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

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

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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.

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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 resveratrol 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](dx.doi.org/10.1016/j.jgeb.2013.04.003)
with cold storage and 24% CO2 + 18% O2 (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
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 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).
Analysis of PGIP and CCR genes in blueberry

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% CO2 + 18% O2 and 18% CO2 + 18% O2 (Fig. 4A). However, no increase in the amount of VcPGIP transcript in blueberry fruits stored at cold storage and 24% CO2 + 18% O2 was observed (Fig. 4A). Genes encoding PGIPs have been isolated from bean [39], soybean [17], pear [15], 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

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Table 1 Changes in antioxidative capacity of the water soluble substances (ACW) of blueberries under various conditions (% CO2 + % O2) at 1 ± 0.5 °C.

<table>
<thead>
<tr>
<th>% CO2 + % O2</th>
<th>Storage periods</th>
<th>3 weeks</th>
<th>6 weeks</th>
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<tr>
<td>00 + 18</td>
<td></td>
<td>4.55 abc</td>
<td>3.21 a</td>
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<tr>
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<td></td>
<td>4.67 ab</td>
<td>3.53 a</td>
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<td>4.58 abc</td>
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<td>4.91 a</td>
<td>3.11 a</td>
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<tr>
<td>24 + 02</td>
<td></td>
<td>4.64 ab</td>
<td>2.70 a</td>
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* 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%.

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% CO2 + 18% O2 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 C. lanceolata, Capsicum annum [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.

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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% CO2 + 18% O2 (6), 18% CO2 + 18% O2 (18), 24% CO2 + 18% O2 (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% CO2 + 18% O2 (6), 18% CO2 + 18% O2 (18), 24% CO2 + 18% O2 (24). (Upper) VcCCR hybridization signals. (Lower) Ethidium bromide-stained rRNA bands (loading control).

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dive 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 delicosa [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.

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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% CO2 + 18% O2 (6), 18% CO2 + 18% O2 (18), 24% CO2 + 18% O2 (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% CO2 + 18% O2 (6), 18% CO2 + 18% O2 (18), 24% CO2 + 18% O2 (24). (Upper) VcCCR hybridization signals. (Lower) Ethidium bromide-stained rRNA bands (loading control).
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 CO2 and O2, 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% CO2 + 18% O2, 18% CO2 + 18% O2, 24% CO2 + 18% O2. Analysis of Northern blot qualitatively indicated that VcCCR was expressed at low levels in blueberry fruits stored at 6% CO2 + 18% O2, 18% CO2 + 18% O2 and cold storage (Fig. 4B). Whereas, a high VcCCR transcript level was detected in freshly harvested blueberry fruits and fruits stored at 24% CO2 + 18% O2 (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% CO2 + 18% O2, 18% CO2 + 18% O2 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% CO2 + 18% O2. 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 CO2 level combined with high O2 (24% CO2 + 18% O2). Decreasing O2 levels did not result in preservation of ACW, although the low O2 level (2%), combined with 6–12% CO2 gave significantly better results than cold storage. The best results were obtained with increasing CO2 levels up to 12%, but with the high O2 level (18%). Correlations tests over the entire storage period revealed a negative correlation (r = −0.5) between O2 level and ACW, but a slightly positive correlation (r = +0.35) between CO2 and ACW. Correlation tests revealed also a negative correlation between CO2 and ACW, either under high O2 levels (r = −0.28) or under low O2 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 CO2 and/or decreasing O2 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% O2 + 10% CO2) storage in blueberry cultivars ‘Bluecrop’, ‘Hardyblue’, ‘Patriot’, ‘Putte’ and ‘Arón’. According to Stewart et al. [34], antioxidant capacity declined with prolonged storage, possibly due to O2-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 (% CO2 + % O2): 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 CO2 (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% CO₂ and 18% O₂ 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 CO₂ level. Moreover, blueberries were cooled within 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/isooamyl 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% CO₂, 18% CO₂, 18% O₂, 24% CO₂, and 18% O₂, 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-Prome ga). 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’- CTTCCCCGACACGGGATATTAGCTCA-3’ derived from the target of PGIP mRNA and 5’-GAGTGTTATTGGGAGTGACCTTGC-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 10x MOPS (0.2 M MOPS, 20 mM sodium acetate, 10 mM EDTA, DEPC-H₂O, 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 blottter (Schleicher & Schuell) with 20x 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 0.5x SSC, 0.1% SDS and one time with 1x 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% HPO₃ solution; see above) were obtained and diluted with dd H₂O 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 $P \leq 0.05$.

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 storage under various post-harvest 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.

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References

[33] Staff, Blueberry elixir reverses age-related symptoms, Agriculture Research (USDA), 2000, p. 23.