/z 353.08 (caffeoylquinic acid), m/z 179.05 (caffeic acid) and m/z 191.05 (quinic acid) as well as their λ_{max} (326 nm) all corresponded well to that of cynarin. However, the retention time of cynarin (RT = 19.2) did not match with any of the dicaffeoylquinic acid isomers observed in the globe artichoke leaf extracts. It has been described that the main isomer found in globe artichoke corresponds to 1,5-dicaffeoylquinic acid, which can be isomerized to cynarin in warm aqueous media (*23*). We therefore assume the main dicaffeoylquinic acid peak in our chromatograms (peak 6; RT = 28.2) to be 1,5-dicaffeoylquinic acid. As our instrument did not enable further identifications using MS^n experiments, in further descriptions, we refer to these isomers as dicaffeoylquinic acid isomers I (RT = 27.9), II (RT = 28.2), III (RT = 29.0), and IV (RT = 30.5).

Variation in Phenolic Content between Plant Material. Globe artichoke is an outbreeding plant species with a high level of heterozygosity (*1*). For this reason it is important to define properly the starting material for comparison of the effects of

different stress treatments on its (poly)phenolic content and to develop an analytical method ensuring low technical and biological variation. Different sampling strategies were compared to reduce the variation between plant materials and to enable us to study changes, if any, in the levels of chlorogenic acid, dicaffeoylquinic acids, and luteolin 7-glucoside upon plant treatments.

First, the technical reproducibility of metabolite extraction and sample analyses was studied. Leaf samples from several plants of cv. 'Orlando' were frozen, pooled, and ground in liquid nitrogen, and the resulting frozen powder was split into three equal portions. Extraction and analysis of these identical samples were compared and yielded a coefficient of variation (CV, i.e., the standard deviation expressed as a percentage of the mean value) between extracts of no more than 5% for either of the phenolic compound classes (**Table 2**), indicating good technical reproducibility.

Second, the variation in phenolic content between individual leaves from the same plant was established by analyzing three complete young leaves from a single plant. Here, the CV between extracts was 6% for chlorogenic acid and 36% for dicaffeoylquinic acid isomer II (in the analysis only this isomer was considered, as it was by far the most abundant). A similar variation was observed when in the procedure foliar disks were

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used, instead of complete leaves (**Table 2**). We selected young plants (8–14 weeks old) because these are normally more responsive to stress treatment as they have suffered only a minimal amount of stress.

Third, variation between plants from a single cultivar ('Orlando') was established. This variation appeared to be much larger than the variation between leaves of a single plant. Nine different genotypes, divided in three groups of age (8, 12, and 14 weeks old), were analyzed (**Table 2**). Whereas the average content of dicaffeoylquinic acid isomer II, chlorogenic acid, and luteolin 7-glucoside increased strongly (20–30-fold) during the period from 8 to 14 weeks after germination, the CV between plants of the same age was very high. For example, in 14-week-old plants, the content of dicaffeoylquinic acid isomer II varied between 0.07 and 7.4 mg/g. The CV for the phenolic content of leaves from different plants of 8–14 weeks old was in all cases more than 100%.

We concluded from these data that the use of material from different plants was not suitable for our experiments, because the variation between plants would be too high to enable the detection of significant effects of our experimental treatments. In contrast to the high variation between plants, the CV within plants was relatively low (about 30%; **Table 2**). Therefore, leaf disks from single plants (i.e., the same genotype) were used in our further studies aimed to monitor the effect of stress-inducing treatments on the phenolic content of globe artichoke leaves.

Effect of Stress Treatments on Phenolic Content. As a first experiment, different treatments were compared on leaf disks collected from a single plant of F1 hybrid 'Orlando'. The experiment was performed in three biological replicates. To circumvent the variation between leaves of different plants, each of the three replicates of a single treatment was performed on foliar disks collected from three different leaves of the same plant. This experimental design needed only three leaves for covering the complete experiment and allowed standardized conditions. The experiment was performed with 12-week-old plant, because at this age the highest rate of response to stress application was observed. After sampling, leaf disks were either incubated for 24 h with 100 μM methyl jasmonate (mj) or 50 μM salicylic acid (sa) or exposed to UV-C (254 nm) light for 20 min and then transferred to normal light for 24 h (uv) in Petri dishes. Control disks were incubated in water at normal light for 24 h (ctr). Hereafter, leaf disks were immediately frozen, ground, and analyzed for the major (poly)phenolic compounds (**Figure 3**).

Most prominently, a significant increase (4-fold compared to the control, $p = 0.017$) was observed in the content of the main dicaffeoylquinic acid isomer II upon UV-C treatment (**Figure 3D**). The same treatment also slightly influenced the levels of chlorogenic acid, caffeoylquinic acid isomer II, the minor dicaffeoylquinic acid isomer I, and luteolin 7-glucoside, but the observed increases were not statistically significant (chlorogenic acid, $p = 0.33$; luteolin 7-glucoside, $p = 0.457$). Remarkably, solutions of methyl jasmonate or salicylic acid, for which activity had been demonstrated in other plant species (not shown), did not significantly change the content of any of the measured phenolic compounds (**Figure 3**), in contrast to UV-C.

In another experiment, the specific effect of UV-C irradiation on dicaffeoylquinic acids synthesis was confirmed in other genotypes of 'Orlando' and 'Violetto di Sicilia'. A marked induction of chlorogenic acid and the main dicaffeoylquinic acid isomer II was observed in all tested genotypes (**Figure 4**). The 'Orlando' plants were used for further analyses.
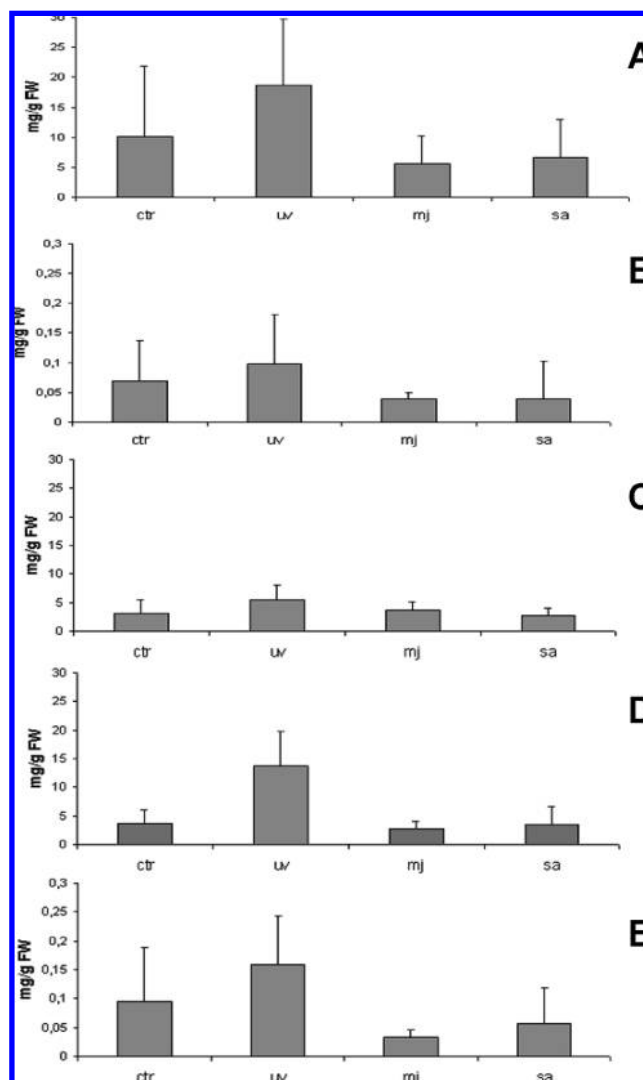

Figure 3. Content (mg/g of FW) of chlorogenic acid (**A**), caffeoylquinic acid II (**B**), luteolin 7-glucoside (**C**), dicaffeoylquinic acid II (main isomer) (**D**), and dicaffeoylquinic acid I (**E**) in globe artichoke foliar disks exposed to abiotic stresses (methyl jasmonate, salicylic acid, UV-C). The effect has been evaluated on one genotype of F1 hybrid 'Orlando' (12 weeks). The error bar indicates the standard deviation, calculated from three observations in each treatment. ctr (control), uv (UV-C), mj (methyl jasmonate), sa (salicylic acid).

The production of phenolic compounds upon UV-C exposure was monitored in time. Leaf disks of 'Orlando' were exposed to UV-C light for 20 min and harvested after 0, 12, 24, and 36 h of incubation in water (**Figure 5**). The experimental design was similar to that described previously, but seven foliar disks were used from each of the three leaves. A slight increase in both chlorogenic acid and dicaffeoylquinic acid isomer II was observed after 12 h, reaching a maximum after 24 h (although the increase was significant for only the dicaffeoylquinic acid) and then decreasing again after 36 h. The UV-C induction of dicaffeoylquinic acids, reaching a level of about 4 times that in the control plants (**Figure 5B**), was highly significant ($p = 0.001$). This increase was much more than observed for chlorogenic acid ($p = 0.123$).

Online Antioxidant Analysis. We investigated which antioxidant compounds are present in globe artichoke leaves by HPLC coupled to an online post-column antioxidant detection system. An example of antioxidant analysis of globe artichoke leaf extract is shown in **Figure 6**. The chromatogram shows
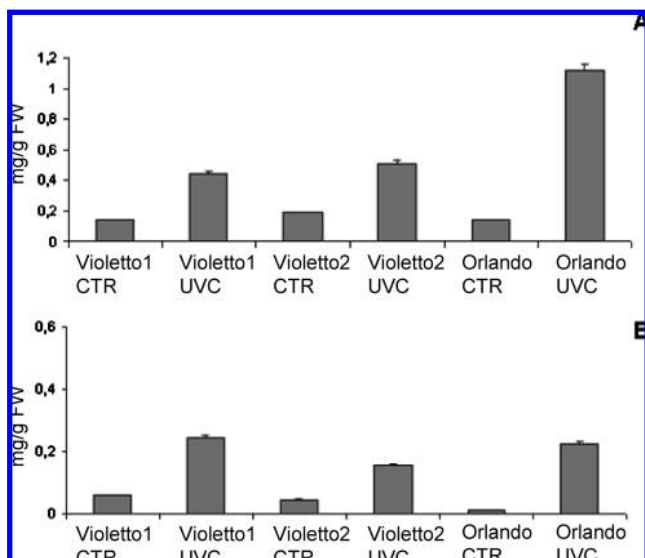
Figure 4. UV-C-induced chlorogenic acid (**A**) and dicaffeoylquinic acid isomer II (**B**) accumulation in three artichoke genotypes: F1 hybrid 'Orlando' and 'Violetto di Sicilia' (Violetto 1 and 2). Ctr (control, 10 weeks plant), uv (UV-C, 10-week plants). Values are expressed in mg/g of FW material. The error bar indicates the standard deviation, calculated from three technical replications in each treatment.
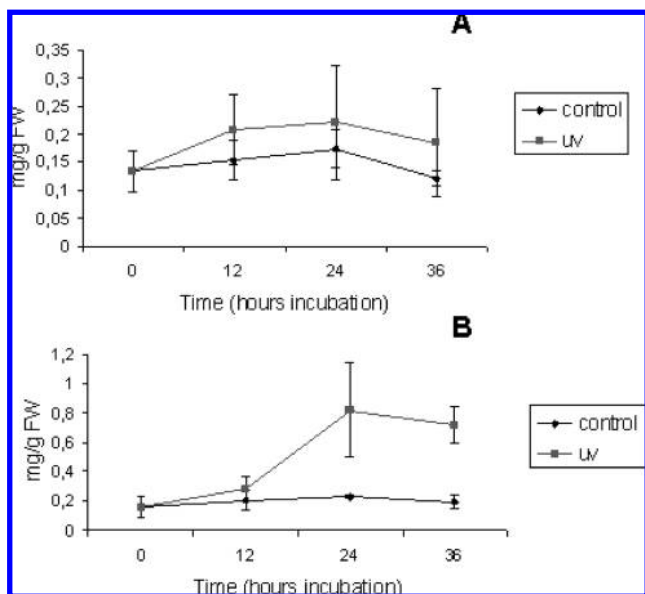

Figure 5. Time curve incubation of the effect of UV stress on chlorogenic acid (**A**) and dicaffeoylquinic acid isomer II (**B**) in globe artichoke (F1 hybrid 'Orlando', 10 weeks). Stress application: 254 nm, 20 min, 16 W, and after different times of incubation (0, 12, 24, 36 h). Values are expressed in mg/g FW material. The error bars indicate standard deviation, calculated from three observations in each treatment.

the ABTS$^{\bullet+}$ radical quenching activities of globe artichoke metabolites present in the aqueous methanol extracts and compares control and UV-C-treated samples, both harvested 24 h after exposure.

By integrating the areas of all (negative) peaks in the antioxidant detector chromatogram, the antioxidant activity of each separated compound can be calculated as a percentage of total antioxidant activity of plant extract. The analysis of the control extract indicates that phenolic acids (chlorogenic acid and dicaffeoylquinic acid isomer II) make a major contribution (60−80%) to the total antioxidant activity of the globe artichoke leaf extract.

The UV treatment not only resulted in an increase of the main two antioxidant compounds but also led to a significant increase in the total antioxidant capacity of tissue, measured as Trolox equivalents (**Table 3**). Chlorogenic acid contributed 57% of total antioxidant activity of globe artichoke leaf extract in UV-treated plants, whereas the dicaffeoylquinic acid contributed 28% of the antioxidant activity.

DISCUSSION

The first aim of this study was to identify standardized techniques for evaluating the effect of different stress treatments on the metabolism of phenolic compounds in globe artichoke. An experimental system based on foliar disks was developed for monitoring the biochemical response of globe artichoke leaves. Whereas hormonal treatments including methyl jasmonate or salicylic acid did not provoke a significant change in phenolic compounds, the dicaffeoylquinic acid content increased by UV-C treatment in several repeated experiments, performed on different globe artichoke genotypes.

Caffeoylquinic acids such as chlorogenic acid and dicaffeoylquinic acids are the dominant phenolic compounds in globe artichoke, which is one of the richest sources of these compounds found in nature. Especially cynarin (1,3-dicaffeoylquinic acid) has been reported to occur at high levels in this plant species (*20*). In our globe artichoke leaf extracts we did not detect a peak corresponding to the cynarin standard 1,3-dicaffeoylquinic acid, but instead dicaffeoylquinic acid isomer II was the main compound. Isomerization of dicaffeoylquinic acids has been reported in warm aqueous media, which could lead to a variation in the extract composition (*23*). It may be relevant that our globe artichoke leaf disks were extracted with near-anhydrous methanol. Therefore, we assume that under our extraction and chromatographic conditions, the main isomer (II) will be 1,5-dicaffeoylquinic acid, followed by the 3,4, 3,5, and 4,5 isomers (*16*).

Chlorogenic acid is known to be involved in responses to different biotic and abiotic stresses in several plant species. As a response to UV-B treatment, chlorogenic acid is known to increase in leaves from tobacco (*27*) and silver birch (*32*), whereas in apple peel, a significant induction by daylight exposure on this compound was observed (*33*). In a close relative of the globe artichoke, the cultivated cardoon, it was observed that blanching (i.e., no exposure to light for a month before harvesting) provokes a reduction in content of phenolic acids and flavonoids (*34*). Apart from light exposure, also wounding and pathogen attack are known to induce chlorogenic acid synthesis, for instance, in lettuce (*26*) and tomato (*28*). One could envisage that dicaffeoylquinic acids are subject to the same regulation as chlorogenic acid, but no information is available on the regulation of these compounds in any plant species. In this study on globe artichoke, we therefore applied three treatments known to induce polyphenol synthesis: UV-C, which represents light stress; methyl jasmonate, which is involved in signaling of wound responses; and salicylic acid, a hormone that is produced in response to pathogen infection.

The specific induction of dicaffeoylquinic acids in response to UV-C exposure suggests a role of these compounds in globe artichoke in protection from UV radiation damage. UV-C, which can be used for sterilization, is a high-energy radiation with a wavelength shorter than 280 nm. UV-C is part of solar light, but it is strongly absorbed by oxygen and ozone in the stratosphere, to such an extent that none of this radiation is present in terrestrial sunlight (*35*). Nevertheless,
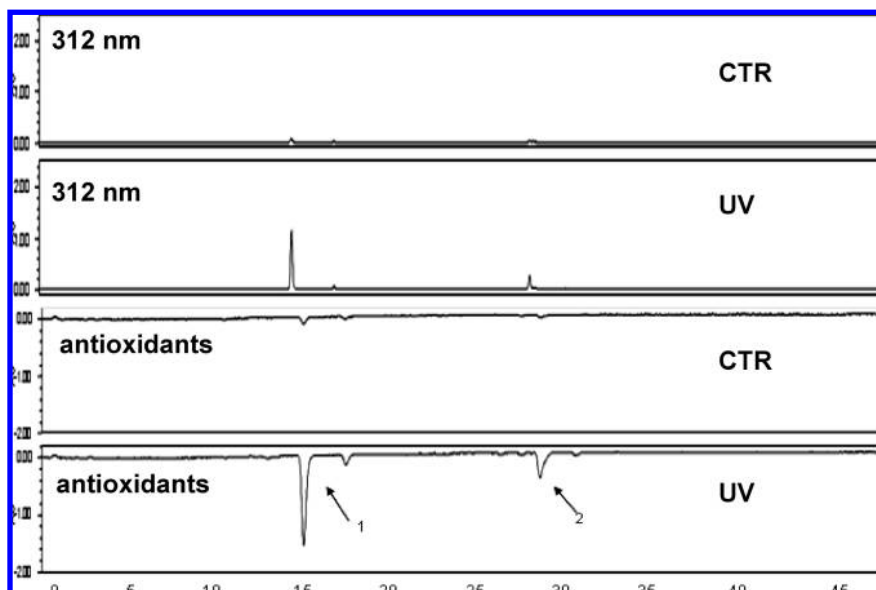
Figure 6. HPLC UV−vis chromatogram (top panels) and analysis of antioxidant compounds (bottom panels) of globe artichoke leaf extract (control sample and UV treated sample). The chromatograms are recorded at 312 nm (HPLC UV−vis) and at 412 nm after reaction with ABTS$^{\bullet+}$(antioxidant analysis). Note that there is a shift of 30s between the first (PDA) and second (antioxidant) detectors. Peak 1 is chlorogenic acid, and peak 2 is dicaffeoylquinic acid isomer II.

Table 3. Effect of UV-C Exposure on Antioxidant Capacity, Expressed as Trolox Equivalents (Micromoles of Trolox per Gram of FW), and Contribution of Phenol Acids (Chlorogenic Acid and Dicaffeoylquinic Acid Isomer II) to the Total Antioxidant Activity (Measured as the Total Peak Area of the Chromatogram), Expressed in Trolox Equivalents (Percentage Value Is Presented in Parentheses)

	chlorogenic acid	dicaffeoylquinic acid	total antioxidants
control	1.26 (50%)	0.44 (18%)	2.5
UV	4.42 (57%)	2.14 (28%)	7.7

UV-C is generally considered to have similar but more intense effects as compared to UV-B and is therefore applied as a convenient source of light stress (*36*). In plant cells, UV-C activates antioxidant activity and increases the levels of polyamines and free radicals such as reactive oxygen species (ROS) (*37*). Chlorogenic acid is known to interact with ROS in vitro (*38*). We monitored and identified individual antioxidant compounds in globe artichoke leaf extract by means of an HPLC system with online antioxidant analysis. Our aim was to determine the contribution of phenylpropanoid compounds to the total antioxidant activity in a methanol extract and to assess differences associated with UV treatment (**Figure 6**). The results showed that the antioxidant activity in globe artichoke leaf extract was strongly (80%) determined by the level of phenolic acids (dicaffeoylquinic acid and chlorogenic acid; **Table 3**). Moreover, the UV radiation led to a strong induction of the antioxidant capacity of the globe artichoke leaf extract. Our results confirmed a previous study suggesting that dicaffeoylquinic acids, chlorogenic acid, and luteolin glucosides are the most active free radical scavengers in globe artichoke leaves (*17*). Phenolic acids localize in chloroplasts of young leaves (*39*). Chloroplasts are known to produce large quantities of ROS under adverse environmental stresses such as high light exposure (*40*). Likely, the production of antioxidant compounds such as dicaffeoylquinic acids in chloroplasts upon UV-C exposure serves to protect young globe artichoke leaf tissue from light damage.

The effect of UV-C exposure on biosynthesis of phenolic compounds is likely mediated by ROS, which may have a role as second messengers, acting upstream of a number of pathways involving the plant hormones salicylic acid and jasmonic acid (*41*). Studies in tomato indicated that regulation of a number of wound-inducible genes, such as proteinase inhibitors, in response to UV-B and UV-C irradiation, depends on jasmonate signaling (*36*). Surprisingly, in our study we did not observe any effect of methyl jasmonate or salicylic acid on accumulation of dicaffeoylquinic acids or chlorogenic acid. Possibly the signal leading to accumulation of dicaffeoylquinic acids in globe artichoke is not mediated by methyl jasmonate or salicylic acid and differs in this sense from other defensive pathways, although we cannot exclude the possibility that higher concentrations of these compounds may be required to up-regulate phenylpropanoid biosynthesis in globe artichoke.

The mechanism underlying the biosynthesis of dicaffeoylquinic acids in plants is still unclear. Globe artichoke may represent a very interesting plant for studying dicaffeoylquinic acid biosynthesis, due to the exceptionally high content of these compounds in its leaves. In most systems studied, phenylpropanoid synthesis is the result of increased transcription of genes encoding the corresponding biosynthetic enzymes. Therefore, the UV-C-based induction system described here will be applied for identifying the genes(s)/enzyme(s) involved in their biosynthesis. Furthermore, polymorphisms in gene sequences may help to explore the relationship between genotype, developmental stage, and content of caffeoylquinic acids.

The content of polyphenols in globe artichoke appears strongly influenced by genetic factors. Molecular studies demonstrated that the varieties of globe artichoke in cultivation are highly heterogeneous (*1, 2*). This reflects their multiclonal composition, which is a direct consequence of the limited selection criteria applied by farmers. In recent years a number of seed-propagated cultivars and F1 hybrids have been developed, which, however often lack uniformity and show significant element of within genetic variation (*42, 43*).

The importance of genetic control of the metabolic composition of plants has been pointed out by previous studies (*44*). Reddivari et al. demonstrated that the polyphenolic composition of potato differed significantly among

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genotypes and that this genotypical effect was dominant over the effect of the plant environment on the metabolic composition (*45*). An interesting and challenging aspect for future research will be to clarify the effect of genotype in relation to environmental interaction on the different classes of (poly)phenols.

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