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Low-Temperature Conditioning of "Seed" Cloves Enhances the Expression of Phenolic Metabolism Related Genes and Anthocyanin Content in 'Coreano' Garlic (*Allium sativum*) during Plant Development

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ABSTRACT: Low-temperature conditioning of garlic "seed" cloves accelerated the development of the crop cycle, decreased plant growth, and increased the synthesis of phenolic compounds and anthocyanins in the outer scale leaves of the bulbs at harvest time, leading to 3-fold content increase compared with those conditioned at room temperature. Cold conditioning of "seed" cloves also altered the anthocyanin profile during bulb development and at harvest. Two new anthocyanins are reported for the first time in garlic. The high phenolics and anthocyanin contents in bulbs of plants generated from "seed" cloves conditioned at 5 °C for 5 weeks were preceded by overexpression of some putative genes of the phenolic metabolism [6-fold for phenylalanine ammonia lyase (PAL)] and anthocyanin synthesis [1-fold for UDP-sugar:flavonoid 3-*O*-glycosyltransferase (UFGT)] compared with those conditioned at room temperature.

KEYWORDS: *Allium sativum, cold conditioning, anthocyanin, phenolic, PAL*

INTRODUCTION

Garlic (*Allium sativum*) is one of the most accepted and widely grown vegetables in the world. It is used in food preparation as either spice or seasoning or to impart flavor as well as for its medicinal value.[1] Garlic is the second most important species in the genus *Allium* after onion. A garlic bulb consists of a disc-shaped stem or basal plate that supports the bulbils or cloves, which are a series of functional leaves. These are organs of propagation of the species, considered vegetative buds in a dormancy stage, which can activate growth to generate a new plant under certain environmental conditions. Covering each of the bulbils is a flaky membranous sheet called inner scale leaves, while externally a number of similar protective sheets cover the whole bulbils, called outer scale leaves, cataphylls, or "coats".[2] The outer scale leaves consist only of a sheath leaf that is lignified and dry. These are responsible for the characteristic color of the bulb, which characterizes different cultivars and varies from white to purple. The color of the bulb is an important quality factor during marketing of the product. For example, in Mexico and some Latin American countries there is a preference for purple or marbled purple garlic, in part because of the strong association between pungency and color intensity.[3] The purple or reddish color of garlic and onion varieties is due to anthocyanins, some of which have already been reported.[4−6] However, there is only limited information about anthocyanins in garlic, among which cyanidin-3-*O*-glucoside (C3G) is considered the main anthocyanin present.[7] Additionally, the existence of aliphatic acylation derivatives in the sugar moiety of C3G in garlic has been suggested.[8] Because the spread of this crop is of the clonal type, storage conditions of the "seed" cloves markedly influence the behavior of the crop after planting in the field. Conditioning of "seed" cloves at 5 °C for 15 to 30 days before planting accelerates the initiation, development, and maturity of the bulbs relative to those of cloves conditioned at room temperature or 20 °C.[9−11] In the last three seasons, our laboratory has shown that conditioning "seed" cloves from 'Coreano' variety at 5 °C during five weeks before planting allowed an earlier harvest of ∼6 weeks ahead of time and increased the purple color of the bulb during growth in the field in comparison to those obtained from bulbs conditioned previously at room temperature (RT). Furthermore, it is unknown how the storage conditioning temperature of the "seed" cloves affects the phenolic and anthocyanin content during its development. To date there have been no reports about the increase of anthocyanins after sowing related to conditioning of seeds at low temperatures. However, there are reports indicating that environmental stress during crop development and postharvest storage stimulates the biosynthesis of anthocyanin along with corresponding increases in the activity of phenylalanine ammonia lyase (PAL) as well as their associated gene expression.[12,13] Recently, the molecular cloning and characterization of PAL and cinnamate 4-hydroxylase (C4H) in garlic have been described.[14] In addition, the

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complete transcriptome in bud garlic cloves stored at RT using an Illumina sequencing system has been reported; 127 933 unigenes were found and categorized into 25 groups of different physiological functions, where the authors gave more emphasis to those genes involved in the biosynthesis of aroma compounds.[15]

Therefore, the objective of this study was to measure changes in the content of phenolic and anthocyanin compounds in garlic bulbs cv. 'Coreano' obtained from bulbs conditioned at RT and 5 °C before sowing and to correlate such changes with the gene expression during low-temperature conditioning of "seed" cloves.

MATERIALS AND METHODS

Materials. Seed bulbs of garlic (*A. sativum*, L.) cv. 'Coreano' were provided by the Garlic Producer Association of Aguascalientes and cultivated at Cosio, Aguascalientes, Mexico, during the summer of 2010. The SV Total RNA Isolation System Kit and Pure Yield Plasmid Miniprep System Kit were purchased from Promega (Madison, WI, USA). The In-Fusion SMARTer Directional cDNA Library Construction Kit, CROMA-SPIN 1000 column, and Stellar Competent Cells were purchased from Clontech Laboratories (Mountain View, CA, USA). *Eco*RI restriction enzyme was purchased from Invitrogen (Carlsbad, CA, USA). The Biotin Chromogenic Detection Kit was purchased from Fermentas (Vilnius, Lithuania). Methanol, formic acid, acetonitrile, and acetic acid of HPLC-MS grades were purchased from Sigma (St. Louis, MO, USA), as were Folin−Ciocalteu reagent (2 N), gallic acid, sodium carbonate, potassium chloride, and sodium acetate.

Low-Temperature Conditioning and Plant Growth Conditions. This study employed 150 bulbs of garlic cv. 'Coreano' harvested in the 2009−2010 season. The bulbs were separated into two sets of 75 bulbs; one set was stored at RT, and the other was conditioned at 5 °C for 5 weeks. After conditioning, the cloves were separated and classified by size selecting those cloves with masses of 5 to 8 g. Three sets of 200 cloves for each temperature (three replicates) were treated with fungicides, nematicides, and bactericides to avoid attacks of different plagues and diseases in the field. Each set of cloves was sown during the 2010−2011 season in a commercial orchard at Cosio, Aguascalientes, Mexico (22° 21′ N, 102° 18′ W), with a density of 336 000 plants per hectare. All cultural practices used were those used commercially and recommended by the Garlic Producer Association of Aguascalientes.

Sampling. The gene expression analysis was carried out in the sprouts (leaf primordia) of different cloves conditioned for 5 weeks at both temperatures; the sprouts were frozen in liquid nitrogen and stored at −70 °C until their analysis. For the determination of anthocyanin and phenolic compounds, two sampling dates were selected, one at the fourth development month [122 days after sowing (DAS)] and other when bulbs were harvested (158 and 200 DAS for the plants from bulbs stored at 5 °C and RT, respectively) and after a curing period in the field for 10 days. The harvest time was decided on the basis of senescence leaves and complete clove formation. Five plants or bulbs (experimental unit) of each replicate were taken from each treatment, placed on ice, and transported to the laboratory, where the cataphylls were frozen in liquid nitrogen, freeze-dried, and stored at −70 °C until their analysis.

Indicative Parameters of Plant and Bulb Growth of Garlic. Cold-conditioning effects during development were evaluated by determining the number of leaves, plant height, leaf width, bulb diameter, plant weight, and bulbification index (bulb diameter/pseudostem diameter) using five plants of each replicate from each treatment.

Total Phenolic and Anthocyanin Compound Extraction. Two different extracts were prepared. The first extract was obtained for the quantification of total phenolic compounds and total anthocyanins as described in the literature[10] with slight modifications. One gram of cataphylls powder was mixed with 15 mL of 5% acetic acid/methanol for 24 h in darkness. The extract was filtered (Whatman no. 4 filter) and kept at −20 °C until quantification of the compounds. The second extract was obtained for anthocyanin identification by LC-MS/MS. A 0.15 g sample of cataphylls powder was added to 0.6 mL of methanol (HPLC grade). The mix was sonicated for 20 min, kept at 4 °C for 24 h in darkness, and then centrifuged at 10621*g* for 10 min at 10 °C, after which the supernatant was filtered (0.22 μm nylon filter) before the quantification and LC-MS/MS analysis.

Total Phenolic Quantification. The experimental procedure was adapted from the literature.[16] A 50 μL aliquot of the extract was mixed with 125 μL of Folin−Ciocalteu reagent (2 N) in a test tube during 5 min. Next, 625 μL of sodium carbonate (20% w/v) was added, and the mixture was incubated for 2 h at room temperature. The absorbance of the solution at 760 nm was measured with a Spectramax 190 UV/vis microplate reader (Molecular Devices, Sunnyvale, CA, USA) against a blank (deionized water plus reagents). The quantification was carried out using a standard curve of gallic acid (0.0−125.0 mg/L), and the results were expressed as milligrams of gallic acid per gram of dry matter (mg GAE/g).

Total Monomeric Anthocyanin Quantification. The pH differential method was followed as described previously.[17] Two 50 μL extract samples were prepared, one with 3 mL of potassium chloride buffer (0.025 M, pH 1) and the other with 3 mL of sodium acetate buffer (0.025 M, pH 4.5). Both dilutions were equilibrated for 15 min at room temperature, and the absorbance was read at 510 nm (maximum absorbance) and 700 nm (reading of the degradation degree of the compounds and correction by interfering substances) with a Lambda 40 UV/vis spectrophotometer (PerkinElmer, Norwalk, CT, USA). A blank with deionized water was used. The results were expressed as milligrams of cyanidin-3-*O*-glucoside per gram (mg C3G/g).

Analysis of Anthocyanins by LC-MS/MS. Individual compounds were identified on the basis of retention time, UV spectra, and their mass-to-charge ratio using LC-MS/MS. Chromatographic separations were performed on a LCQ Deca XP Max LC-MS/MS system (Thermo Finnigan, San Jose, CA, USA) equipped with an autosampler, a Surveyor 2000 quaternary pump, and a UV 2000 PDA detector using a 150 mm × 2.00 mm Synergi 4μ Hydro RP 80A column (Phenomenex, Torrance, CA) and a guard column of the same chemistry. Elution gradients were performed with solvent A (1% formic acid/water) and solvent B (acetonitrile). Separations were achieved by a linear gradient with A and B as follows: 0 min 87% A, 3 min 85% A, 18 min 75% A, 28 min 60% A, 35 min 100% A. The flow rate was 200 μL min^{-1}. The injection volume was 10 μL.

Samples were delivered to the LCQ MS by an electrospray ionization (ESI) source. Conditions for analysis in positive ion mode were as follows: spray voltage, 5.0 KV; sheath gas flow rate, 50 arbitrary units; auxiliary gas flow rate, 3.0 arbitrary units; capillary temperature, 301.4 °C; capillary voltage, 46.25 V. Spectra were scanned over a range of m/z 180−2000 at 3 scans per second. Helium was used as the collision gas, and the collision energy was set at 24%. MS^2 analysis was used during the identification.

Isolation of Total RNA. An SV Total RNA Isolation System Kit was used to extract the total RNA from 30 mg of sprouts from both storage conditioning temperatures. RNA purified by spin column assembly was analyzed for integrity and size by formaldehyde agarose gel electrophoresis, and quantification and purity of RNA were assessed by the $OD_{260/280}$ value.

Synthesis, Amplification, and Purification of cDNA. A 1 μg sample of total RNA of each storage temperature was used as a template to synthesize the first strand of cDNA using an In-Fusion SMARTer Directional cDNA Library Construction Kit. Next, cDNA was amplified by LD-PCR with 15, 18, 21, and 24 cycles separately and analyzed through 1.2% agarose gel electrophoresis in order to determine the optimal number of cycles to collect a suitable amount rather than a superfluous one to build the subtractive library. Placental total RNA was performed as a control. A CROMA-SPIN 1000 column was used to purify the cDNA.

Cloning of cDNA Fragments and Slot-Blotting Hybridization. cDNA fragments were inserted in linearized pSMART21FD Stellar Competent Cells, which were used for transformation and

Table 1. Parameters of Plant and Bulb Growth of Garlic cv. 'Coreano' During the Crop Cycle Obtained from Previously Conditioned "Seed" Bulbs Stored at 5 °C and Room Temperature[a]

storage temperature	days after sowing	number of leaves	plant height (cm)	leaf width (cm)	bulb diameter (mm)	plant weight (g)	bulbification index
RT[b]	56	6.5 d	29.6 f	1.3 e	13.9 e	8.5 f	1.7 d
	87	8.4 c	29.6 e	1.8 c	19.2 de	19.4 ef	1.9 cd
	122	9.1 bc	53.9 c	2.2 b	25.1 d	46.6 d	1.9 cd
	158	12.7 a	84.7 a	3.1 a	40.3 c	131.5 a	2.4 cd
	200	12.5 a	85.2 a	3.0 a	65.8 a	153.1 a	4.2 b
5 °C	56	6.7 d	33.9 ef	1.6 d	16.2 e	9.6 f	1.8 d
	87	8.6 c	48.3 d	1.8 c	24.6 d	27.8 e	2.4 cd
	122	9.8 b	69.3 b	2 bc	36.4 c	63.6 c	3.1 bc
	158	9.2 bc	68.8 b	2.1 b	53.5 b	96.3 b	7.7 a
	200	–	–	–	–	–	–

[a]Each value is the average of $n = 15$ samples. Means with different letters within columns are significantly different at $P \leq 0.05$. [b]RT: room temperature.

Figure 1. Visual appearance of (A, B) garlic plants 122 days after sowing and (C, D) bulbs harvested from plants obtained from "seed" bulbs conditioned for 5 weeks at (A, C) 5 °C and (B, D) room temperature.

further cDNA fragments screening. Plasmid was screened for the presence of inserts using *Eco*RI restriction enzyme. For screening, plasmid DNA was extracted using a Pure Yield Plasmid Miniprep System Kit. A 5 μg sample of each clone was transferred using a manifold device and cross-linked in a nylon membrane. Total cDNAs originally synthesized (from both storage temperatures) were labeled using a Biotin Chromogenic Detection Kit.

Storage of Libraries. Selected white colonies containing recombinant plasmid were inoculated separately into 5 mL LB/ampicillin/kanamycin solution, which was shaken at 37 °C overnight. Next, 500 μL of each culture was added into a 2 mL cryogenic vial containing 500 μL of 100% glycerol and kept at −80 °C.

DNA Sequencing and Database Comparison. The nucleotide sequences of differentially expressed fragments were determined using an ABI PRISM 310 genetic analyzer (PerkinElmer, Norwalk, CT, USA). Online database comparisons were performed using the BLASTX algorithm from the National Center for Biotechnology Information (NCBI).

Statistical Analysis. All determinations were done in triplicate. Analysis of variance (ANOVA) was performed using JMP 8.0 software (SAS Institute, Cary, NC, USA), and means were compared using Student's t test at $P \leq 0.05$.

RESULTS AND DISCUSSION

Effect of Low-Temperature Conditioning on Plant Growth and Visual Aspect of Garlic Bulbs. Plants from bulbs conditioned at 5 °C showed a shorter development cycle (158 DAS) than plants from RT-conditioned bulbs (200 DAS), which meant an ealier harvest time with a time difference of ∼6 weeks. However, there were also some differences in the growth of the two crop cycles. The number and width of leaves, the weight and height of the plants, and the bulb diameter were higher in plants from RT-stored bulbs (Table 1). Indeed, the bulbing index changed faster (values >2 from day 87) in plants from 5 °C-conditioned bulbs with respect to the changes showed by plants from RT storage bulbs, which started their differentiation process until 158 days. The yield per hectare (∼11 ton) and the bulb size were lower than for the RT-conditioned bulbs (∼15 ton) but within commercial standards. Probably the genetic expression changes due to cold conditioning modified the physiological patterns of development, such as reducing the number of cells with larger size in the cloves, accelerating the senescence process, and limiting the growth of plant and bulbs.[10,11] Although similar behavior has

been observed in other varieties, the response is variety-dependent.[18−20]

These data confirmed that it is possible to shorten the crop cycle through cold conditioning of the "seed" bulbs for specific periods. However, it is necessary to understand how this process occurs to take advantage of it and design better strategies to improve the visual quality of the bulbs, their biochemical changes, and the general genetic response to cold treatment.

Content of Phenolic Compounds and Anthocyanins. Cold conditioning at 5 °C for 5 weeks before sowing increased the purple color of the bulb during plant growth and in the harvested bulbs (Figure 1), indicating that the anthocyanin accumulation process was affected by the conditioning applied. This color change is considered a positive quality trait because the purple color is highly appreciated in different markets.

Table 2 shows the total phenolic contents and total anthocyanin contents of two different sampling dates in the cataphylls of bulbs obtained from plants of previously conditioned "seed" cloves at two temperatures. On both sampling dates, the total phenolic content was higher in the samples obtained from cloves conditioned at 5 °C (3.25 and 1.87 mg GAE/g of freeze-dried cataphylls, respectively) than in samples obtained from cloves stored at RT (2.61 and 0.61 mg GAE/g of freeze-dried cataphylls, respectively). These changes represented increases of ∼24 and 205% for the 5 °C treatment relative to the RT treatment, respectively. Similar behavior was also observed for the anthocyanin content, where the increases were ∼30 and 73%, respectively, at the same sampling dates.

Table 2. Total Phenolic Contents and Total Anthocyanin Contents in Garlic Cataphylls of Plants Obtained during the Crop Cycle from Previously Conditioned "Seed" Bulbs Stored at 5 °C and RT[a]

days after sowing	storage temperature	total phenolics (mg GAE/g)	total anthocyanins (mg C3G/g)
122	5 °C	3.249 ± 0.020 a	1.156 ± 0.032 a
122	RT	2.614 ± 0.019 b	0.892 ± 0.031 b
158	5 °C	1.873 ± 0.039 a	0.380 ± 0.004 a
200	RT	0.613 ± 0.040 b	0.103 ± 0.005 b

[a]Means with different letters within columns are significantly different at $P \leq 0.05$.

The results in the present study are higher than those in previous reports that analyzed whole bulbs or cloves of the plant.[14,21,22] It has also been reported that cataphylls of onion bulbs have higher phenolic and anthocyanin content with respect to other plant organs.[23] In general, these data showed that cold conditioning of "seed" cloves has an important effect on the metabolism of phenolic compounds and anthocyanins, promoting their biosynthesis during plant growth in the field. In addition, it is important to point out that the contents of these compounds were higher in growing bulbs (122 DAS) than in harvested bulbs (158 and 200 DAS), suggesting an apparent loss of these compounds, very likely during the curing process or bulb drying on the field for an additional 10 days after harvest (Table 2). These losses represented ∼42 and 67% of the phenolic and anthocyanin contents, respectively, in samples from 5 °C-conditioned seed bulbs and ∼76 and 79%, respectively, in samples from RT-conditioned seed bulbs, suggesting a larger loss for the latter conditioning treatment. Another possible reason for the observed decrease in phenolic content would be related to a dilution effect during growth due to an increase in bulb diameter in the late stage of development (and the corresponding cataphylls), such as a ∼46% increase in bulb diameter from day 122 to 158 for samples conditioned at 5 °C and a ∼63% increase from day 158 to 200 for samples conditioned at RT (Table 1). However, the exact mechanism for this apparent loss is still unknown and deserves further investigation.

On the other hand, it is known that the biosynthesis of anthocyanin and phenolics may be controlled by the photoperiod and low temperature[12,19,25,26] and that changes in phenolic content may take place as well.[22,24]

In the present study, higher biosynthesis was observed in the first stage of development, which coincided with the prevalence of low temperatures during the development of the plant (720 h with temperatures below 5 °C during the first 120 days of plant development). Furthermore, other studies have shown that a long photoperiod induces the synthesis of these compounds.[13,25] In the present study, however, the temperature and the photoperiod for the two sets of experimental conditions were the same since the treatments were sown in the same area. This observation supports the idea that the temperature conditioning treatments before planting were the key factor in anthocyanin synthesis in the present study.

In addition to the above observations, it is known that PAL activity as well as the content of free phenolics and their ester- and glycoside-bound forms increase during cold stress while the levels of phenolics and acids ester-bound to cell walls decrease, which could be associated with an increase in the cell wall stiffness and extensibility but also could be limiting the plant growth.[28,29] Thus, the smaller size of bulbs for 5 °C conditioning treatments may be explained by this effect, and the presence of caffeic acid, ferulic acid, vanillic acid, *p*-hydroxybenzoic acid, and *p*-cumaric acid reported in garlic [27] may contribute to the process.

In general, a shorter crop cycle with enhanced visual color of the bulb could facilitate the commercialization of garlic when production for the market is low. In addition, a short crop cycle can diminish the effects of higher temperatures normally seen in extended crop cycles that provoke the formation of sprouts.

All of these data show the high impact that cold conditioning of the "seed" cloves can potentially have on the physiology during garlic plant development.

Analysis of Anthocyanins by LC-MS/MS. The HPLC chromatogram of the extract obtained from cataphylls of garlic bulbs of plants from RT-conditioned seed bulbs (Figure 2) revealed six major anthocyanin peaks, corresponding to compounds shown in Table 3.

The identification of the anthocyanins in the samples was based on mass spectrometry data (Table 3), taking into account the fact that fragmentation pattern of anthocyanins occurs almost exclusively in the glycosidic linkages between the flavylium cation and the adjacent sugar and the breaking of ester bonds between the glycosides and existing acyl groups.[31] The acyl groups were determined by calculating the possible combinations of aliphatic and aromatic acids previously reported in acylated anthocyanins within the genus *Allium*.[8]

In general, there is little information available about the identification of anthocyanins in garlic, with only reports concerning the external layers of the cloves but not the cataphylls of the bulbs.[7,8] In the present study, five compounds were identified as cyanidin derivatives, showing a fragment of m/z 287, corresponding to the mass of cyanidin aglycone. Peak

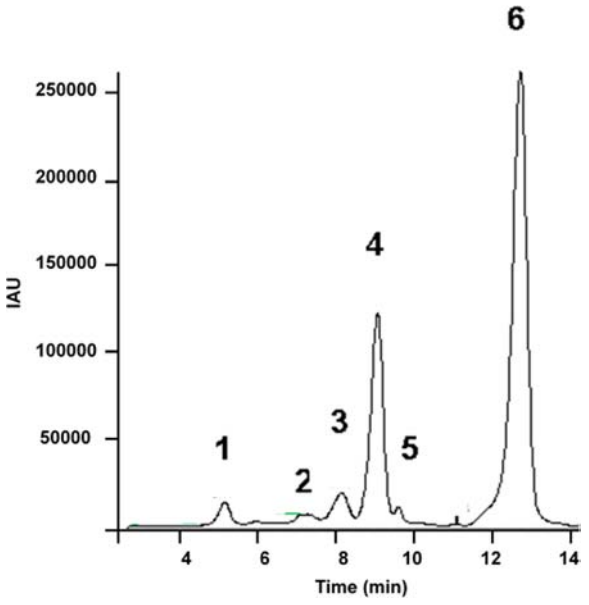

Figure 2. Typical HPLC chromatogram of anthocyanin extracts from cataphylls of garlic bulbs obtained from plants of RT-conditioned seed bulbs: peak 1, cyanidin-3-*O*-glucoside; peak 2, cyanidin-3-*O*-(3″-malonyl) glucoside; peak 3, cyanidin-3-*O*-(3″-acetoyl) glucoside; peak 4, cyanidin-3-*O*-(6″-malonyl) glucoside; peak 5, not identified; peak 6, cyanidin-3-*O*-(6″-malonyl acetoyl) glucoside.

Table 3. Chromatographic and Mass Spectrometry Characteristics of Anthocyanins in Outer Scale Leaves of Garlic Bulbs cv. 'Coreano' at Harvest Time Obtained from Previously Conditioned "Seed" Bulbs Stored at Room Temperature

peak	retention time (min)	λ_{max} (nm)	$[M]^+$ (m/z)	fragments MS^2 (m/z)	identity
1	4.28	281, 515	449	287	cyanidin-3-*O*-glucoside
2	6.25	281, 517	535	449, 287	cyanidin-3-*O*-(3″-malonyl) glucoside
3	7.06	281, 516	491	449, 287	cyanidin-3-*O*-(3″-acetoyl) glucoside
4	7.82	280, 517	535	491, 449, 287	cyanidin-3-*O*-(6″-malonyl) glucoside
5	8.39	269, 507	645		not identified
6	11.58	281, 518	577	533, 491, 449, 287	cyanidin-3-*O*-(6″-malonyl acetoyl) glucoside

1 (t_R = 4.28 min) was identified as cyanidin-3-*O*-glucoside, with a typical mass spectrum of this compound ($[M]^+$ m/z 449.00, MS/MS m/z 287 [M − glucose]$^+$ or [cyanidin]$^+$). Peaks 2 and 4 (t_R = 6.25 and 7.82 min, respectively) are malonated isomers of cyanidin-3-*O*-glucoside ($[M]^+$ m/z 535) and were identified as cyanidin-3-*O*-(3″-malonyl) glucoside (MS/MS m/z 449 [M − malonyl]$^+$, 287 [cyanidin]$^+$) and cyanidin-3-*O*-(6″-malonyl) glucoside (MS/MS m/z 491 [M − 44]$^+$, 449 [M − malonyl]$^+$, 287 [cyanidin]$^+$), respectively. The m/z 44 loss corresponds to the breakdown of an acetyl group of malonic acid. Peak 3 (t_R = 7.06 min) was identified as cyanidin-3-*O*-(3″-acetoyl) glucoside ($[M]^+$ m/z 491, MS/MS m/z 449 [M − acetyl]$^+$, 287 [cyanidin]$^+$). Peak 5 (t_R = 8.39 min) could not be identified with the information obtained ($[M]^+$ m/z 645), but it may correspond to an adduct formed during the anthocyanin extraction process. Peak 6 (t_R = 11.58 min) was tentatively identified as cyanidin-3-*O*-(6″-malonyl acetoyl) glucoside ($[M]^+$ m/z 577, MS/MS m/z 533 [M − acetyl]$^+$, 491 [M − 42]$^+$, 449 [M − malonyl]$^+$, 287 [cyanidin]$^+$), in which, as for peak 4, the loss of about m/z 42 corresponds to the rupture of an acetyl group of malonic acid. This is the first report of the presence of cyanidin-3-*O*-(3″-acetoyl) glucoside and cyanidin-3-*O*-(6″-malonyl acetoyl) glucoside in garlic. Although these compounds have been identified in red onions, which had a similar fragmentation pattern, as cyanidin-3-*O*-(3″-acetoyl) glucoside ($[M]^+$ m/z 491; MS/MS m/z 287) and cyanidin-3-*O*-(6″-malonyl acetoyl) glucoside ($[M]^+$ m/z 577; MS/MS m/z 491/287).[32,33]

The type of anthocyanin did not change during the development of plant or bulb, but there were quantitative differences associated with the development and also the cold treatment applied to the "seed" bulbs. The cataphylls of bulbs of immature plants (122 DAS) from 5 °C-conditioned bulbs showed higher contents of all anthocyanins compared with plants from RT storage bulbs (Figure 3), confirming that the

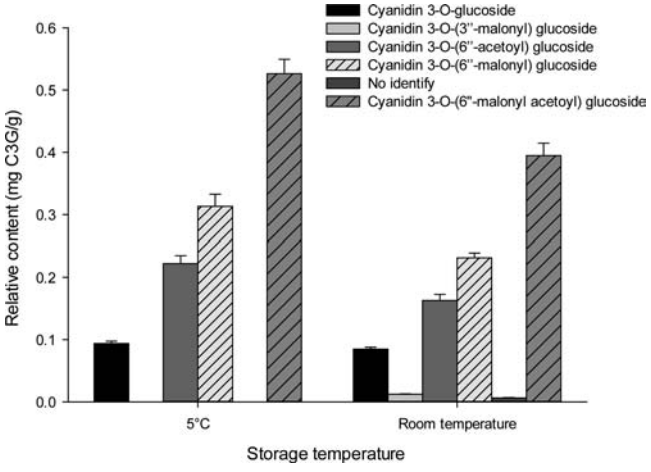

Figure 3. Individual content of anthocyanins from HPLC analysis in outer scale leaves of garlic bulbs cv. 'Coreano' obtained 122 days after sowing from previously conditioned "seed" bulbs stored at 5 °C and room temperature.

cold treatment before planting increased the biosynthesis of these compounds during the development stage. However, cyanidin-3-*O*-(3″-malonyl) glucoside and the nonidentified anthocyanin (peak 5) were present only in the samples from RT storage bulbs and not in those subject to cold conditioning. On the other hand, the harvested and cured bulbs showed an important loss of individual anthocyanins in relation to immature bulbs (Figure 4). These losses observed in bulbs of plants from cold-conditioning treatments were ∼81, 71, 59, and 13% for cyanidin-3-*O*-(6″-malonyl acetoyl) glucoside, cyanidin-3-*O*-(6″-acetoyl) glucoside, cyanidin-3-*O*-(6″-malonyl) glucoside, and cyanidin-3-*O*-glucoside, respectively. On the other hand, the losses in bulbs of plants from RT conditioning treatments were higher for the same compounds (∼94, 86, 82, and 80.5%, respectively). The enhanced biosynthesis of individual anthocyanins in immature bulbs of plants from cold-conditioning treatments allowed a higher content of the compounds to be retained, despite an apparent decrease after the harvest and curing phase, and the color of the harvested bulbs to be maintained in comparison with bulbs of plants from RT storage treatments. This effect enhances the quality of the

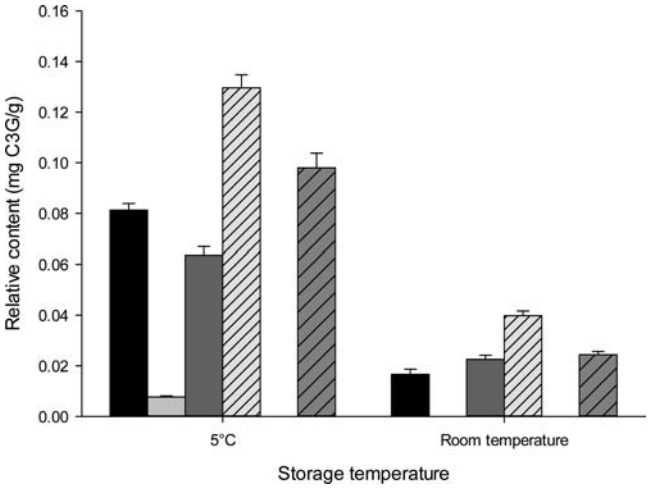

Figure 4. Individual content of anthocyanins from HPLC analysis in outer scale leaves of garlic bulbs cv. 'Coreano' obtained at harvest time from previously conditioned "seed" bulbs stored at 5 °C and room temperature.

bulbs, representing an advantage during the marketing process of this garlic variety.

In general, the data confirm that cold conditioning of "seed" bulbs before planting changes the accumulation and composition of anthocyanins in the cataphylls of garlic. This is the first report to demonstrate this effect in garlic cataphylls. These results of anthocyanin biosynthesis by low temperature add to those previously reported in other plants such as grape skin and winter oilseed.[26,30,34]

Changes in Gene Expression Associated with Phenolic and Anthocyanin Pathways. The differential hybridization of plasmid DNA between samples conditioned at 5 °C and room temperature generated 85 clones corresponding to 19 overexpressed genes for samples conditioned at 5 °C, while the other 66 genes remained without significant expression changes (data not shown). Table 4 shows the expression levels of those putative genes involved in the biosynthesis of anthocyanins and other phenolic compounds that were identified among the insert fragments obtained in the library.

Table 4. Overexpressed Garlic Genes with Putative Phenolic Biosynthesis Activity in Garlic Sprouts Conditioned at 5 °C Compared with Those Conditioned at Room Temperature

plant gene homology (putative)	accession number	*e* value	identity	arbitrary overexpressed value[a]
phenylalanine ammonia lyase (PAL)	ADO24189.1	0.078	85	5.9
cinnamate 4-hydroxylase (C4H)	ADO24190.1	0.018	50	1.7
4-coumarate-CoA ligase (4CL)	AEM44785.1	0.360	66	1.1
UDP-sugar:flavonoid 3-*O*-glycosyltransferase (UFGT)	AAP88405.1	0.012	54	0.8
sucrose:sucrose 1-fructosyltransferase (1-SST)	AAM21931.1	0.180	42	3.3

[a]Times of change based on an arbitrary value of "0" for the expression level of samples conditioned at room temperature.

This study points out that during cold conditioning there was greater genetic activity that was not visually expressed during early development of the garlic plant and that the changes associated with that activity could be observed during the development and at the final stage of plant development, when the formation and differentiation of the garlic bulb occurred.

The expression pattern showed that putative PAL genes were most affected by the cold conditioning. A similar effect for the putative genes that encode cinnamate 4-hydroxylase (C4H) and 4-coumarate-CoA ligase (4CL) enzymes was observed. These three enzymes belong to the phenylpropanoid pathway, which is the first part of the biosynthesis of phenolic compounds, where conversion of phenylalanine into cinnamic acid takes place with subsequent conversion into 4-coumaric acid and 4-coumaroyl-CoA, which results finally in lignin formation and flavonoid and/or anthocyanin biosynthesis.[12,35]

The accumulation of anthocyanins and the increase in PAL gene expression due to low temperatures is associated with the time of exposure and has already been reported in other species.[13,36] In the present study, we report that 5 °C conditioning treatments for 16 days (data not shown) did not affect plant growth or the color of the harvested bulbs, in constrast to the 5 week 5 °C conditioning treatment that did induce those effects associated with the changes in gene expression discussed above, confirming the need of a time window of exposure.

The enhanced expression of the PAL, C4H, 4CL, and UFGT genes suggests that there was an early signal that induced the biosynthesis of phenolics and anthocyanins in the tissues. There is evidence that low temperatures or heat stress increase the activity of PAL and the synthesis of phenolic compounds.[37−39] For example, the breaking of dormancy in onions is associated with changes of phenolic compounds and oligosaccharides promoted by storage at 0 and 9 °C.[40] It is important to note that in the present study the induction of phenolic and anthocyanin biosynthesis likely began in the sprout, with their expression ending during the development of the plant.

Our results differ from previous studies in which low gene expression was reported in garlic seed cloves.[14] In those studies, the bulb was not conditioned at low temperature, nor were the sprouts of individual seed cloves analyzed. These differences are key since the sprout is the structure of high multiplying activity and gene expression that will generate the new plant and thus very likely is the site of major sensitivity to abiotic stress factors such as temperature.

In summary, our findings indicate that the conditioning of "seed" cloves of garlic at 5 °C before planting increased the amount of anthocyanins and total phenolic content of garlic bulbs during plant development until harvest time. Furthermore, the conditioning allowed an early harvest time in the field of about 6 weeks earlier than RT-conditioned samples. Five different anthocyanins were identified in the outer scale leaves or cataphylls of garlic cv. 'Coreano' that are derivatives of cyanidin-3-*O*-glucoside, two of them reported in garlic for the first time. All of them were present in higher concentrations in samples from 5 °C conditioning treatments relatvie to RT conditioning. The conditioning of seed cloves at 5 °C increased the biosynthesis of anthocyanins that began in the sprouts with a high number of transcripts of putative genes encoding enzymes of the anthocyanin biosynthetic pathway (phenylalanine ammonia lyase, cinnamate 4-hydroxylase, 4-coumarate-CoA ligase, and UDP glucose:flavonoid-3-*O*-glucosyl transferase) in comparison with RT samples. These results confirm

that low-temperature conditioning of "seed" cloves stimulates anthocyanin biosynthesis during development of the garlic plant. Currently, RNA-Seq and proteomic strategies are being studied under both temperature conditioning treatments to elucidate the metabolic mechanisms involved in the synthesis of these compounds in garlic.

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Notes

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ABBREVIATIONS USED

1-SST, sucrose:sucrose 1-fructosyltransferase; 4CL, 4-coumarate-CoA ligase; C3G, cyanidin-3-*O*-glucoside; C4H, cinnamate 4-hydroxylase; cDNA, complementary DNA; DAS, days after sowing; ESI, electrospray ionization; GAE, gallic acid equivalents; LC-MS/MS, liquid chromatography−mass spectrometry/mass spectrometry; NCBI, National Center for Biotechnology Information; PAL, phenylalanine ammonia lyase; RNA-Seq, RNA sequencing; ROS, reactive oxygen species; RT, room temperature; UFGT, UDP-sugar:flavonoid 3-*O*-glycosyltransferase.

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