ResearchGate

See discussions, stats, and author profiles for this publication at: https://www.researchgate.net/publication/236944903

Molecular cloning and characterization of Polygalacturonase-Inhibiting Protein and Cinnamoyl-Coa...

Article · June 2013

DOI: 10.1016/j.jgeb.2013.04.003

CITATIONS
0

READS
41

3 authors, including:

Basel Khraiwesh
New York University Abu Dhabi
36 PUBLICATIONS **786** CITATIONS
SEE PROFILE

Jamil Harb
Birzeit University
40 PUBLICATIONS **151** CITATIONS
SEE PROFILE

Some of the authors of this publication are also working on these related projects:

Organic Farming in Palestine View project

All content following this page was uploaded by Basel Khraiwesh on 05 January 2017.

The user has requested enhancement of the downloaded file.

Journal of Genetic Engineering and Biotechnology (2013) xxx, xxx–xxx

Academy of Scientific Research & Technology and National Research Center, Egypt

Journal of Genetic Engineering and Biotechnology

www.elsevier.com/locate/jgeb

ARTICLE

Molecular cloning and characterization of Polygalacturonase-Inhibiting Protein and Cinnamoyl-Coa Reductase genes and their association with fruit storage conditions in blueberry (*Vaccinium corymbosum*)

Basel khraiwesh [a,*], **Jamil Harb** [b], **Enas Qudeimat** [a]

[a] *Center for Desert Agriculture, Division of Biological and Environmental Sciences and Engineering, King Abdullah University of Science and Technology, Thuwal 23955-6900, Saudi Arabia*
[b] *Department of Biology and Biochemistry, Birzeit University, P.O. Box 7, Birzeit, West Bank, Palestine*

Received 6 January 2013; revised 27 March 2013; accepted 16 April 2013

KEYWORDS

Blueberry
Polygalacturonase-Inhibiting Protein
Cinnamoyl-Coa Reductase
Gene expression
Fruit storage

Abstract Blueberry is a widely grown and easily perishable fruit crop. An efficient post-harvest handling is critical, and for that purpose gene technology methods have been part of ongoing programmes to improve crops with high food values such as blueberry. Here we report the isolation, cloning, characterization and differential expression levels of two cDNAs encoding Polygalacturonase-Inhibitor Protein (*PGIP*) and Cinnamoyl-Coa Reductase (*CCR*) from blueberry fruits in relation to various storage conditions. The open reading frame of *PGIP* and *CCR* encodes a polypeptide of 329 and 347 amino acids, respectively. To assess changes in the expression of blueberry *PGIP* and *CCR* after harvest, a storage trial was initiated. The northern blots hybridization showed a clear differential expression level of *PGIP* and *CCR* between freshly harvested and stored fruits as well as between fruits stored under various storage conditions. Although the prospects of exploiting such a strategy for crop improvement are limited, the results provide further insight into the control of the quality over the storage period at the molecular level.

© 2013 Production and hosting by Elsevier B.V. on behalf of Academy of Scientific Research & Technology.

* Corresponding author. Tel.: +966 (2) 808 2923; fax: +966 (2) 802 0103.
E-mail address: Basel.khraiwesh@gmail.com (B. khraiwesh).

Peer review under responsibility of National Research Center, Egypt.

Production and hosting by Elsevier

1. Introduction

Blueberries are members of the genus *Vaccinium* and belong to the Rhododendron family (*Ericaceae*). The production of blueberries had increased during the last decade from around 256 hundred thousand tones to reach 356 hundred thousand tones

1687-157X © 2013 Production and hosting by Elsevier B.V. on behalf of Academy of Scientific Research & Technology.
http://dx.doi.org/10.1016/j.jgeb.2013.04.003

worldwide [16].This increase corresponds to 69% increase in the area harvested. The *Vaccinium* genus contains several species of economic importance e.g. the highbush blueberry (*Vaccinium corymbosum*) and the lowbush blueberry (*Vaccinium angustifolium*).

A breakthrough in value-added marketing came in the late 1990s, when scientific research indicated special health benefits associated with blueberry consumption [16,33]. More farmers are looking at marketing blueberries as a healthy "functional" food that contains flavonoids, vitamin C, anthocyanins, and phenolic acids [19]. Scientists have found blueberries to contain high levels of resveratol which they believe can reduce the risk of heart disease.

Since blueberries are easily perishable crops efficient post-harvest handling is critical. Storage of blueberry fruits can negatively affect the fruit quality due to the development of physiological disorders. Recently Harb et al. [19,20] showed reduction in levels of blueberry monodehydroascorbate reductase (MDAR) gene, which is the enzymatic component involved in the regeneration of reduced ascorbate, upon storage of fruits under various conditions. Additionally, in the same study the antioxidative capacity of water soluble antioxidants (ACW) had been reduced upon storage of fruits. One way to overcome quality loss over the period of storage and to improve crop post-harvest conditions is to apply gene technology.

In the world of post-harvest biology, plant cell wall is an important aspect to study. During plant growth and development, the composition of cell walls changes considerably, and these changes are caused to a great extent by the activity of hydrolytic enzymes, including the pectolytic ones. Plant cell wall Polygalacturonase-Inhibitor Protein (*PGIP*) gene has attracted the attention of many researchers; because PGIP plays a key role in processes important for both plants themselves and humans using plant products. PGIP is involved in the transformations of pectin substances during the growth of plants and ripening of fruits [6]. It is also believed to be one of the factors of plant tolerance towards fungal diseases, which hinders fungal penetration into the plant by inhibiting the fungal endo-PGIP proteins [2,7]. PGIP was found virtually in all plant organs and tissues. It was isolated from suspension-cultured plant cells as well as from the callus tissue [11]. Various plant tissues differ in their PGIP activity, and its changes depend on the developmental stage and environmental effects [17]. In particular, this activity changes during fruit maturation like pear [1]. However, the role of PGIP in plants is not clearly understood, and its activity in blueberries is not yet investigated.

Lignin is a phenolic cell wall polymer closely linked to cellulose and hemicelluloses, and is, second to cellulose, the most abundant biopolymer on earth [5,9]. In the past decade, cloning and characterization of genes involved in lignin biosynthesis and modification of lignin content and composition in plants have provided new insight into the lignin biosynthesis pathway [5]. Two known effectors involved in the biosynthesis of monolignols and the production of reactive oxygen species (ROS) are Cinnamoyl-CoA reductase (CCR) and NADPH oxidase, respectively [14,22]. Both are being associated therefore with lignification. CCR is responsible for the CoA ester conversion into aldehyde in monolignol biosynthesis, which diverts phenylpropanoid-derived metabolites into the biosynthesis of lignin. The cloning of a cDNA encoding CCR in eucalyptus [14] has allowed the investigation of CCR downregulation on lignin profiles in tobacco [21]. It was shown that downregulation of CCR activity exhibited a strong reduction in lignin content together with altered development (reduced size, abnormal morphology of the leaves, collapsed vessels) [21]. CCR cDNAs also have been isolated from maize [29] and wheat [26]. Downregulation of CCR in tomato induces dramatic changes in soluble phenolic pools [40]. There have been only few reports on cloning lignin genes from fruit crops. Due to the role that CCR plays in lignin composition, it will be interesting to study how this gene is expressed in response to different storage conditions.

Little is known about fruit gene expression in response to post-harvest storage conditions, analysis of gene expression changes associated with cell wall can be useful in defining the cellular processes that affect blueberry quality during post-harvest. To address this issue cDNAs of blueberry encoding *PGIP* and *CCR* were isolated, and differential expression levels of *PGIP* and *CCR* were studied in blueberry fruits stored under various post-harvest conditions.

2. Results and discussion

2.1. Isolation and characterization of PGIP gene in blueberry

Comparison of mRNA pools of freshly harvested blueberry fruits and stored fruits under various storage conditions by differential display reverse transcription-polymerase chain reaction (DDRT-PCR) facilitated the identification of a cell wall gene induced upon storage treatments: 6% CO_2 + 18% O_2 and 18% CO_2 + 18% O_2 (Fig. 1A). One arbitrary primer gave rise to a cDNA fragment derived from the cDNA pool of blueberry fruits at harvest and treated fruits with 6% CO_2 + 18% O_2 and 18% CO_2 + 18% O_2, which was absent in the RT-PCR reaction using the cDNA from blueberry fruits treated

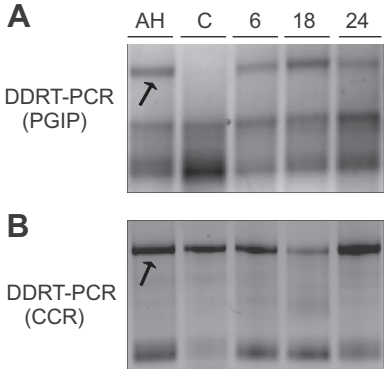

Figure 1 Identification of induced gene expression via differential display reverse transcription-polymerase chain reaction (DDRT-PCR). DDRT-PCR was performed using the GeneFishing DEG Kit, with total RNA derived from the blueberry fruits: At harvest (AH), cold storage (C), 6% CO_2 + 18% O_2 (6), 18% CO_2 + 18% O_2 (18), 24% CO_2 + 18% O_2 (24). (A) The *VcPGIP* cDNA fragment was amplified with the provided arbitrary primer ACP/dTACP1 (arrow indicates a *VcPGIP* band). (B) The *VcCCR* cDNA fragment was amplified with the provided arbitrary primer ACP/dTACP2 (arrow indicates a *VcCCR* band).

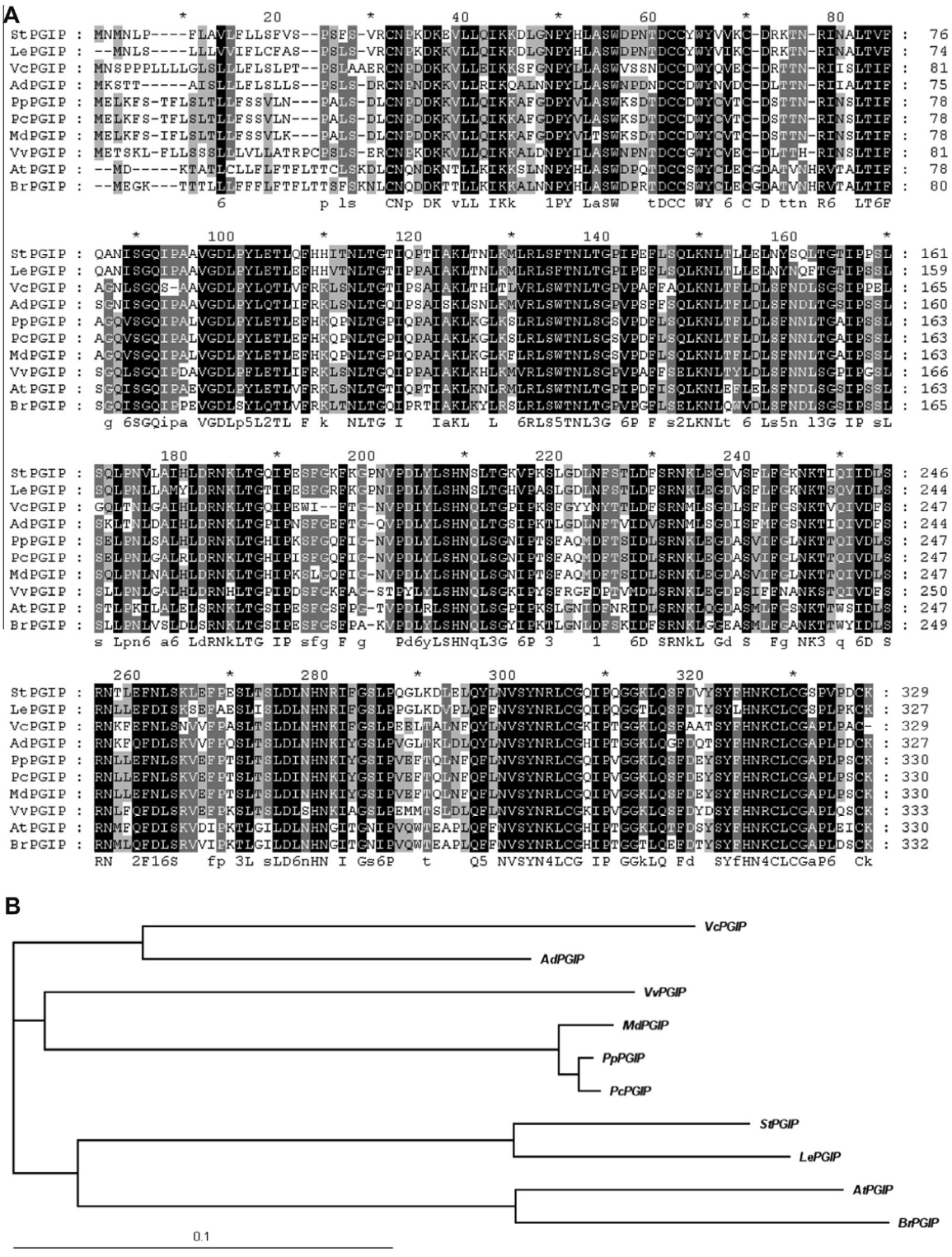

Figure 2 Analysis of the Polygalacturonase-Inhibiting Protein (VcPGIP). (A) Multiple protein sequence alignment of VcPGIP homologues from plants (B) Neighbour-joining tree showing the phylogenetic relationships between plant PGIP proteins. Species abbreviations are Ad (*Actinidia deliciosa*), At (*Arabidopsis thaliana*), Br (*Brassica rapa*), Le (*Lycopersicon esculentum*), Md (*Malus domestica*), Pc (*Pyrus communis*), Pp (*Pyrus pyrifolia*), St (*Solanum torvum*), Vc (*Vaccinium corymbosum*) and Vv (*Vitis vinifera*).

with cold storage and 24% CO_2 + 18% O_2 (Fig. 1A). The PCR fragment was cloned, sequenced and used for the identification of a 990-bp full-length cDNA sequence, containing an open reading frame coding for a protein of 329 amino acids, with a predicted molecular mass of 36.26 kDa and termed as VcPGIP (accession number FJ347133). BLAST searches with

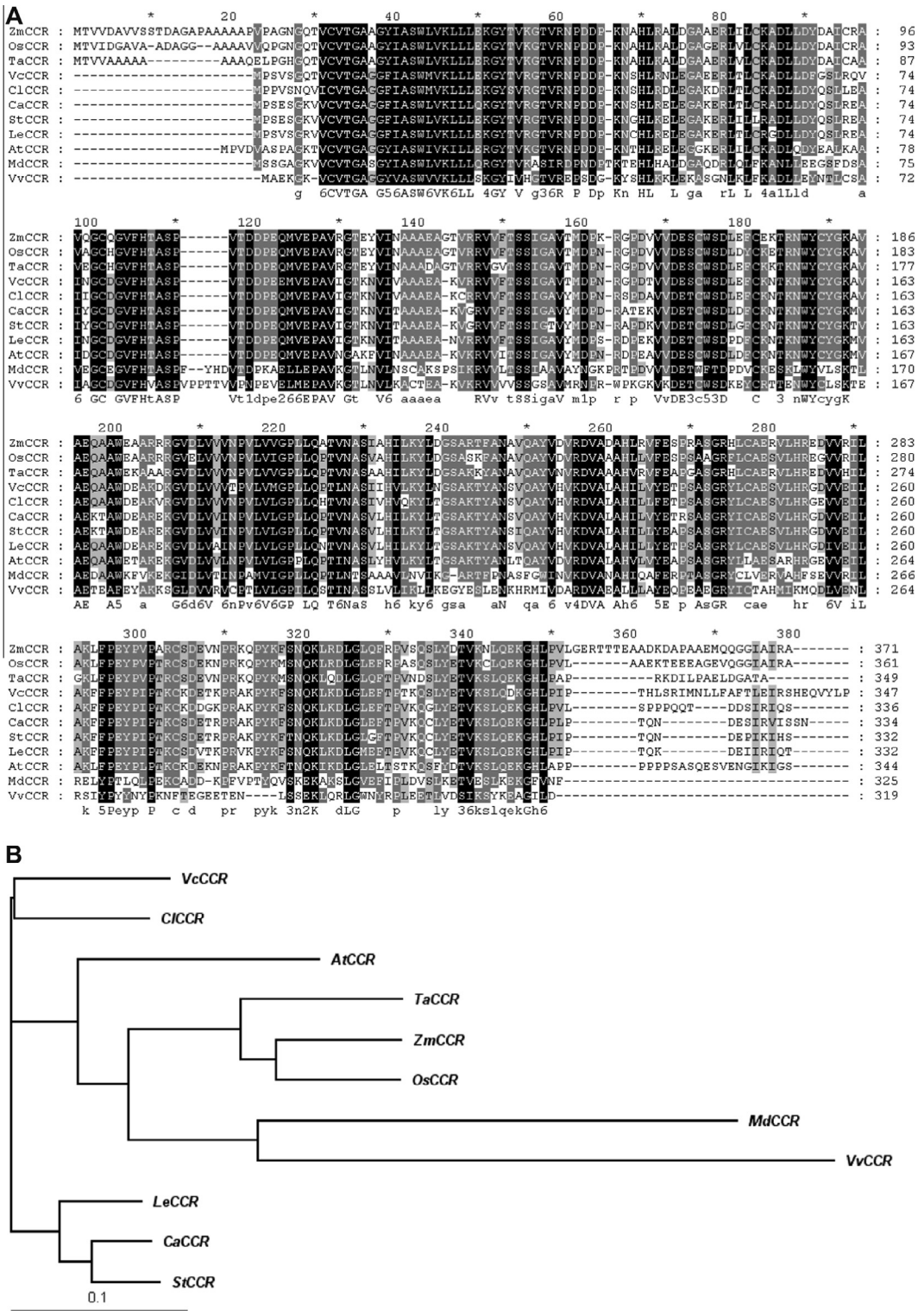

Figure 3 Analysis of the Cinnamoyl-Coa Reductase (VcCCR). (A) Multiple protein sequence alignment of VcCCR homologues from plants (B) Neighbour-joining tree showing the phylogenetic relationships between plant CCR proteins. Species abbreviations are At (*Arabidopsis thaliana*), Ca (*Capsicum annuum*), Cl (*Codonopsis lanceolata*), Le (*Lycopersicon esculentum*), Md (*Malus domestica*), Os (*Oryza sativa*), Ta (*Triticum aestivum*), St (*Solanum torvum*), Vc (*Vaccinium corymbosum*), Vv (*Vitis vinifera*) and Zm (*Zea mays*).

the predicted amino acid sequence revealed homology to proteins from other plant species. Prediction of protein domains in the Pfam database [4] revealed the existence of all functional domains present in the plant PGIP proteins. Most important family is leucine rich repeat N-terminal domain. These are short sequence motifs present in a number of proteins with

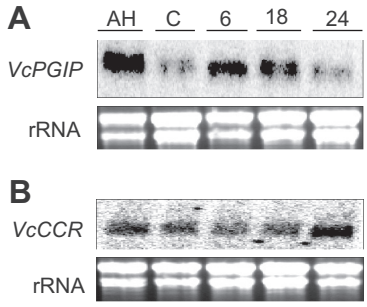

Figure 4 Analysis of *VcPGIP* and *VcCCR* gene expression in blueberry. (A) RNA gel blot from the blueberry plant treatments: at harvest (AH), cold storage (C), 6% CO_2 + 18% O_2 (6), 18% CO_2 + 18% O_2 (18), 24% CO_2 + 18% O_2 (24). (Upper) *VcPGIP* hybridization signals. (Lower) Ethidium bromide-stained rRNA bands (loading control). (B) RNA gel blot from the blueberry plant treatments: At harvest (AH), cold storage (C), 6% CO_2 + 18% O_2 (6), 18% CO_2 + 18% O_2 (18), 24% CO_2 + 18% O_2 (24). (Upper) *VcCCR* hybridization signals. (Lower) Ethidium bromide-stained rRNA bands (loading control).

diverse functions and cellular locations. Leucine rich repeats are often flanked by cysteine rich domains. This domain is often found at the N-terminus of tandem leucine rich repeats [23]. However, these sequence motifs have been found to be involved mainly in protein–protein or protein–ligand interactions. An alignment of homologous proteins was performed using CLUSTALW multiple sequence alignment program [38] as shown in Fig. 2A followed by neighbour-joining tree showing the phylogenetic relationships between plant PGIP proteins (Fig. 2B).

This clone showed 77%, 69%, 69%, 69%, 69%, 68%, 64% and 61% identities at the amino acid sequence level with those from *Actinidia deliciosa* [32], *Malus domestica* [41], *Pyrus pyrifolia* [15], *Pyrus communis* [35], *Vitis vinifera*, *Solanum torvum*, *Lycopersicon esculentum* [36] and *Brassica rapa*, respectively, suggesting that the enzyme is highly conserved between various plant species. Other *PGIP* genes cloned from various plant species have become available in the databases.

Table 1 Changes in antioxidative capacity of the water soluble substances (ACW) of blueberries under various conditions (% CO_2 + % O_2) at 1 ± 0.5 °C.

% CO_2 + % O_2	Storage periods	
	3 weeks	6 weeks
00 + 18	4.55 abc*	3.21 a
06 + 18	4.67 ab	3.53 a
18 + 18	4.58 abc	3.40 a
24 + 18	3.79 d	3.12 a
06 + 02	3.64 d	3.52 a
18 + 02	4.91 a	3.11 a
24 + 02	4.64 ab	2.70 a

* Mean values in the same column that are not followed by the same letter are significantly different using Duncan's Multiple Range Test at 5%.

2.2. Isolation and characterization of CCR gene in blueberry

A comparison of mRNA pools of freshly harvested blueberry fruits and stored fruits under various storage conditions by DDRT-PCR facilitated the identification of another cell wall gene induced in the freshly harvested blueberry fruits and fruits stored at 24% CO_2 + 18% O_2 compared to fruits stored at other storage conditions (Fig. 1B). A cDNA fragment derived from the cDNA pool of freshly harvested blueberry fruits was cloned and sequenced. The full-length of the identified cDNA sequence comprises 1044 bp and contains an open reading frame coding for a protein of 347 amino acids. BLAST searches with the identified amino acid sequence revealed homology to proteins from other plant species. With a predicted molecular mass of 38.26 kDa, the identified protein is termed as VcCCR (accession number FJ197338). Prediction of protein domains in the Pfam database [4] revealed the existence of all functional domains present in the plant CCR proteins. Most important family is NAD-dependent epimerase–dehydratase family. This family of proteins utilizes NAD^+ as a cofactor by which nucleotide-sugar substrates are being used for a variety of chemical reactions [37].

An alignment of homologous proteins from different plant species performed with the CLUSTALW multiple sequence alignment program [38] is shown in Fig. 3A followed by neighbour-joining tree showing the phylogenetic relationships between plant CCR proteins (Fig. 3B). This clone showed 85%, 85%, 84%, 84%, 75%, 75% and 69% identities at the amino acid sequence level with those from *Codonopsis lanceolata*, *Capsicum annuum* [28], *L. esculentum* [40], *Solanum tuberosum* [24], *Zea mays* [29], *Triticum aestivum* [26] and *Arabidopsis thaliana* [18,25], respectively, suggesting that the enzyme is highly conserved between various plant species. Other CCR genes cloned from various plant species have become available in the databases.

2.3. Molecular analysis

2.3.1. Expression level of PGIP in relation to fruit storage

PGIPs are encoded by small gene families in most plant species studied. PGIP belongs to a group of proteins containing leucine rich repeats, with diverse function and cellular location [23]. As we cloned and sequenced *VcPGIP* gene, we next analysed its transcript level by RNA gel blot. *VcPGIP* transcript level varied in freshly harvested blueberry fruits, and stored fruits under various storage conditions. Relatively high *VcPGIP* transcript level was detected in blueberry fruits at harvest and fruits stored at 6% CO_2 + 18% O_2 and 18% CO_2 + 18% O_2 (Fig 4A). However, no increase in the amount of *VcPGIP* transcript in blueberry fruits stored at cold storage and 24% CO_2 + 18% O_2 was observed (Fig. 4A). Genes encoding *PGIPs* have been isolated from bean [39], soybean [17], pear [35], tomato [36] and kiwifruit [32]. In pear and tomato fruits, it has been reported that *PGIP* genes were constitutively expressed and were not induced by wounding or pathogen challenge [30]. Nevertheless, tomato fruit from transgenic plants overexpressing the pear *PGIP* showed higher resistance to *B. cinerea* infection [30]. Variations in the *PGIP* transcript levels in fruits at different

developmental stages were reproducibly observed [41]. It has been demonstrated that *PGIP* gene products are accumulated in bean in response to wounding, elicitors and fungal infection [13]. Transcriptional activation of *PGIP* in fruit tissue can be induced by several environmental stresses such as percentage of CO_2 and O_2, mechanical wounding and fungal infection. In some cases, such as fruit ripening and pathogen penetration, a decrease in the PGIP activity brings about maceration of cell plates and softening of tissues, and impairment of the cell wall integrity which suggests the role of PGIP in preventing the penetration of some pathogenic microorganisms in tissues. A decrease in the PGIP activity enhances pectin hydrolysis and suppresses synthetic processes. These results suggest that plant PGIP may have multiple functions both in normal plant development and in response to biotic and abiotic stresses. The presence of multiple *PGIP* genes in plants such as bean, tomato and pear has been reported [35,36,12].

2.3.2. Expression level of CCR in relation to fruit storage

To investigate the spatial expression of *VcCCR* mRNA, Northern analysis was carried out with RNA extracted from the blueberry fruit treatments: At harvest, cold storage, 6% CO_2 + 18% O_2, 18% CO_2 + 18% O_2, 24% CO_2 + 18% O_2. Analysis of Northern blot qualitatively indicated that *VcCCR* was expressed at low levels in blueberry fruits stored at 6% CO_2 + 18% O_2, 18% CO_2 + 18% O_2 and cold storage (Fig. 4B). Whereas, a high *VcCCR* transcript level was detected in freshly harvested blueberry fruits and fruits stored at 24% CO_2 + 18% O_2 (Fig. 4B). Characterization of CCRs in plants has aroused great interests in improving lignin content through genetic engineering. When tobacco *CCR* is downregulated, all transgenic tobacco lines exhibited important changes in lignin content and composition and some contained unusual cell wall-bound phenolics. Lignin content was significantly decreased; almost 50% reduction was observed without an obvious alteration of plant development [27]. Arabidopsis plants transformed with a vector containing a full-length *AtCCR1* cDNA in an antisense orientation were obtained and characterized [18]. The most severely downregulated homozygous plants showed drastic alterations to their phenotypical features. These plants had a 50% decrease in lignin content accompanied by changes in lignin composition and structure, with incorporation of ferulic acid into the cell wall [18]. *AtCCR1* has higher substrate affinity and is preferentially expressed in tissues undergoing lignification. In contrast, *AtCCR2* is involved in the biosynthesis of phenolics and was transiently induced during pathogen resistance development [25]. As a result of success in decreasing lignin synthesis in transgenic tomato plants (CCR downregulated plants), total phenolic content and antioxidant capacity were markedly increased when compared with control plants [40]. This observation suggests that soluble phenolic compounds are accumulated in vegetative organs as a result of *CCR* downregulation. In agreement with *VcCCR* downregulation in blueberry fruits stored at 6% CO_2 + 18% O_2, 18% CO_2 + 18% O_2 and cold storage, the modification on the soluble phenolic content and antioxidant capacity will be increased in these fruits compared with freshly harvested blueberry fruits and fruits stored at 24% CO_2 + 18% O_2. Cloning and characterization of *VcCCR* will help to clarify how lignifications in blueberry are regulated under various post-harvest conditions and will provide a physical basis of optimal lignin, soluble phenolic and antioxidant contents in blueberry fruits in relation to post-harvest storage conditions.

2.4. Quantitative determination of antioxidative capacity of the water soluble compounds (ACW)

The level of ACW decreased under all storage conditions (Table 1). Extending the storage period for another 3 weeks resulted in a further significant loss in ACW, in particular with fruits stored under cold storage (1 ± 0.5 °C), and under the highest CO_2 level combined with high O_2 (24% CO_2 + 18% O_2). Decreasing O_2 levels did not result in preservation of ACW, although the low O_2 level (2%), combined with 6–12% CO_2 gave significantly better results than cold storage. The best results were obtained with increasing CO_2 levels up to 12%, but with the high O_2 level (18%). Correlations tests over the entire storage period revealed a negative correlation ($r = -0.5$) between O_2 level and ACW, but a slightly positive correlation ($r = +0.35$) between CO_2 and ACW. Correlation tests revealed also a negative correlation between CO_2 and ACW, either under high O_2 levels ($r = -0.28$) or under low O_2 levels ($r = -0.85$). Northern blot hybridization confirmed, although partially, the quantitative assessment of ACW (Fig. 4). The concentration of water soluble antioxidants in fruits decreased with prolonged storage, and irrespective of the specific impact of each storage condition. It is clear that increasing CO_2 and/or decreasing O_2 partial pressures within the storage atmosphere did not decisively change this loss. Results obtained by various researchers on the impact of various keeping conditions on water soluble antioxidants were contradictory. Connor et al. [10] found that the antioxidant activity of immature harvested blueberries cv. Elliott, demonstrated an increase in antioxidant activity, total phenolic and anthocyanin content during the first 3 weeks of storage. While Remberg et al. [31] found that the total antioxidant capacity decreased considerably during refrigerated storage as well as controlled atmosphere (10% O_2 + 10% CO_2) storage in blueberry cultivars 'Bluecrop', 'Hardyblue', 'Patriot', 'Putte' and 'Aron'. According to Stewart et al. [34], antioxidant capacity declined with prolonged storage, possibly due to O_2-promoted oxidation of the main antioxidants including anthocyanins and other phenolic compounds. This may explain the decline of water soluble antioxidants of cold-stored blueberries in our experiment.

3. Material and methods

3.1. Fruit materials and storage conditions

Blueberries (cv. 'Bluecrop') were obtained from a cooperative packing house in the Lake Constance area-Southwest Germany. Fruits were picked, selected for uniformity and were free of decay or external injuries. They were cooled to 1 ± 0.5 °C within 12 h, and stored in small CA chambers under the following conditions (% CO_2 + % O_2): 00 + 18 (cold storage), 06 + 18, 12 + 18, 18 + 18, 24 + 18, 06 + 02, 12 + 02, 18 + 02, and 24 + 02. These storage conditions were selected to combine the increasing concentrations of CO_2 (from 0% up to 24%) with high (18%) and low (2%) oxygen to assess the specific effects of each gas on storability and

quality changes, although under commercial conditions the storage conditions 12–18% CO_2 and 18% O_2 are widely adopted. However, for the molecular analysis, a number of treatments were reduced to include only storage conditions which have promising results, either for commercial usage or for understanding the metabolism of fruits under the most stressful CO_2 level. Moreover, blueberries were cooled within 12 h (overnight) to simulate the time needed for cooling under commercial conditions. The gas concentrations were measured by a paramagnetic O_2 (Magnos 3K) and an infrared CO_2 analyser (Uras 3G, both Hartmann & Braun, Germany) and regulated to set points by a computerized system using nitrogen from a N_2-separator, CO_2 from a gas cylinder, and O_2 from air.

3.2. RNA extraction

Total RNA was isolated using protocol developed by Chang et al. [8] with slight modifications. In brief, β-mercaptoethanol was added to final concentration of 2% (v/v) to the CTAB buffer and pre-warmed to 65 °C. 10 ml of the extraction buffer was added to 1 g finely ground tissue (peel and flesh combined) and incubated for 10 min at 65 °C. After centrifugation, precipitation, and purification steps were undertaken using chloroform:isoamyl alcohol (24:1), 5 M lithium chloride, and 70% ethanol, respectively. Total RNA in the extract was purified using RNeasy spin columns from QIAGEN GmbH (Hilden, Germany). Quality and quantity of RNA obtained were assessed on a 1% (w/v) agarose gel and according to A260:A280 ratios, respectively.

3.3. Molecular cloning

3.3.1. Genefishing assay

GeneFishing DEG Kit 101 (Seegene, http://www.seegene.com) was used to assess the differentially expressed genes. In brief, differential display reverse transcription PCR (DDRT-PCR) was performed with total RNA derived from the blueberry fruit treatments: at harvest, cold storage, 6% CO_2 + 18% O_2, 18% CO_2 + 18% O_2, 24% CO_2 + 18% O_2, respectively, and reverse transcribed using M-MLV reverse transcriptase enzyme (Fermentas, Germany). The blueberry *PGIP* and *CCR* cDNA fragments were amplified with the provided arbitrary primer dT-ACP1 and dT-ACP2, respectively. The cDNAs were subjected to two-stage PCR using the primers provided in the kit, and PCR products were further electrophoresed on 2% agarose gel containing EtBr. Bands that reflected differentially expressed genes were incised and DNA was extracted using QIAquick Gel extraction kit.

3.3.2. Cloning fragment products from Genefishing PCRs

Each extracted DNA fragments were cloned using the pJET1.2 cloning vector (Fermentas, Germany) and transformed into *Escherichia coli* competent cells. The culture was plated on LB plates supplemented with Ampicillin antibiotic and incubated overnight at 37 °C. The size of insert in the cultured cells was evaluated through colony-PCR. A broth of the selected colonies was incubated in liquid LB media containing Ampicillin antibiotic overnight at 37 °C. DNA was purified using the Wizard Plus SV Minipreps (DNA Purification System-Promega). The purified DNA was sequenced by GATC company (Germany). A *PGIP* and *CCR* full-length cDNAs were sequenced and identified by BLASTN searches [3].

3.3.3. Cloning of the blueberry PGIP and CCR cDNAs

cDNA clones comprising the 3' prime end of *PGIP* and *CCR* were sequenced, and subsequently the synthesis of 5'RACE-ready cDNAs was carried out according to Zhu et al. 2001 [42] using the BD Smart RACE cDNA Amplification Kit (Clontech, Germany). Subsequent PCR reactions were performed using the UPM Primer-Mix supplied with the Kit in combination with gene specific primers: 5'- CTTCCCGCACAGCCGATTATAGCTCA-3'derived from the target of PGIP mRNA and 5'-GAGTGGGTATTGGGAG GTGACCCTTGTC-3' derived from the target of CCR mRNA. The resulted full fragment products of PGIP and CCR were excised from the gel, cloned into the pJET1.2 cloning vector (Fermentas, Germany) and sequenced.

3.4. RNA gel blots and hybridization

20 μg of total RNA was mixed with an equal volume of RNA-denaturing buffer (500 μL deionized formamide, 120 μL formaldehyde (37%), 200 μL 10× MOPS (0.2 M MOPS, 20 mM sodium acetate, 10 mM EDTA, DEPC-H_2O, pH 7.0), 1 μL ethidium bromide) and incubated for 10 min at 65 °C. As a size marker the peqGOLD High Range RNA Ladder (PeqLab, Erlangen) was used. The RNA gels were blotted to Hybond-N^+ nylon membranes (GE Healthcare) using a Turbo blotter (Schleicher & Schuell) with 20× SSC. RNAs were fixed by UV cross-linking. Hybridizations were carried out with an [α-32P]dCTP labelled DNA probes derived from PGIP and CCR. DNA labelling was carried out with the Rediprime II Random Prime Labeling Kit (GE Healthcare). Pre-hybridizations were carried out at 67 °C for 4 h, subsequent hybridizations at 67 °C for overnight. Blots were washed three times with 0.5× SSC, 0.1% SDS and one time with 1× SSC, 0.1% SDS at 67 °C. Signals were detected using the Molecular Imager FX (Bio-Rad).

3.5. Multiple protein sequence alignment and phylogenetic tree construction

To define and extract the *VcPGIP* and *VcCCR* genes we screened the published plant PGIP and CCR proteins by BLAST searches [3] against a database containing all predicted proteins of the plant organisms. As queries, the known genes of the PGIP (accession number FJ347133) and CCR (accession number FJ197338) of the blueberry (*V. corymbosum*) were used. Multiple sequence alignment was performed using the European Bioinformatics Institute CLUSTALW server [38]. The phylogenetic tree was constructed using the MEGA2 program with the neighbour joining method.

3.6. Quantitative determination of antioxidative capacity of the water soluble compounds (ACW)

Determinations were carried out using the PHOTOCHEM system (Analytik Jena AG, Germany) with the PCL-method (method of photo-chemiluminescence). Here the photochemical excitation of radical forms is combined with luminometric detection. Standard kits from Analytik Jena AG were used to measure water soluble antioxidants. Homogenized fruit samples (in 3% HPO_3 solution; see above) were obtained and diluted with dd H_2O at a ratio of 1:100. Ten microliters of the

diluted sample was added to the reagents provided by the kit and readings were read by PHOTOCHEM system.

3.7. Statistical analysis

Results of the parameters above were subjected to analysis of variance (ANOVA) using the CoStat-software (CoHort Software, Monterey, CA, USA), and mean separations were calculated by Duncan's Multiple Range Test at $P \leqslant 0.05$.

4. Conclusions

In this study, we reported the isolation and characterization of *PGIP* and *CCR* cDNAs from blueberry. In addition, the results of this study indicate that molecular changes during fruit under various post-harvest storage conditions involve changes in the expression of genes associated with cell wall metabolism. Although the prospects of exploiting such a strategy for crop improvement are limited, the results provide further insight into the control of the quality over the storage period at the molecular level. Overall, results reported here provide an initial characterization of the *VcPGIP* and *VcCCR* expression activity of blueberry fruits under different post-harvest treatments.

Acknowledgements

We thank the German Academic Exchange Service (DAAD) for the financial support for J. Harb, and Spargelbauer Landerer, Meckenbeuren for the delivery of the fruit.

References

[1] A.A. Abu-Goukh, L.L. Strand, J.M. Labavitch, Physiol. Plant Pathol. 23 (1983) 101–109.
[2] P. Alghisi, F. Favaron, Eur. J. Plant Pathol. 101 (1995) 365–375.
[3] S.F. Altschul, T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller, D.J. Lipman, Nucleic Acids Res. 25 (1997) 3389–3402.
[4] A. Bateman, L. Coin, R. Durbin, R.D. Finn, V. Hollich, S. Griffiths-Jones, A. Khanna, M. Marshall, S. Moxon, E.L.L. Sonnhammer, D.J. Studholme, C. Yeats, S.R. Eddy, Nucleic Acids Res. 32 (2004) D138–141.
[5] M. Baucher, C. Halpin, M. Petit-Conil, W. Boerjan, Crit. Rev. Biochem. Mol. Biol. 38 (2003) 305–350.
[6] E.A. Bulantseva, N.T.E. Tkhang, N.L. Buza, A.A. Krinitsyna, M.A. Protsenko, Appl. Biochem. Microbiol. 41 (2005) 251–253.
[7] D. Cantu, A.R. Vicente, L.C. Greve, F.M. Dewey, A.B. Bennett, J.M. Labavitch, A.L.T. Powell, Proc. Nat. Acad. Sci. U.S.A. 105 (2008) 859–864.
[8] S. Chang, J. Puryear, J. Cairney, Plant Mol. Biol. Reporter 11 (1993) 113–116.
[9] C. Chapple, N. Carpita, Curr. Opin. Plant Biol. 1 (1998) 179–185.
[10] A.M. Connor, J.J. Luby, J.F. Hancock, S. Berkheimer, E.J. Hanson, Journal of Agricultural and Food Chemistry 50 (2002) 893–898.
[11] L. Degrá, G. Salvi, D. Mariotti, G. De Lorenzo, F. Cervone, J. Plant Physiol. 133 (1988) 364–366.
[12] A. Desiderio, B. Aracri, F. Leckie, B. Mattei, G. Salvi, H. Tigelaar, J.S.C. Van Roekel, D.C. Baulcombe, L.S. Melchers, G. De Lorenzo, F. Cervone, Mol. Plant Microbe Interact. 10 (1997) 852–860.
[13] A. Devoto, A.J. Clark, L. Nuss, F. Cervone, G.D. Lorenzo, Planta 202 (1997) 284–292.
[14] L. Eric, H. Simon, D. Jan Van, P. Joël, G. Deborah, P. Odile, M.B. Alain, G.-P. Jacqueline, Plant J. 11 (1997) 429–441.
[15] M. Faize, T. Sugiyama, L. Faize, H. Ishii, Physiol. Mol. Plant Pathol. 63 (2003) 319–327.
[16] FAOSTAT, Food and Agriculture Organization of the United Nations Crops, FAO, 2013, http://faostat3.fao.org, pp. Statistics.
[17] F. Favaron, R. D'Ovidio, E. Porceddu, P. Alghisi, Planta 195 (1994) 80–87.
[18] T. Goujon, V. Ferret, I. Mila, B. Pollet, K. Ruel, V. Burlat, J.-P. Joseleau, Y. Barrière, C. Lapierre, L. Jouanin, Planta 217 (2003) 218–228.
[19] J. Harb, B. Khraiwesh, J. Streif, R. Reski, W. Frank, Sci. Hortic. 125 (2010) 390–395.
[20] J. Harb, B. Khraiwesh, J. Streif, R. Reski, W. Frank, Acta Hortic. (ISHS) 858 (2010) 131–136.
[21] P. Joël, L. Catherine, M. Kate, O.c. Ann, S. Wolfgang, G.p. Jacqueline, M.B. Alain, Plant J. 13 (1998) 71–83.
[22] T. Kawasaki, H. Koita, T. Nakatsubo, K. Hasegawa, K. Wakabayashi, H. Takahashi, K. Umemura, T. Umezawa, K. Shimamoto, Proc. Nat. Acad. Sci. U.S.A. 103 (2006) 230–235.
[23] B. Kobe, J. Deisenhofer, Trends Biochem. Sci. 19 (1994) 415–421.
[24] K. Larsen, J. Plant Physiol. 161 (2004) 105–112.
[25] V. Lauvergeat, C. Lacomme, E. Lacombe, E. Lasserre, D. Roby, J. Grima-Pettenati, Phytochemistry 57 (2001) 1187–1195.
[26] Q.-H. Ma, J. Exp. Bot. 58 (2007) 2011–2021.
[27] C. Matthieu, B. Abdellah, L. Catherine, M.M. Jane, R. John, P. Michel, D. Saïda, H. Claire, G.-P. Jacqueline, B. Alain Michel, Plant J. 28 (2001) 257–270.
[28] M. Mazourek, A. Pujar, Y. Borovsky, I. Paran, L. Mueller, M.M. Jahn, Plant Physiol. 150 (2009) 1806–1821.
[29] M. Pichon, I. Courbou, M. Beckert, A.M. Boudet, J. Grima-Pettenati, Plant Mol. Biol. 38 (1998) 671–676.
[30] A.L. Powell, J. van Kan, A. ten Have, J. Visser, L.C. Greve, A.B. Bennett, J.M. Labavitch, Mol. Plant Microbe Interact. 13 (2000) 942–950.
[31] S. Remberg, K. Haffner, R. Blomhoff, Acta Hortic. (ISHS) 600 (2003) 595–598.
[32] C.G. Simpson, E. MacRae, R.C. Gardner, Plant Physiol. 108 (1995) 1748.
[33] Staff, Blueberry elixir reverses age-related symptoms, Agriculture Research (USDA), 2000, p. 23.
[34] D. Stewart, J. Oparka, C. Johnstone, P. Iannetta, H. Davies, Scottish Crop Research Institute, Invergowrie, Dundee, Scotland, 2000, pp. 119–124.
[35] H.U. Stotz, A.L.T. Powell, S.E. Damon, L.C. Greve, A.B. Bennett, J.M. Labavitch, Plant Physiol. 102 (1993) 133–138.
[36] H.U. Stotz, J.J.A. Contos, A.L.T. Powell, A.B. Bennett, J.M. Labavitch, Plant Mol. Biol. 25 (1994) 607–617.
[37] J.B. Thoden, A.D. Hegeman, G. Wesenberg, M.C. Chapeau, P.A. Frey, H.M. Holden, Biochemistry 36 (1997) 6294–6304.
[38] J.D. Thompson, D.G. Higgins, T.J. Gibson, Nucleic Acids Res. 22 (1994) 4673–4680.
[39] P. Toubart, A. Desiderio, G. Salvi, F. Cervone, L. Daroda, G. De Lorenzo, Plant J. 2 (1992) 367–373.
[40] B. van der Rest, S. Danoun, A.-M. Boudet, S.F. Rochange, J. Exp. Bot. 57 (2006) 1399–1411.
[41] C. Yao, W. Conway, R. Ren, D. Smith, G. Ross, C. Sams, Plant Mol. Biol. 39 (1999) 1231–1241.
[42] Y.Y. Zhu, E.M. Machleder, A. Chenchik, R. Li, P.D. Siebert, Biotechniques 30 (2001) 892–897.