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Physiological and molecular changes associated with prevention of woolliness in peach following pre-harvest application of gibberellic acid

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ABSTRACT

Peach [*Prunus persica* (L.) Batsch, cv. Chiripá] fruit harvested from plants sprayed with gibberellic acid (GA_3), at the beginning (T1) and end of pit hardening (T2), kept under cold storage (CS) and controlled atmosphere (CA/CS), and from plants not sprayed with GA_3 (Control) and kept under CS, were evaluated in terms of fruit size and mass, ripening, occurrence of woolliness and expression of supposedly related genes and proteins. Peaches not sprayed with GA_3 and submitted to CS had a high incidence of woolliness, high mRNA abundance of vesicle transport genes and low mRNA abundance of genes associated with cell wall loosening, ethylene biosynthesis and heat shock proteins (*HSPs*). Early GA_3 spraying did not delay ripening but induced an increase in fruit size. In addition, it also induced a climacteric rise and prevented the occurrence of woolliness after CS. Woolliness prevention as a result of either GA_3 or CA/CS treatments resulted in higher abundance of mRNAs associated with cell wall metabolism, mitochondrial *HSPs* and 1-aminocyclopropane-1-carboxylic acid oxidase (*ACCO*). A unique GA_3 response consisted of a high mRNA abundance of genes and/or proteins such as *HSP40-1er*, *HSP40-2er*, *HSPCTR2*, β-mannosidase (*β-Man*) and α-L-arabinofuranosidase (*α-Ara*).

1. Introduction

Chiripá peach [*Prunus persica* (L.) Batsch], a late maturing cultivar grown in Southern Brazil, yields medium size white melting flesh fruit highly appreciated by consumers. The fruit is harvested in summer, from mid December through mid January, and requires cold storage (CS) to achieve an extended shelf-life. However, the occurrence of woolliness due to a prolonged period of CS is an important constraint for Chiripá peach growers (Rombaldi et al., 2002; Girardi et al., 2005). Woolliness occurs in almost all melting flesh cultivars of peach and nectarine [*P. persica* (L.) Batsch var. nucipersica (Suckow) C.K. Schneid], and the prevention of this disorder is essential in order to maintain their marketability (Lurie and Crisosto, 2005; González-Agüero et al., 2008). This chilling injury, characterized by loss of juiciness, has been associated with abnormal cell wall disassembly during ripening (Zhou et al., 2000; Brummell et al., 2004; Girardi et al., 2005; Lurie and Crisosto, 2005; González-Agüero et al., 2008). Normal peach fruit ripening involves a series of cell wall modifications and increased transcription of genes encoding proteins and enzymes associated with the functionality of the endomembrane system (Trainotti et al., 2003; González-Agüero et al., 2008). Recently, González-Agüero et al. (2008) identified a set of genes differentially expressed in juicy and woolly peaches, including genes putatively involved in intracellular trafficking and cell wall metabolism that were repressed in woolly fruit. In addition, Vizoso et al. (2009) found differential expression of genes associated with plastids, mitochondria, endoplasmic membrane and ribosomes when comparing woolly and juicy fruit. Ogundiwin et al. (2008) also found genes controlling chilling injury differentially expressed between juicy and woolly fruit. In woolly fruit, commonly stress-induced genes, ripening-related genes and genes involved in amino acid transport were up-regulated, while *HSPs* genes were down-regulated.

The use of controlled atmosphere (CA/CS), intermittent warming, or ethylene treatment during CS are effective measures in preventing woolliness in Chiripá peach (Girardi et al., 2005). However, in contrast to CA/CS, the use of intermittent warming or ethylene during storage results in a high incidence of fruit decay (50% and 25%, respectively) (Girardi et al., 2005). One strategy for extending the period of fruit availability and shelf-life is to

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delay the ripening process. For example, GA_3 has been shown to delay ripening in peach (Ju et al., 1999; Martinez-Romero et al., 2000; Amarante et al., 2005), nectarine (Zilkah et al., 1997), persimmon (*Diospyros kaki* Thunb.) (Ben-Arie et al., 1996; Ferri et al., 2004), tangerine (*Citrus reticulata* Blanco) (Marur et al., 1999) and strawberry (*Fragaria annanassa* Duch.) (Martínez et al., 1994) fruit. Responses to GA_3 action seem to vary according to the species and cultivar involved. GA_3 can induce hydrolytic cell wall enzymes, enhancing polysaccharide solubilization and favouring cell expansion (Thomas et al., 2005). GA_3 is also involved in the protection of the endomembrane system, regulating genes involved in its own biosynthetic pathway, or acting in cell wall disassembly (Hu et al., 2008). In other cases, GA_3 inhibits chlorophyll breakdown (Rosenvasser et al., 2006) and delays the onset of climacteric respiration (Ben-Arie et al., 1996) and the ripening cycle (Ferri et al., 2004). In addition, previous studies have indicated the stage of pit development as being pivotal in achieving physiological responses with the application of plant growth regulators such as GA_3 (Zilkah et al., 1997; Ju et al., 1999; Amarante et al., 2005).

The current study tested whether GA_3 application at a definite stage of pit development is effective in increasing fruit size and mass, delaying the ripening cycle, and preventing woolliness in Chiripá peaches. Moreover, in order to gain further understanding of the molecular mechanisms associated with these physiological changes, the relative mRNA abundance of genes putatively associated with cell wall metabolism, intracellular trafficking, heat shock proteins and ethylene synthesis, as well as the expression of a set of proteins involved in cell wall metabolism and ethylene, synthesis were investigated.

2. Materials and methods

2.1. Plant material and experimental design

A preliminary study (2006) was performed on a 6-year-old commercial peach [*P. persica* (L.) Batsch cv. Chiripá] orchard, planted in Farroupilha, Southern Brazil. In order to evaluate the effects of gibberellic acid (GA_3) on fruit growth and ripening, three replicates of twenty trees, selected based on size uniformity, were treated as follows: Control—without GA_3; T1—spraying 400 L ha^{-1} of a GA_3 solution [50 mg L^{-1} of GA_3 (Proggib®) and surfactant (0.05%, v/v) (Silwet®), pH 4.5] at the beginning of the pit hardening stage (45 days after anthesis, DAA); and T2—spraying 400 L ha^{-1} of the same GA_3 solution at the end of the pit hardening stage (75 DAA). A similar stage characterization has been applied in previous studies (Zilkah et al., 1997; Ju et al., 1999; Amarante et al., 2005). For each treatment, 84 kg of peach (12 boxes with 7 kg of fruit) were harvested when fruit colour was light green, corresponding to the pre-climacteric stage, between S3 I and S3 II, as described by Trainotti et al. (2003). Fruit were evaluated in terms of size (equatorial circumference), mass, skin colour, flesh firmness, soluble solids content (SSC), and ethylene production, and then cold stored at 1.0 °C (±1.0 C), 92.0% (±5.0%) relative humidity (RH) for 30 days. Finally, the fruit were exposed to 23.0 °C (±3.0 °C) and 75.0%(±5.0%) RH for 2 days then evaluated for woolliness occurrence. Fruit that upon hand squeezing did not release any juice were considered woolly. For a detailed description of each analysis refer to Girardi et al. (2005).

A second experiment was carried out in the following year (2007), in which Control (without GA_3) and T1 (GA_3 sprayed at the beginning of pit hardening) treatments were applied following the same experimental design on the same set of plants. At harvest, 168 kg of peach (24 boxes with 7 kg of fruit) from Control and 84 kg of peach (12 boxes with 7 kg of fruit) from T1 were collected. Half of the Control fruit were kept under CS (Control CS) and the other half under controlled atmosphere (Control CA/CS) at 2 kPa of O_2, 5 kPa of CO_2, 1.0 °C (±1.0 °C) and 92.0% (±5.0%) RH (same temperature and humidity used for CS), while T1 fruit were stored under CS only (T1 CS). After 30 days of storage under CS or CA/CS conditions, fruit were exposed to 23.0 °C (±3.0 °C) and 75%(±5%) RH, for 6 h, 2, 4 and 6 days and then evaluated for mRNA and protein abundance, ethylene production and occurrence of woolliness.

The experiments were performed in a completely randomized design. Percentage data was normalized according to the equation $f(x) = \arcsin \sqrt{X}$. ANOVA was performed using the *F*-test at the 5% significance level. Means of treatments were compared using Duncan's test at the 5% significance level. SANEST (Zonta and Machado, 1991) was used to perform statistical analyses.

2.2. Quantitative real-time PCR (q-PCR)

RNA was extracted from peach flesh following the protocol described for *PureLinK*™ reagent (*Plant RNA Reagent—Invitrogen*™). Total RNA was treated with DNAse I—*Invitrogen*™ and each sample was reverse-transcribed into cDNAs using the commercial kit *SuperScript First-Strand System for RT-PCR* (*Invitrogen*™). The quantity and quality of the RNA and cDNA was assessed spectroscopically and by electrophoresis in agarose gel.

Genes from peach, tomato (*Lycopersicon esculentum* Mill.) and arabidopsis [*Arabidopsis thaliana* (L.) Heynh.] putatively encoding proteins involved in cell wall metabolism, intracellular trafficking, heat shock proteins, and ethylene synthesis were selected based on previous work that showed an association between these metabolic functions and woolliness (Pratt and Toft, 2003; Trainotti et al., 2003, 2006; González-Agüero et al., 2008; Iwata et al., 2008; Mueller et al., 2008; Su and Li, 2008). Gene-specific primers were designed from sequences deposited in the GeneBank (Benson et al., 2005) using Vector NTI Advance™ 10 (Invitrogen, 2005). The criteria used for primer selection consisted of amplicon size between 100 and 150 bp, CG content between 40% and 60%, 3′ ends with less than two C and G bases in the last five nucleotides and melting temperature ranging from 60 to 65 °C according to Applied Biosystems® recommendations. The sizes of amplification products and their specificity were tested in agarose gels (2%, w/v) prior to q-PCR. Melting curves were evaluated and only primers giving single peaks were used (Table 1). q-PCR was performed with a 7500 Real-Time PCR System (Applied Biosystems®) using SYBR® Green. The amplification reaction was carried out in a total volume of 25 μL, containing 2 μM of each primer, 12.5 μL of PCR Master Mix SYBR® Green, 1 μL of cDNA (diluted 5-fold) and water to make up the final volume. Samples were loaded in 96 well optic plates (Applied Biosystems®) and covered with optic adhesives (Applied Biosystems®). Thermal cycle conditions were as follows: denaturing at 50 °C for 2 min and 95 °C for 10 min, followed by 40 three-step cycles (95 °C for 30 s, 57 °C for 1 min and 72 °C for 1 min) and final extension at 72 °C for 5 min. Relative quantification of each single gene expression was performed using the comparative threshold cycle method, as described by Livak and Schmittgen (2001). For each cDNA, *18S* was used as a reference gene to quantify cDNA abundance (at the same dilution as mentioned above). Threshold cycle (CT) was calculated based on the PCR exponential reaction obtained from the relative expression level (REL) formula, $REL = 2^{-\Delta\Delta CT}$. Results were expressed as mRNA abundance in a colour diagram using the *Multi Experiment Viewer* (TIGR MeV) software (Saeed et al., 2003). mRNA abundance of each gene from Control CS fruit at 6 h served as the baseline for determining relative RNA levels.

Table 1
Specific primers used for quantitative PCR analysis of target genes.

Genes	GI	Forward	Reverse
Ethylene synthesis			
1-Aminocyclopropane-1-carboxylate oxidase (*ACCO*)	33329719	GGATTGGGAGCTTGCTTGCAA	TTGCAAGCCCGTGAGATGAT
Cell wall metabolism			
Cobra (*Cob*)	22478530	ACTCATCCAGGAAGCTGTGTAG	ATGGCTGTATCATTTATTGTCGCA
Glucan Synthase (*GLS*)	22478796	TGGGAAACATGGTGGTATGAGGA	CGA GACATTTGAAGTGAGTGAAC
Galactosyl transferase (*GalT*)	22480478	ATGTGAAAAGTGGATGCGGAA TG	TTGGATGAGAAGCGGGAAGAGA
Cinnamoyl-CoA reductase (*CCR*)	22481300	ATCAAGTCCAAGACCCCGAGAA	CGCCCAACACGGTGCCAGGA
Pectate lyase (*PL*)	22483439	GCCTTGCCGTACGCTCATGTC	CTTCAGCCTCAACCCCTTCCCT
Endopolygalacturonase (*PG*)	110293962	AAAGGGTGCCCTGGTCAGGTAAGATA	GCTCTTCTAGGTGGAAGCCCAAGAAA
Pectin methylesterase (*PME*)	1213628	AGGTGGCCTCCATTCTCTCTCAGTT	GGGAAGCAGAGAGAGACCAGTTCAA
Beta-galactosidase (*β-Gal*)	157313305	CGTGTATGGGGCATTGGAGAA	CTCCCGCATTCCATGTCTCAA
Endo-1,4-beta-mannosidase (*β-Man*)	157313309	ACAAGGTTTTCCCATCCATGTTGAA	ATGGGTCAGAAGCCACATACATCAA
Alpha-L-arabinofuranosidase (*α-Ara*)	145338352	ACCTCCACTCCTCCTCTGTCTGATT	TTCTGAGCAACTTCCAAGACTCCAT
Expansin 1 (*Exp1*)	16305104	AAACGTTGGTGGTGCCGGTGAT	TTGCTTGCCAACCAGTCCTGGA
Exp2	29466640	TCCAGGACTGGTTGGCAAGCAA	TAGGACACCACTGTGCGGCCAT
Exp3	29466642	GGGTGCATGGGAAGCAGCTCAT	CCATGGTGCCAGAGGCATCAGA
Exp4	21901947	TGAGCTGTGGGGCATGCTATGA	AGGATCACACCAGCCACCTGGT
Endomembrane transport			
Golgi transport protein SFT2-like (*SFT2*)	22481199	CACTCA AAGGCCCGAAGAATCA	CAACGTCACCTCCCAAAACATC
SNARE-like protein (*Vap27-2*)	22481415	CCAAAGAAATACTGCGTGCGGC	GGAAGAGGGTGGGCTGATGAG
Dynamin-like protein1A (*ADL1A*)	22481576	GTGAACAAAATCCAAAGAGCTTG	GCCAGTTTCTCGATCTGTCTC
ROC7 cyclophilin (*ROC7*)	22481624	CCAGGCAAA GAAGTCAAAGGAG	TCACCTCCCTGAATCATGAAACT
Rab GTP-binding protein (*Rab5*)	22482805	CCATAGGTGCTGCCTTCTTCTC	CCATGTTTGGATTGCCTTGTGATT
Vesicle-associated membrane protein 722 (*Vamp*)	22483019	GCAAAGCAGGTGGTCTCAGG	TTAAGGCTATTGGCAGGGGCT
Kinesin (*Kin*)	51560894	ATCAAACGACCAAAGCGGGCTTA	AATTTCGCCACGAGAACCCACAT
Clathrin-binding protein b-adaptin (*Cla*)	22483877	CTTGGTGATCTGATTGGCATGG	ACTTGTGGAACCTGAAGGGGTC
Syntasin (*Syn*)	56162812	CTGTGCAAGCTACCTCCCACCTT	GATCACTGCTCAAGCCACCACAA
ER lumen protein retaining receptor 2 (*ERD2*)	22477673	GCCAGTATTTTGGTCCTCCTTC	TCTTGAAATGTGAATTCCTCGTG
RabGTP-binding protein (*Rab11*)	22484722	ATGTTTGTAGGTTATTAGTCGCTTA	CGCTCTTGACCAGTTGTATCCCA
Heat shock proteins (*HSPs*)			
HSP40-1 er resident (*HSP40-1er*)	186512089	TGCACCTCGCCTGCTTGAAGAT	TTGCGGGGTATAACGGCCATCT
HSP70er	240254046	CCGCGGTCCAAGGTGGAGTATT	TCAAAGGCGCAACATCAAGCAG
HSP40-2er	79318135	CGCCAGCAGCTTCTGCACACAA	TTCTCCGTCGAGCCACGCAAAT
HSP calnexin 1 (*HSPCNX1*)	145359541	TGTCCTCGTCGCCATTGTGGTT	CTTCTTTTCCACAGGTGCCCGCC
HSP calreticulin 2 (*HSPCRT2*)	145335312	TCTCCTCGCGGTATATATAAGC	AGACGAGGCTAGGAATCATTTT
HSP70 chloroplast resident (*HSP70ch*)	145359060	CCAACCTCCGCCTTCCTTCGTA	ACCAACGGTGTATCGGGAAGCG
HSP17.8ch	30680121	CCGAGTCACCAGCTTTGCTCAGAA	TGTTGTTGCCGAAGAAGCTTGGAA
HSP60 mitochondria resident (*HSP60mi*)	20466255	ACCTCGCCTCCAAGGCAAGGAT	TTCTGCTGGAAACCTGGCGAGC
HSP26.5mi	186490445	CAATGGCTCTAGCTCGTCTGGCTTT	TTCACCAGCCGAAGTAGCCATGAAT
HSP60-3Bmi	145338881	CCTCGCCTCCAAGGCAAGGATT	TTCTGCTGGAAACCTGGCGAGC
Endogenous control			
18S	66627320	AAAACGACTCTCGGCAACGGATA	ATGGTTCACGGGATTCTGCAATT

2.3. Protein immunodetection

Immunodetection of proteins was performed by Western blotting using mouse polyclonal antibodies produced against the recombinant proteins endopolygalacturonase (anti-PG, gi110293962), pectin methylesterase (anti-PME, gi1213628), β-galactosidase (anti-β-Gal, gi157313305), pectate lyase (anti-PL, gi22483439), endo-1,4-β-mannase (anti-β-Man, gi157313309) and α-arabinofuranosidase (anti-α-Ara, gi145338352) expressed by vector pAE (Ramos et al., 2004) and ACC oxidase (anti-*ACCO*, gi33329719) as previously described by Rombaldi et al. (1994). Frozen peach flesh (3.0 g) was ground into powder and extracted with a solution containing 1.5 mL of 280 mM Tris–HCl (pH 8.3), 0.5 M DTT, glycerol (20%, v/v) and SDS (4%, w/v). β-Mercaptoethanol (10%, v/v) was added just before heating the samples at 80 °C for 10 min. Samples were then cooled at 4 °C and centrifuged at 14,000 × *g* for 30 min. Protein quantification was performed according to Bradford's (1976) method following precipitation with trichloroacetic acid and solubilization with 0.1 M sodium hydroxide. Equal amounts of total protein (30 μg) were loaded per gel slot onto a denaturing, 1 mm thick, polyacrylamide gel (12%, w/v), according to Sambrook et al. (1989), and run for 30 min at 90 V followed by 1 h at 150 V. Proteins were transferred from the polyacrylamide gel to a nitrocellulose membrane (Hybond ECL, GE Healthcare®) using a gel blotter (Bio-Rad), at 4 °C (±2 °C). The efficacy of the transfer was monitored by colour using Ponceau's reagent. The membrane was washed with a Tris-buffered saline Tween solution [TBS-T, 20 mM Tris, 137 mM NaCl and Tween-20 (0.1%, v/v), pH 7.6] for 1 h at room temperature (RT) with agitation and blocked with ECL Advance Blocking® agent (2%, w/v) in TBS-T. Primary antibodies were diluted in TBS-T at 1:2000 (anti-β-Man and anti-α-Ara), 1:5000 (anti-PL, anti-β-Gal and anti-*ACCO*) and 1:10,000 (anti-PG and anti-PME) and incubated for 1 h at RT. Membranes were washed three times for 10 min with TBS-T and incubated for 1 h at RT with a 1:50,000 dilution of the peroxidase-labelled rabbit anti-mouse antibody (GE Healthcare®). Membranes were then washed with water and developed using the GE Healthcare® Kit (ECL Advance Western® blotting detection reagents), with an exposure time of about 10 s.

2.4. Preparation of antibodies

Total RNA was extracted from 50 mg of flesh from Chiripá peach at S1, S2, S3 and S4 ripening stages using Concert Plant RNA Reagent (Invitrogen, USA, catalogue # 12323-012) to produce polyclonal antibodies. The RNAs were mixed and 5 μg was reverse-transcribed using SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, USA, catalogue # 18080-051).

The first strand cDNA was amplified using forward primers including a *Bam*HI restriction site and reverse primers including a

Table 2
Characteristics of Chiripá peach fruit, immediately after harvest from trees sprayed with GA_3 at the beginning (T1) and end (T2) of pit hardening, and from untreated trees (Control)[a,b].

Treatment	Fruit equatorial circumference (cm)	Fruit mass (g)	Fruit colour (hue angle)	Flesh firmness (N)	Soluble solids content (%)	Ethylene production ($nL\,g^{-1}\,h^{-1}$)	Woolly fruit (%)
Control	19.14 b[c]	102.12 b	98.12 a	50.25 a	12.01 a	1.26 a	85.0 a
T1	26.80 a	150.14 a	102.01 a	48.14 a	11.82 a	1.09 a	12.3 b
T2	18.15 b	100.04 b	100.07 a	52.25 a	12.23 a	0.86 a	90.3 a

[a] The peaches were harvested when the fruit colour was light green, corresponding to the pre-climacteric stage, between S3 I and S3 II, described by Trainotti et al. (2003).
[b] Three replicates of ten trees, selected based on size uniformity, were treated as follows: Control—without GA_3; T1—spraying 400 L ha^{-1} of a GA_3 solution [50 mg L^{-1} of GA_3 (Proggib®) and 0.05% (v/v) of surfactant (Silwet®), pH 4.5] at the beginning of the pit hardening stage; T2—spraying 400 L ha^{-1} of the same GA_3 solution at the end of the pit hardening stage.
[c] Means of treatment followed by different letters are statistically different according to Duncan's test ($p \leq 0.05$). For the variates fruit equatorial circumference, fruit mass, fruit colour, flesh firmness, soluble solids content and woolly fruit, $n = 120$, and for the variate ethylene production, $n = 3$.

*Kpn*I restriction site and also the stop codons TGA and TCA. PCR conditions were: denaturing at 95 °C for 3 min, followed by 30 cycles at 95 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 90 s, followed by one cycle at 72 °C for 10 min. Fragments amplified by PCR were purified using GFX™ PCR DNA system and Gel Band Purification system (Amersham Bioscience®) and analyzed by electrophoreses in agarose gel (0.8%, w/w). The purified product was sequenced (Applied Biosystems Automated 3730 DNA Analyzer), cloned using the vector pCR2.1-TOPO-TA (Invitrogen®) and transfer ·ed to the expression vector pAE (Ramos et al., 2004). Each cloning was carried out in duplicate and resequenced prior to *Escherichia coli* BL21 pLyss transformation. Recombinant proteins were affinity-purified on a HisTrap column ÄKTA prime (Amersham Biosciences®) then inoculated into mouse to obtain the polyclonal antibodies. A solution containing 100 μg of each purified protein in 0.05 mL of sterile 200 mM phosphate buffer (pH 8.0), containing NaCl 0.5 M and imidazole 0.005 M was mixed with aluminium hydroxide (15%, v/w) prior to intramuscular injection. The second dose was given 21 days later. Serum was collected 63 days after the second injection and immunopurified against the respective recombinant proteins. The specificity and sensitivity of the antibodies was tested against recombinant proteins and total peach protein extracts from the S1, S2, S3 and S4 ripening stages. Antibodies recognized their recombinant protein and reacted specifically with one or few background bands in 30 μg of total peach proteins. Antibody recognition occurred at the same conditions of Western blot, 0.005 pg of recombinant PME, PG, β-Gal, PL, *ACCO* and 0.025 pg of β-Man and α-Ara protein.

3. Results

In order to extend the ripening cycle and increase fruit size, Chiripá peaches were treated with gibberellic acid (GA_3) at the beginning and end of the pit hardening stage. Pre-harvest GA_3 spray did not delay fruit ripening (Table 2). Fruit from all treatments reached the same ripening stage at the same time without showing any differences in colour (hue angle from 98.12 to 102.01), flesh firmness (ranging from 48.14 to 52.25 N), soluble solids content (ranging from 11.82% to 12.23%) or ethylene production. At harvest, fruit underwent pre-climacteric ripening equivalent to the S3 I or S3 II stage (Trainotti et al., 2003), with low ethylene production ranging from 0.86 to 1.26 $nL\,g^{-1}\,h^{-1}$. Pre-harvest spraying of GA_3 at the beginning of pit hardening (T1) resulted in a significant increase in fruit size and mass compared to untreated fruit (Control), leading to a 47% gain in productivity per plant. However, postponing GA_3 application to the end of pit hardening (T2) did not contribute to an increase in fruit size or mass.

Larger fruit, from the early GA_3 treatment (T1), had a lower incidence of woolliness when compared to untreated fruit (Control) or the late GA_3 treatment (T2) (Table 2). Incidence of woolliness in peach from the Control treatment (without GA_3) was high after 30 days of cold storage (CS), reaching 85% of the fruit after 2 days of ripening at room temperature. In contrast, fruit that received GA_3 treatment at the beginning of pit hardening (T1) and after 30 days of CS had lower levels of woolliness (12.3%). However, woolliness incidence in peaches treated with GA_3 at the end of pit hardening (T2) did not differ statistically from the untreated control.

Storage under controlled atmospheres (Control CA/CS), as expected, prevented woolliness (Table 3). Woolliness occurrence in GA_3-treated peach in cold storage (T1 CS) was as low as 29% and did not differ statistically from that in untreated peaches stored under CA/CS. The highest percentage of woolly fruit (94.2%) was observed in untreated peaches stored at low temperature during the second and fourth days of ripening. Upon extension of the ripening period, woolly fruit became juicy again.

Fruit from treatments with lower occurrence of woolliness (Control CA/CS and T1 CS) developed classical climacteric behaviour during ripening, while fruit with a high occurrence of woolliness (Control CS) did not develop climacteric production of ethylene (Table 4).

In order to assess the effects of pre-harvest GA_3 treatment and storage under CA/CS on woolliness prevention, mRNA abundance of a set of 36 genes associated with cell wall structure and disassembly (14 genes), intracellular transport (11 genes), heat shock proteins (10 genes) and ethylene biosynthesis (1 gene) were studied (Fig. 1).

T1 CS and Control CA/CS peaches had higher relative mRNA abundance of expansins (*Exp1*, *Exp2* and *Exp3*), *PME*, *PG*, *PL*, *β-Gal*, *β-Man* and heat shock proteins (*HSP40-1er*, *HSP40-2er*, *HSP70er*, *HSPCNX1*, *HSP17.8ch*, *HSP60mi*, *HSP60-3Bmi* and *HSP26.5mi*) than Control CS peaches.

Table 3
Woolliness incidence in Chiripá peach fruit, harvested from untreated trees (Control) and from trees sprayed with GA_3 at the beginning of pit hardening (T1), stored for 30 days, under cold storage (CS) or controlled atmosphere (CA/CS), and finally exposed to 23.0 °C (±3.0 °C) and 75.0%(±5.0%) RH for 6 h, 2, 4 and 6 days[a].

Treatment		Woolly fruit (%)			
		Period at 23.0 °C (±3.0 °C) and 75.0% (±5.0%) RH			
GA_3	Storage	6 h	2 days	4 days	6 days
Control	CS[b]	24.5 a[c]	94.2 a	94.2 a	48.5 a
Control	CA/CS[d]	0.0 b	23.5 b	23.2 b	0.0 c
T1	CS	0.0 b	28.6 b	9.5 b	19.8 b

[a] Peaches were harvested when the fruit colour was light green, corresponding to the pre-climacteric stage, between S3 I and S3 II, as described by Trainotti et al. (2003).
[b] Cold storage (CS): 1.0 °C (±1.0 °C), 92.0% (±5.0%) relative humidity (RH).
[c] Means of treatment ($n = 120$) in the same column, followed by different letters, are statistically different according to Duncan's test ($p \leq 0.05$).
[d] Controlled atmosphere (CA/CS): 2 kPa of O_2, and 5 kPa of CO_2, 1.0 °C (±1.0 °C), 92.0% (±5.0%) RH.

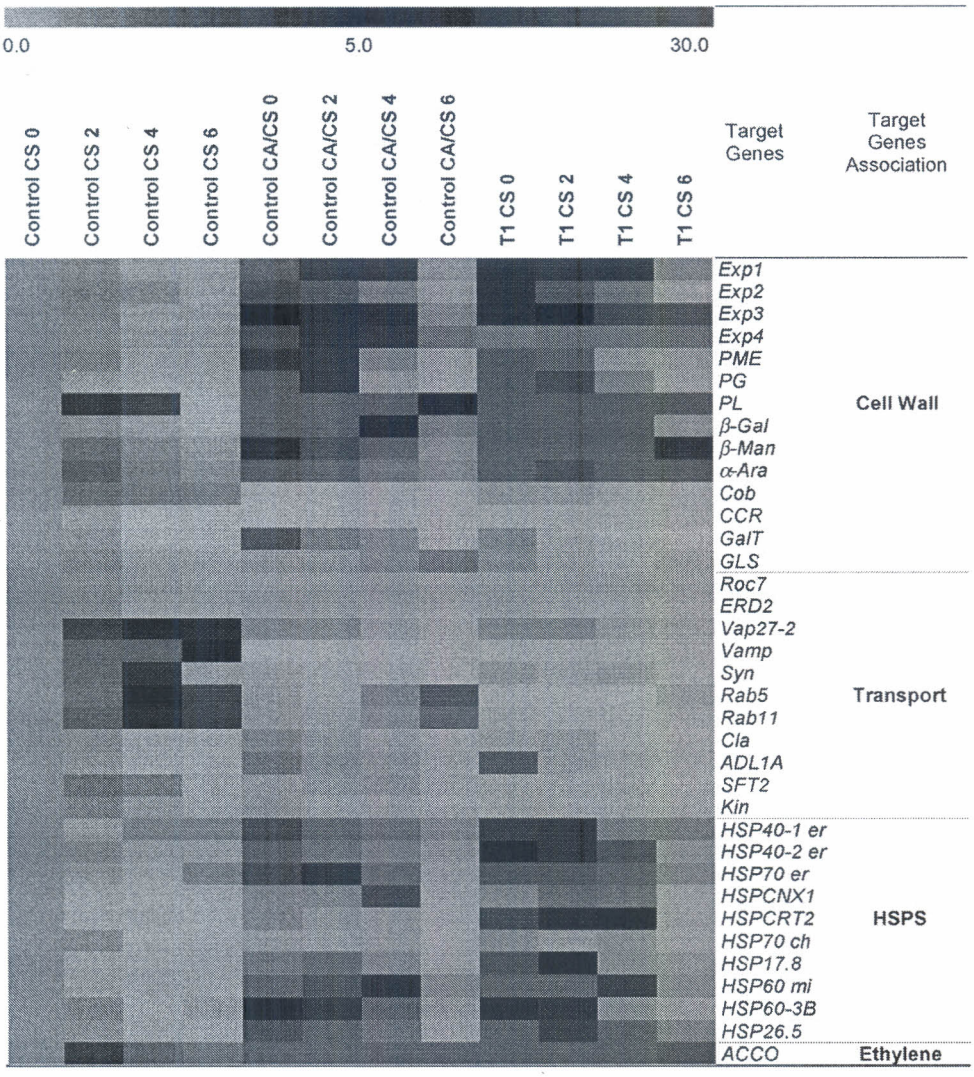

Fig. 1. Relative mRNA abundance in Chiripá peach fruit, harvested from untreated trees (Control) and from trees sprayed with GA_3 at the beginning of pit hardening (T1), stored for 30 days, under cold storage (CS) or controlled atmosphere/cold storage (CA/CS), and finally removed from storage and exposed to 23.0 °C (±3.0 °C) and 75.0% (±5.0%)RH, for 6 h (0), 2, 4 and 6 days. mRNA abundance is represented on a scale of 0–30, using the *Multi Experiment Viewer* (TIGR MeV) software (Saeed et al., 2003). The lower end of the scale (light green colour) indicates the lowest mRNA abundance; black colour in the middle of the scale represents mRNA abundance 5 times higher than the light green end; and red colour in the upper end of the scale represents the highest mRNA abundance, 30 times higher than the lowest. mRNA abundance of each gene from Control CS fruit at 6 h served as the baseline for determining relative RNA levels. Cold storage (CS): 1.0 °C (±1.0 °C), 92.0% (±5.0%) relative humidity (RH). Controlled atmosphere (CA/CS): 2 kPa of O_2, and 5 kPa of CO_2, 1.0 °C (±1.0 °C), 92.0% (±5.0%) RH.

mRNA abundance of *HSP40-1er*, *HSP40-2er*, *HSPCRT2*, *HSP17.8ch*, *Exp4*, *β-Man*, *α-Ara*, *GalT* and *ADL1A*, positively related to woolliness prevention, had different mRNA abundance profiles between Control CA/CS and T1 CS. mRNA abundance of *HSPs*, *ADL1A*, *α-Ara* and *β-Man* was higher in GA_3-treated peaches, whereas, mRNA abundance of *Exp4* and *GalT* was higher in Control CA/CS peaches.

mRNA abundance of transport associated genes *Roc7*, *ERD2*, *Cla*, *SFT2*, *Kin*, of *HSP70ch* and of cell wall structure associated genes *Cob*, *GLS* and *CCR*, were generally unaffected by GA_3 treatment or by storage condition during ripening, but *Vap27-2*, *Vamp*, *Syn*, *Rab5* and *Rab11*, vesicle transport associated genes, had higher relative abundance of mRNAs in woolly fruit (Control CS).

In woolly fruit (Control CS), *ACCO* mRNA abundance was lower than in untreated CA/CS and T1 CS fruit (Fig. 1) and protein was not detected (Fig. 2). In contrast, high relative abundance of *ACCO* mRNA and protein were detected and classic climacteric behaviour was observed in Control CA/CS and T1 CS peaches.

In woolly fruit all measured proteins were detected in lower amounts (Fig. 2). PME, PG, β-Gal and PL proteins (Fig. 2) were detected during ripening at room temperature in treatments preventing woolliness (Control CA/CS and T1 CS) before the onset of the climacteric stage (Table 4). The differences between protein abundance between Control CA/CS and T1 CS rely on PL which started to accumulate later in Control CA/CS when compared to T1 CS, and PME which was absent during day 4 in T1 CS and present in Control CA/CS. Moreover β-Man and α-Ara proteins were only detected during days 4 and 6 during ripening in gibberellic acid treated fruit (Fig. 2).

4. Discussion

Pre-harvest application of GA_3 to peach (Zilkah et al., 1997; Ju et al., 1999; Martinez-Romero et al., 2000; Amarante et al., 2005) and other fruit, such as persimmon (*D. kaki* L.) (Ben-Arie et al., 1996;

Table 4
Ethylene production of Chiripá peach fruit, harvested from untreated trees (Control) and from trees sprayed with GA_3 at the beginning of pit hardening (T1), stored for 30 days, under cold storage (CS) or controlled atmosphere (CA/CS), and finally removed from storage and exposed to 23.0 °C (±3.0 °C) and 75.0%(±5.0%) RH, for 6 h, 2, 4 and 6 days[a].

Treatment		Ethylene production ($nL\,g^{-1}\,h^{-1}$)			
		Period at 23.0 °C (±3.0 °C) and 75.0%(±5.0%)RH			
GA_3	Storage	6 h	2 days	4 days	6 days
Control	CS[b]	0.93 a[c]	1.23 c	1.24 c	0.59 b
Control	CA/CS[d]	0.87 a	5.52 b	15.21 b	3.36 a
T1	CS	1.06 a	11.65 a	24.53 a	4.80 a

[a] The peaches were harvested when the fruit colour was light green, corresponding to the pre-climacteric stage, between S3 I and S3 II, as described by Trainotti et al. (2003).
[b] Cold storage (CS): 1.0 °C (±1.0 °C), 92.0% (±5.0%) relative humidity (RH).
[c] Means of treatment ($n = 3$) in the same column, followed by different letters, are statistically different according to Duncan's test ($p \leq 0.05$).
[d] Controlled atmosphere (CA/CS): 2 kPa of O_2, and 5 kPa of CO_2, 1.0 °C (±1.0 °C), 92.0% (±5.0%) RH.

Ferri et al., 2004), tangerine (*C. reticulata* L.) (Marur et al., 1999) and cherry (*Prunus avium* L.) (Kappel and MacDonald, 2002; Usenik et al., 2005), can extend the ripening cycle and as a consequence in some cases increase fruit size. In this study, a pre-harvest spray with GA_3 did not extend the ripening cycle of Chiripá peaches but induced an increase in fruit size and mass. Fruit from all treatments reached the same ripening stage at the same time without showing any differences in physicochemical characteristics (Table 2). At harvest, fruit showed pre-climacteric ripening with an ethylene production ten times less than that found for peaches at the climacteric stage (Girardi et al., 2005).

However, an important technological improvement was obtained here, with the increase in fruit size and mass upon GA_3 application, leading to a 47% gain in productivity per plant (51–75 kg per plant) equivalent to increasing productivity from 25,500 to 37,740 kg ha^{-1}. It has been shown that agronomic practices that contribute to an increase in fruit size in Chiripá peaches (i.e. thinning) result in higher susceptibility to woolliness (Rombaldi et al., 2002). Interestingly, the increase in fruit size did not negatively affect peach preservation, with low levels of woolliness after CS.

Gibberellic acid has been associated with the prevention of many physiological postharvest disorders (Zilkah et al., 1997; Ju et al., 1999; Martinez-Romero et al., 2000; Kappel and MacDonald, 2002; Amarante et al., 2005; Usenik et al., 2005). According to Yamaguchi and Kamiya (2000), responses derived from exogenous GA supply are associated with synthesis deficiency and/or higher sensitivity to signal reception and transduction. Physiological responses observed in Chiripá peaches upon GA_3 treatment indicate a possible deficit of this hormone. In addition, Chiripá peaches were only responsive to GA_3 when sprayed prior to pit hardening, leading to a size increase and preventing woolliness upon cold storage.

Woolliness occurrence in GA_3-treated peaches (T1 CS) was as low as in untreated control peaches stored under CA/CS (Table 3). Upon extension of the ripening period, it was observed that woolly fruit (Control CS) became juicy again (Table 3). According to Lurie and Crisosto (2005), this apparent restoration of free juice is due to tissue breakdown and senescence. Therefore, a decrease in occurrence of woolliness seen in late ripening does not represent an actual reduction of the problem but a further decay of the fruit. In addition, signs of internal browning observed 6 days after CS confirmed the senescence of the fruit at that point.

In order to understand the molecular mechanisms associated with the physiological responses to GA_3 application and storage conditions, relative mRNA abundance of a set of genes putatively involved in endomembrane transport via endoplasmic reticulum (ER), Golgi complex (GC) and vesicles, cell wall structure and disassembly, stress response (heat shock proteins) and ethylene synthesis were investigated.

Fruit from treatments with lower occurrence of woolliness (Control CA/CS and T1 CS) developed a classical climacteric behaviour during ripening, while fruit with high occurrence of woolliness (Control CS) did not develop climacteric production of ethylene (Table 4). This response might be related to low relative abundance of *ACCO* mRNA (Fig. 1) and absence of *ACCO* protein observed in woolly fruit (Fig. 2). In contrast, high relative abundance of *ACCO* mRNA and protein were detected and classic climac-

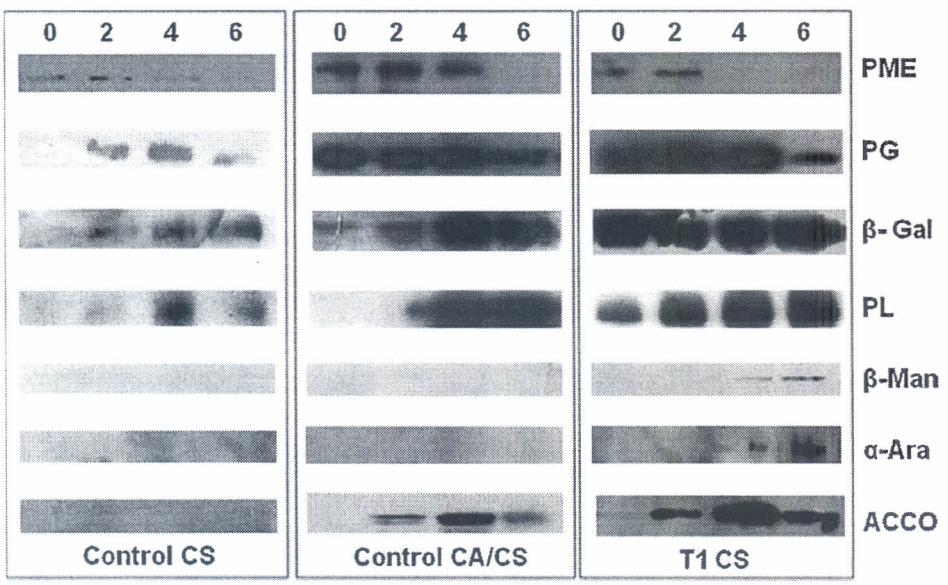

Fig. 2. Immunodetection of pectin methyl esterase (PME), endopolygalacturonase (PG), β-galactosidase (β-Gal), pectate lyase (PL), endo-1,4-β-mannase (β-Man), α-arabinofuranosidase (α-Ara) and ACC oxidase (*ACCO*) in total protein extracts of Chiripá peach fruit, harvested from untreated trees stored for 30 days under cold (Control CS) or controlled atmosphere conditions (Control CA/CS), and from trees sprayed with GA_3 at the beginning of pit hardening followed by 30 days under cold storage (T1 CS), and finally removed from storage and exposed to 23.0 °C (±3.0 °C) and 75.0% (±5.0%) RH, for 6 h (0), 2, 4 and 6 days.

teric behaviour was observed in Control CA/CS and T1 CS peaches, suggesting ethylene involvement in woolliness prevention. These findings agree with Giehl et al. (2008) who demonstrated that juiciness in peach is an ethylene-dependent process, with CA/CS storage allowing normal ethylene production and reduced woolliness. Moreover, Girardi et al. (2005) observed that supplying ethylene during CS prevents woolliness, while inhibiting ethylene with 1-MCP increases this chilling injury. In this study, the transcription and translation of genes encoding cell wall proteins were not completely ethylene-dependent. *PME*, *PG*, *β-Gal* and *PL* mRNAs (Fig. 1) and proteins (Fig. 2) were detected before the onset of the climacteric stage (Table 4), corroborating transcript findings of Trainotti et al. (2003). In addition, Nishiyama et al. (2007) found that two out of three *PGs* are ethylene-dependent in climacteric melons.

All genes studied here, putatively coding for cell wall associated proteins, have a signal peptide, suggesting that transport to the apoplast occurs via ER and GC, as predicted using iPSORT (Bannai et al., 2002), TargetP (Emanuelsson et al., 2000) and SignalP (Nielsen et al., 1997) software. Therefore, it is expected that protection of the ER, GC and vesicular endomembrane system is essential for the appropriate transport and folding of proteins to occur, in agreement with recent results by Vizoso et al. (2009) and Ogundiwin et al. (2008). The higher relative expression profile of ER genes (*HSP40-1er*, *HSP40-2er*, *HSP70er*, *HSPCNX1*) in peach from Control CA/CS and T1 CS agrees with this hypothesis. Moreover, recently, Sun et al. (2010) found three cytosolic small heat shock proteins with an important role in chilling tolerance in stonefruit. The potential role of these genes in regulating cell homeostasis under abiotic stresses has been previously observed in other species (Pratt and Toft, 2003; Iwata et al., 2008; Mueller et al., 2008; Su and Li, 2008). GA_3 treatment also induced high relative expression of expansins (*Exp1*, *Exp2* and *Exp3*), *PME*, *PG*, *PL*, *β-Gal*, *β-Man* and *α-Ara* genes (Fig. 1) coding for cell wall targeted proteins transported via ER and GC (Nakashima et al., 2004; Lycett, 2008). Moreover, preharvest GA_3 treatment contributed to a higher mRNA abundance of *HSP* genes, associated with the defense of chloroplasts (*HSP17.8ch*) (Millar et al., 2006; Jarvis, 2008; Kahlau and Bock, 2008) and mitochondria (*HSP60mi*, *HSP60-3Bmi* and *HSP26.5mi*) (Lister et al., 2004; Radhamony and Theg, 2006), indicative of better homeostasis of the metabolism of these organelles in peaches with adequate ripening. According to González-Agüero et al. (2008), peaches with normal ripening generally show up-regulation of genes encoding proteins associated with vesicle transport. In contrast, in Chiripá peaches, vesicle transport associated genes *Vap27-2*, *Vamp*, *Syn*, *Rab5* and *Rab11* showed high relative mRNA abundance in woolly fruit (Control CS) (Fig. 1). *Rab11* involvement in PME and PG transport and fruit softening has been previously demonstrated in tomato (Lu et al., 2001). Similarly, Lycett (2008) cited *Rab5* participation in the formation of endosomes, with increased transcription during fruit ripening. Additionally, Appezzato-da-Glória et al. (2004) in cytological studies of woolly fruit, observed a macroendocytosis process with endosome individualization containing cell wall materials, probably pectins, suggesting an endocytic transport associated with woolliness.

Although an inverse relationship between the expression of some vesicular genes and the prevention of woolliness was observed, a direct relationship between the mRNA abundance of genes associated with ethylene synthesis, ER, mitochondria and chloroplast protection, and of cell wall metabolism occurred (Fig. 1). The lower relative abundance of proteins involved in cell wall metabolism observed in Control CS (Fig. 2) is in agreement with the low relative mRNA abundance (Fig. 1), indicating a coordination between transcription and translation of these genes.

mRNA abundance of *Cob*, *GLS*, *CCR*, *Roc7*, *ERD2*, *Cla*, *SFT2*, *Kin* and *HSP70ch* genes was not affected by GA_3 treatment or by storage condition during ripening (Fig. 1). *HSP40-1er*, *HSP40-2er*, *HSPCRT2*, *HSP17.8ch*, *Exp4*, *β-Man*, *α-Ara*, *GalT* and *ADL1A*, positively related to woolliness prevention, had different profiles between Control CA/CS and T1 CS. While *HSPs*, *ADL1A* and *α-Ara* and *β-Man* showed higher relative expression in GA_3-treated peach, *Exp4* and *GalT* expression was high in Control CA/CS peach. Although both treatments prevented woolliness, differential mRNA and protein abundance suggest differences in the molecular and biochemical mechanisms of woolliness prevention induced by GA_3 and CA/CS.

In summary, GA_3 treatment followed by cold storage (T1 CS) proved as efficient as storage of peach under controlled atmosphere conditions (Control CA/CS) in providing normal ripening and preventing woolliness in Chiripá peaches. GA_3 treatment, despite not delaying ripening, induced an increase in fruit size and mass when applied at the beginning of pit hardening. The molecular responses associated with woolliness prevention in common between GA_3 and CA/CS treatments, involved high relative mRNA abundance of genes associated with cell wall metabolism, ER, chloroplast and mitochondria *HSPs*, and *ACCO* and lower relative mRNA abundance of vesicle transport related genes (*Vap27-2*, *Vamp*, *Syn*, *Rab5* and *Rab11*) when compared to woolly fruit. Although both treatments prevented woolliness, differential abundance of mRNA (*HSP40-1er*, *HSP40-2er*, *HSPCTR2*, *HSP17.8ch*, *Exp4*, *β-Man*, *α-Ara*, *GalT* and *ADL1A*) and protein (β-Man and α-Ara) suggest different molecular and biochemical mechanisms induced by GA_3 and CA.

Studies should follow to further characterize the protective mechanisms induced by GA_3 treatment and controlled atmosphere storage of peach. Currently, a pre-harvest evaluation of gene expression in peach fruit treated with GA_3 is under way to characterize early changes leading to woolliness or woolliness prevention.

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