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Oxidative stress and senescence-like status of pear calli co-cultured on suspensions of incompatible quince microcalli

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Summary This work presents a simple in vitro system to study physiological, biochemical and molecular changes occurring in a pear callus (*Pyrus communis* L., cv. Beurré Bosc) grown in close proximity to spatially separated undifferentiated homologous (pear) or heterologous (quince; *Cydonia oblonga* Mill., East Malling clone C) cells in its neighboring environment. After a 7-day co-culture period, the presence of heterologous cells produced negative effects on the pear callus, whose relative weight increase and adenylate energy charge decreased by 30 and 24%, respectively. Such behavior was associated with a higher O_2 consumption rate (+125%) which did not seem to be coupled to adenosine triphosphate synthesis. Analyses of alternative oxidase and enzymatic activities involved in reactive oxygen species (ROS) detoxification strongly suggested that the higher O_2 consumption rate, measured in the pear callus grown in the heterologous combination, may probably be ascribed to extra-respiratory activities. These, in turn, might contribute to generate metabolic scenarios where ROS-induced oxidative stresses may have the upper hand. The increase in the levels of 2-thiobarbituric acid reactive metabolites, considered as diagnostic indicators of ROS-induced lipid peroxidation, seemed to confirm this hypothesis. Moreover, reverse transcription polymerase chain reaction analysis revealed that the expression levels of a few senescence-associated genes were higher in the pear callus grown in the heterologous combination than in the homologous one. Taken as a whole, physiological and molecular data strongly suggest that undifferentiated cells belonging to a pear graft-incompatible quince clone may induce an early senescence-like status in a closely co-cultured pear callus.

Keywords: AEC, AOX, graft incompatibility, O_2 consumption rate, TBA-reactive compounds.

Introduction

Grafting is a widely adopted practice in fruit tree propagation. It involves joining vegetative parts of two different genotypes in order to integrate the advantageous traits of both of the original partners in a new chimeric individual (Hartmann et al. 2002). The use of genetically divergent partners may lead to incompatibility responses which result in unsuccessful graft unions. Since both causes and effects of graft incompatibility seem to be numerous and closely related to the partner combination, several typologies of this phenomenon may be observed (Andrew and Serrano-Marquez 1993, Hartmann et al. 2002, Pina and Errea 2005). In some combinations of pear/quince (Ermel et al. 1997, 1999), as well as of apricot/peach and apricot/plum (Errea et al. 1994), incompatibility results in defective root–shoot relationships which are caused by limited and/or not fully functional vascular reconnection between scion and rootstock at the graft interface. As an extreme case, partner disagreement may result in breakage at the union even some years after the occurrence of early healing between scion and rootstock (Gur et al. 1978, Andrew and Serrano-Marquez 1993).

The basic steps required for a successful graft are: (i) the proliferation of callus cells originating from both rootstock and scion that soon intermingle and interlock, filling up the spaces between the two components; (ii) the differentiation of new cambial cells from the newly formed callus, to form a continuous cambial join between rootstock and scion; (iii) the production of new xylem and phloem in order to permit an effective vascular connection between the partners (Andrew and Serrano-Marquez 1993, Pina and Errea 2005).

Although the formation of the callus bridge occurs independently of (in)compatibility relationships existing between the partners, some responses affecting the graft success may already be triggered during the earliest interaction steps

between callus cells. Such a hypothesis is supported by previous studies performed using in vitro callus graft systems, in which biochemical (Errea et al. 2001, Mng'omba et al. 2008) and molecular (Pirovano et al. 2002, Pina and Errea 2005) responses, as well as callus growth (Moore 1984, 1986) and cell ultrastructures (Errea et al. 2001, Pina et al. 2009), have been functionally related to (in)compatibility relationships. Interaction between interfacing cells could be triggered by either cell–cell surface contact or cell chemical cross-talking. This last hypothesis is supported by experimental evidence obtained by co-growing pear/quince callus masses separated by a membranous filter or growing in vitro pear cultures on media previously used for quince culture growth (Moore 1984, 1986). On the basis of these findings, it appeared possible that the pear incompatibility-like responses were probably determined by cyanide release due to the hydrolytic activity of a β-glycosidase, particularly abundant in pear cells, on the cyanogenic glycoside prunasin, mainly present in quince cells (Gur et al. 1968, Moore 1986).

The general aim of the present work was to evaluate whether it is possible to study the physiological and molecular effects of the cell chemical cross-talk occurring during the interaction between homologous or heterologous undifferentiated proliferating cells in order to gain preliminary information about the nature of intrinsic factors which may compromise the success of a graft union between two partners. For this purpose, co-cultures of homologous (pear/pear) and heterologous (pear/quince) undifferentiated cells, which can simulate compatible and incompatible scion/rootstock combinations, were used.

Material and methods

Plant material and experimental setup

Calli were obtained from cut shoots of pear (*Pyrus communis* L., cv. Beurré Bosc; B) and quince (*Cydonia oblonga* Mill., East Malling clone C; EMC) and multiplied by cell suspension cultures in a liquid proliferation medium with the following composition: 3.2 g l^{-1} Schenk and Hildebrandt basal salt mixture, 3% (*w*/*v*) sucrose, 555 μM myo-inositol, 4 μM nicotinic acid, 26.6 μM glycine, 2.96 μM thiamine hydrochloride, 2.4 μM pyridoxine hydrochloride, 0.25 mg l^{-1} kinetin and 1 mg l^{-1} 2,4-dichlorophenoxyacetic acid (pH 5.80). Cell suspension subcultures were maintained in flasks for 14 days in the dark, at 21°C and constant stirring speed, until suspensions of microcalli were obtained. Callus co-cultures were set up in Magenta® vessels (GA-7, Sigma), where B callus (1.5 g) was placed on a membrane raft (Durapore 5 μm pore size, 47 mm ∅, low protein adsorption, Millipore) floating on two different microcalli suspensions (1.5 g of either B or EMC microcalli in 70 ml of proliferation medium). Each Magenta® vessel was sealed, with its hermetic lid and wrapped in a plastic film to preserve sterility, and maintained in the dark at 21°C for 7 days, under constant stirring speed. At the end of the growing period, the B callus, placed on the membrane, was washed with distilled water, blotted with a paper towel, weighed and immediately used for experiments or frozen in liquid N_2 to be stored at −80°C for further analyses.

Adenylate energy pool and 2-thiobarbituric acid-reactive metabolites

For the determination of the adenylate energy pool, samples of about 500 mg of frozen callus were homogenized in a chilled mortar with 2.5 ml of ice-cold 0.5 M perchloric acid (PCA). The homogenate was centrifuged (4°C) at 13,000*g* for 10 min, and the pellet was washed with the same volume of 0.5 M PCA. K_2CO_3 was then added to the collected supernatants to remove PCA.

Adenosine triphosphate (ATP) was determined by measuring the bioluminescence emission with a 1250 LKB-Wallac luminometer (Wallac) after the addition of 200 μl of the LKB 1242-200 ATP monitoring reagent to 800 μl of a reaction mixture containing 25 or 50 μl of the extract, 125 mM Tris-acetate (pH 7.75), 2.5 mM Na_2-EDTA and 25 mM K-acetate. Adenosine diphosphate (ADP) and adenosine monophosphate (AMP) were determined by difference after enzymatic conversion to ATP with pyruvate kinase and adenylate kinase (Lundin et al. 1986). Internal ATP standards were included in order to correct the ATP, ADP and AMP determined values. The adenylate energy charge (AEC) values were calculated as AEC = (ATP + 0.5ADP)/(ATP + ADP + AMP).

The levels of 2-thiobarbituric acid (TBA)-reactive metabolites were determined according to Hodges et al. (1999).

Oxygen consumption

Fresh callus pieces (500 mg) were transferred into a thermoregulated (26°C) airtight cuvette containing 2 ml of 1 mM MES-BTP (pH 6.20) and 0.5 mM $CaSO_4$, and their oxygen consumption rate was measured using a Clark-type electrode (YSI Incorporated). The callus oxygen consumption rates were measured for 5 min after a 5-min period of pre-incubation. The O_2 consumption rate of B calli remained constant for more than 20 min.

Ethylene levels

To determine the ethylene levels within the in vitro system, the original Magenta® vessel lids had been modified by inserting in the middle a rubber septum to allow the sampling (through a syringe) of gas developed inside the system during the co-culture growth. Ethylene was determined by gas chromatography according to Bregoli et al. (2002). Data were normalized on the fresh weight reached by the B callus after the 7-day co-culture period.

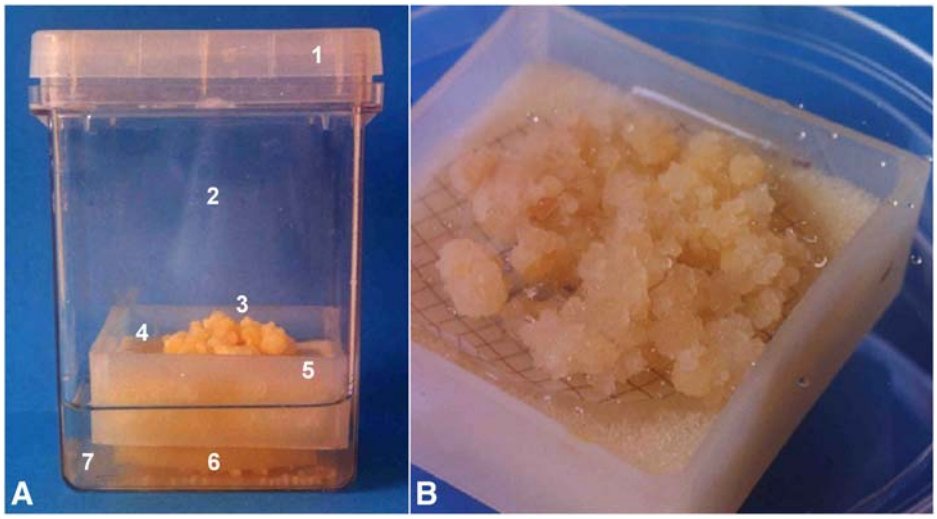

Figure 1. In vitro system for studying the physiological and molecular interaction between homologous or heterologous proliferating undifferentiated cells (A); detail of the B callus grown for 7 days on the membrane (B). 1, Magenta® lid; 2, Magenta® vessel; 3, B callus; 4, membrane; 5, membrane plastic raft; 6, foam rubber support; 7, microcalli suspension.

Enzyme assays

For the evaluation of enzyme activities, 1 g of callus was finely powdered in liquid N_2 using a pestle and mortar with 2% (*w*/*w*) polyvinylpolypyrrolidone (PVPP) and homogenized in 4 ml of a buffer containing 50 mM Na-phosphate (pH 7.00), 5 mM β-mercaptoethanol and 2 mM Na_2-EDTA. For ascorbate peroxidase activity, 1 mM ascorbic acid was added to the homogenization buffer. The homogenates were centrifuged at 10,000*g* for 10 min (4°C). The supernatants were further centrifuged at 48,000*g* for 20 min, and the newly obtained supernatants were frozen at −80°C. The protein content was determined by the Bradford procedure using γ-globulin as the standard (Bradford 1976). Glutathione reductase (GR; EC: 1.8.1.7), ascorbate peroxidase (APX; EC: 1.11.1.7), catalase (CAT; EC: 1.11.1.6), superoxide dismutase (SOD; EC 1.15.1.1) and dehydroascorbate reductase (DHR; EC: 1.8.5.1) activities were determined according to Schaedle and Bassham (1977), Nakano and Asada (1981), Aebi (1983), Elstner et al. (1983) and Asada (1984), respectively. Spectrometric measures were carried out with a Cary-50 spectrophotometer (Varian) at different wavelengths.

Immunoblot analysis

One gram of frozen B callus was finely powdered in liquid N_2 using a pestle and mortar with 1% (*w*/*w*) PVPP and homogenized with 3 ml of extraction buffer [50 mM Tris HCl pH 7.00, 10 mM Na_2-EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mg ml^{-1} Pefabloc (Fluka), 0.2% (*v*/*v*) β-mercaptoethanol]. The extract was then centrifuged at 13,000*g* at 4°C for 20 min, and the supernatant was collected. Protein concentration was determined by Bradford assay (Bradford 1976). To each sample, an equal volume of 2× sodium dodecyl sulfate (SDS) buffer [300 mM Tris HCl pH 6.80, 4% SDS, 20% (*v*/*v*) glycerol, 4% (*v*/*v*) β-mercaptoethanol] was added. The samples were finally incubated at 95°C for 5 min.

Thirty micrograms of proteins were separated by tricine–sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE, Schägger and von Jagow 1987) and electrophoretically transferred to a polyvinylidene difluoride filter as described by Espen et al. (2004). Filters were incubated overnight at 4°C with monoclonal antibodies against *Sauromatum guttatum* AOX (Elthon et al. 1989) fractions. The filters were incubated for 2 h at room temperature with antimouse immunoglobulin G as a secondary antibody. The blot was developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (FAST BCIP/NBT; Sigma-Aldrich). Blot images were scanned, and signals were quantified by ImageQuant 5.2 software (Molecular Dynamics).

RNA extraction, cDNA cloning and gene expression analysis

Total RNA was extracted from B calli using Trizol Reagent (Invitrogen), and first-strand cDNA synthesis was carried out using the SuperScript III first-strand synthesis system for reverse transcription polymerase chain reaction (RT-PCR) (Invitrogen), according to the manufacturer's instructions.

The partial cDNA of *P. communis* metallothionein 2b (*PcMT2b*) was isolated by RT-PCR using oligonucleotide primers designed on the sequence of the partial cDNA of *Pyrus pyrifolia* metallothionein-like protein (GenBank accession no. AF195206): PcMTfor 5′-ACAGAGTTGTGGTTGTCTGC-3′ and PcMTrev 5′-CCATAGAAGCCCAGCGATTC-3′. However, the oligonucleotide primers used for the isolation of all the other genes examined in this work were designed on the *P. communis* sequences already available in GenBank, as follows: pathogenesis-related protein 1 (*PcPR-1*; GenBank accession no. AF498321; PcPR1for 5′-GTCCCTTGACGTGGGATGAC-3′; PcPR1rev 5′-GTTACGCCAAACCACCTGTG-3′),

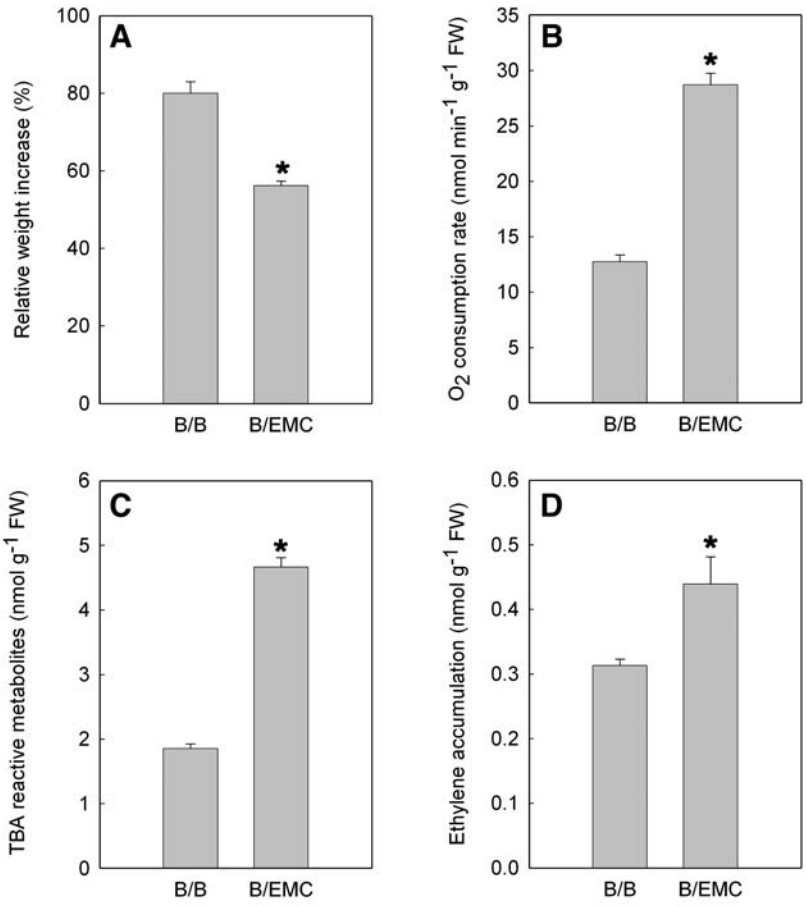

Figure 2. Relative weight increase (A), O_2 consumption rate (B) and TBA-reactive metabolites (C) of the B callus grown in the presence of homologous (B/B) or heterologous (B/EMC) microcalli and ethylene accumulation (D) inside the homologous (B/B) or heterologous (B/EMC) in vitro system. Relative weight increase was determined by measuring the fresh weight of the B callus before and after the 7-day co-culture period. O_2 consumption rate (B) was measured using a Clark-type electrode incubating fresh callus pieces in a thermoregulated airtight cuvette containing 2 ml of 1 mM MES-BTP (pH 6.20) and 0.5 mM $CaSO_4$. TBA-reactive metabolites were evaluated according to Hodges et al. (1999). Ethylene accumulation was determined by measuring gas developed inside the system during the 7-day co-culture period. Bars and error bars are means and SE of three independent experiments ($n = 9$). *$P \leq 0.05$, significant differences between the two co-culture conditions.

1-aminocyclopropane-1-carboxylic acid synthase (*PcACS*; GenBank accession no. AF386823; PcACSfor 5′-AGCCTCCATTTGCACAGCAG-3′; PcACSrev 5′-AAGCCAGGGAACCCCATGTC-3′), 1-aminocyclopropane-1-carboxylic acid oxidase (*PcACO*; GenBank accession no. X87097; PcACOfor 5′-TTCCCAGTTGTTGACTTGAGC-3′; PcACOrev 5′-AGAAGCTGGAGGCCGCTGAC-3′) and actin (*PcACT*; GenBank accession no. AF386514; PcACTfor 5′-CTTAACCCCAAGGCCAATCG-3′; PcACTrev 5′-TAGTGGGACCTCCACTGAGG-3′).

PCR was carried out on the first-strand cDNA using *Pfu* DNA polymerase (Promega), and the amplified fragments were cloned into the pCR®-Blunt II-TOPO® vector (Invitrogen). The identity of the PCR products was verified by sequencing both of the strands, and the *PcMT2b* partial cDNA sequence was submitted to GenBank (accession no. GU220357).

For semiquantitative RT-PCR analyses, first-strand cDNA deriving from 150 ng of total RNA was used for the amplification of *PcMT2b*, *PcPR-1*, *PcACS*, *PcACO* and *PcACT* partial cDNAs. PCR was carried out for 24 cycles, where cDNAs were exponentially amplified by *Pfu* DNA polymerase (Promega) using the same gene-specific primers as described before. PCR products were separated in agarose gels and stained with Vistra green (GE Healthcare). Signals were detected using a laser scanner (Typhoon 9200, GE Healthcare) with a 532-nm laser and a 526-nm filter, quantified by means of ImageQuant 5.2 software (Molecular Dynamics) and normalized using *PcACT* as an internal control.

Biological replication and statistical analysis

Data reported in Figure 2, Table 1 and Table 2 are mean values obtained in three independent experiments run in triplicate (number of observed calli = 9); RT-PCR and immunoblot analyses were carried out three times using a pool of three calli for each sample. Student's *t*-test was used to assess the significance of the observed differences. Statistical

Table 1. ATP, ADP and AMP levels and adenylate energy charge in the B callus grown in the presence of homologous (B/B) or heterologous (B/EMC) microcalli.

	Adenylate level (μmol g^{-1} FW)			AEC
	ATP	ADP	AMP	
B/B	12.0 ± 0.3	4.0 ± 0.1	1.4 ± 0.1	0.80 ± 0.04
B/EMC	6.7 ± 0.2*	7.9 ± 0.2*	2.9 ± 0.1*	0.61 ± 0.03*

Data are means ± SE of three experiments run in triplicate ($n = 9$).
*$P \leq 0.05$, significant differences between the two co-culture conditions.

Table 2. Specific activity of antioxidant enzymes in the B callus grown in the presence of homologous (B/B) or heterologous (B/EMC) microcalli.

	B/B	B/EMC
APX (μmol min^{-1} mg^{-1} protein)	0.18 ± 0.01	0.47 ± 0.02*
DHR (μmol min^{-1} mg^{-1} protein)	2.00 ± 0.12	3.60 ± 0.11*
GR (nmol min^{-1} mg^{-1} protein)	45.21 ± 1.32	87.43 ± 2.84*
SOD (unit min^{-1} mg^{-1} protein)[1]	45.01 ± 2.71	115.20 ± 7.11*
CAT (nmol min^{-1} mg^{-1} protein)	11.13 ± 0.42	21.26 ± 0.63*

APX, ascorbate peroxidase; DHR, dehydroascorbate reductase; GR, glutathione reductase; SOD, superoxide dismutase; CAT, catalase. Data are means ± SE of three experiments run in triplicate ($n = 9$).
[1]SOD activity is expressed as arbitrary units, according to Elstner et al. 1983.
*$P \leq 0.05$, significant differences between the two co-culture conditions.

analyses were performed using SigmaPlot 11.0 software (Systat Software Inc.).

Results and discussion

In vitro systems have been shown to be powerful tools to study some aspects of the incompatibility responses resulting in unsuccessful graft unions (Moore 1984, Espen et al. 2005, Pina et al. 2009). In this work, we present a simple in vitro system in which a pear callus (cv. Beurré Bosc, B) was grown on a membrane raft floating on a pear (B) or quince (East Malling clone C, EMC) microcalli suspension. Such a system allowed us to study the physiological changes of the pear callus induced by chemicals released by other undifferentiated cells (either pear or quince microcalli) proliferating in its neighboring environment, since the presence of the permeable membrane assured the spatial separation between the cells, while at the same time allowing an adequate nutrient supply to the pear callus and permitting cell chemical cross-talking between the co-cultures (Figure 1).

B callus floating on either B (B/B or homologous combination) or EMC (B/EMC or heterologous combination) microcalli suspensions grew without revealing any apparent symptoms of stress. Nevertheless, the B callus relative weight increase, measured after a 7-day period, was about 30% lower in the heterologous combination than in the homologous one (Figure 2A). Such behavior was in agreement with previous results obtained by Moore (1984) using a callus versus callus experimental system.

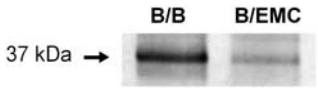

Figure 3. Western blot analysis of AOX extracted from the B callus grown in the presence of homologous (B/B) or heterologous (B/EMC) microcalli. Analysis was performed using antibodies directed against a *S. guttatum* AOX protein. Data are representative of one typical experiment repeated three times with similar results.

The value of the adenylate energy pool of the B callus was similar in B/B and B/EMC combinations (17.4 ± 0.5 and 17.5 ± 0.5, respectively; Table 1). However, considering the adenylate energy charge, which represents the relative saturation of the adenylate pool in phosphoanhydride bonds [AEC = (ATP + 0.5ADP)/(ATP + ADP + AMP)], a significant difference between the two combinations was found (0.80 versus 0.61, in B/B and B/EMC, respectively). AEC has largely been accepted as a convenient means of describing the cell energy status of a tissue in relation to a number of physiological events (Pradet and Raymond 1983). Usually, AEC values ≥0.8 are typical of 'actively metabolizing' tissues and also indicate a 'normal' balance between energy-consuming and energy-producing metabolic activities. Considering our data, it appeared that the presence of EMC microcalli in the neighboring environment of the B callus negatively affected its energy status by limiting ATP synthesis through mitochondrial respiration. However, the O_2 consumption rate did not seem to be coupled to the energy status reached by the B callus in the heterologous combination (Figure 2B), since it combined the highest value of O_2 consumption rate (+125% with respect to B/B) with the lowest ATP level (−38% with respect to B/B) measured during the experiments. Such a peculiar metabolic picture might have been the result of a high rate of both uncoupled mitochondrial respiration through the alternative pathway and/or extra-respiratory O_2 consumption which specifically involved the B callus in the heterologous combination. To better elucidate this point, we measured the level of the 37-kDa peptide corresponding to the homodimeric alternative oxidase (AOX), since the steady state level of this protein has been shown to be related to the actual capacity of alternative respiration in plant mitochondria (Vanlerberghe and McIntosh 1997, Arnholdt-Schmitt et al. 2006). Western blot analysis showed that the presence of EMC microcalli resulted in a decrease in the steady state level of the AOX [(−63 ± 2)%] in the mitochondria of the B callus (Figure 3). This finding allowed us to rule out the hypothesis that the higher O_2 consumption rate measured in the heterologous combination might have been due to an increase in the electron flux along the mitochondrial alternative respiration pathway. Increases in the extra-respiratory O_2 consumption have been largely described in nonphotosynthetic plant tissues under different stresses that may result in secondary oxidative damage due to the accumulation of

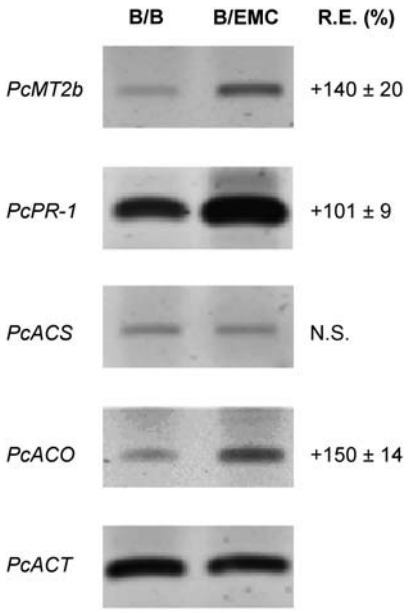

Figure 4. RT-PCR analysis of SAG expressions in the B callus grown in the presence of homologous (B/B) or heterologous (B/EMC) microcalli. PCR was carried out for 24 cycles where cDNAs were exponentially amplified. PCR products were separated in agarose gels and stained with Vistra green. Signals were detected using a laser scanner with a 532-nm laser and a 526-nm filter and quantified with ImageQuant 5.2 software. *PcMT2b*, metallothionein 2b; *PcPR-1*, pathogenesis-related protein 1; *PcACO*, 1-aminocyclopropane-1-carboxylic acid oxidase; *PcACS*, 1-aminocyclopropane-1-carboxylic acid synthase; *PcACT*, actin; R.E., relative expression (B/EMC versus B/B). Quantitative data were normalized on the signal of *PcACT*, used as an internal control of RT-PCR. Images are representative of one typical experiment repeated three times ($n = 3$) with similar results; data are means ± SE; N.S., no significant difference ($P \leq 0.05$).

reactive oxygen species (ROS, Arora et al. 2002). Since AOX is considered to be crucial in containing the mitochondrial generation of ROS (Maxwell et al. 1999, Arnholdt-Schmitt et al. 2006), it is interesting to note that the lower level of AOX protein observed in the B callus grown in the heterologous combination may have contributed to generating metabolic scenarios where ROS-induced oxidative stresses may have had the upper hand.

Taken as a whole, these data strongly suggest the hypothesis that the presence of heterologous cells in the neighboring environment of the B callus can promote an oxidative stress-like status through an increase in O_2 consuming activities. Starting from this hypothesis, we measured in both combinations some of the enzymatic activities of the cell antioxidant defense system of the B callus (Arora et al. 2002, Mittler 2002). As reported in Table 2, the specific activities of APX, DHR, GR, SOD and CAT were significantly higher in the B callus grown in the heterologous combination when compared with the homologous one. The increase in antioxidant defenses provides support for the suggested hypothesis, since this response is typical of plant tissues experiencing increases in ROS production (Arora et al. 2002, Mittler 2002). Moreover, considering the levels of TBA-reactive metabolites (Figure 2C), we can reasonably suppose that the rate of ROS production overcomes the antioxidant capacity of the B callus grown in the heterologous combination and therefore generates an oxidative stress condition. In fact, the level of these compounds—diagnostic indicators of ROS-induced lipid peroxidation (Heath and Packer 1968, Hodges et al. 1999)—was significantly higher in the heterologous combination (+152% with respect to B/B).

It has been widely shown that metabolic conditions in which defense mechanisms fail to protect plants from ROS accumulation may eventually result in cell death (Van Breusegem and Dat 2006). However, in many cases plants exhibit symptoms similar to those observed during leaf senescence before cell death occurs (Buchanan-Wollaston 1997, Navabpour et al. 2003). Such behavior is normally associated with changes in the expression pattern of genes, referred to as senescence-associated genes (SAGs; Buchanan-Wollaston 1997, Miller et al. 1999, Quirino et al. 1999, John et al. 2001, Navabpour et al. 2003), which can be used as diagnostic molecular tools. With these considerations in mind, we compared the expression level of a few SAGs in the B callus in the presence of homologous or heterologous microcalli (Figure 4). The expression analysis was carried out by semi-quantitative RT-PCR techniques, comparing in the two conditions the relative transcript levels of genes codifying for metallothionein 2b (*PcMT2b*), pathogenesis-related protein 1 (*PcPR-1*), 1-aminocyclopropane-1-carboxylic acid synthase (*PcACS*) and 1-aminocyclopropane-1-carboxylic acid oxidase (*PcACO*), of which the expression may be related to the occurrence of a cell senescence-like status (Buchanan-Wollaston 1997). Results show that, although the transcripts of all the marker genes were detectable in both conditions, the steady state levels of *PcMT2b*, *PcPR-1* and *PcACO* were significantly higher in B/EMC as compared to B/B; no obvious differences were found for *PcACS* (Figure 4).

PcMT2b encodes a metallothionein-like protein very similar in sequence to LSC210 and MT2b from *Brassica napus* and *Arabidopsis thaliana* (Zhou and Goldsbrough 1995, Buchanan-Wollaston and Ainsworth 1997). The *B. napus* metallothionein-like genes have been described as SAGs since their transcript levels increase during leaf senescence (Buchanan-Wollaston and Ainsworth 1997). Although the functions of this type of protein in senescence are still not clear, it is possible to hypothesize a role for these proteins in sequestering metal ions released by protein breakdown during senescence (Buchanan-Wollaston and Ainsworth 1997).

PcPR-1 encodes a member of the pathogenesis-related protein group, normally synthesized in response to pathogen infection (Van Loon and Van Strien 1999, Edreva 2005). Notwithstanding the fact that their biological roles are not yet clearly defined, some of these proteins may be involved in senescence, since the relative transcripts accumulate in healthy senescing tissues (Hanfrey et al. 1996).

PcACS and *PcACO* encode isoforms of the last two enzymes along the ethylene biosynthetic pathway,

1-aminocyclopropane-1-carboxylic acid synthase and 1-aminocyclopropane-1-carboxylic acid oxidase, respectively (Argueso et al. 2007). The ACS protein is thought to catalyze the rate-limiting step in ethylene biosynthesis, and the level of this enzyme has been shown to be controlled at both transcriptional and post-translational levels in response to various stimuli (Chae and Kieber 2005, Argueso et al. 2007). However, several studies indicate that ACO expression may also be regulated, especially when its activity becomes limiting to sustaining a high rate of ethylene evolution (Kende 1993, Blume and Grierson 1997, Argueso et al. 2007). Measurements of ethylene levels reached inside the in vitro system revealed that the heterologous combination (B/EMC) evolved more ethylene (+40%) than the homologous one (Figure 2D). Such behavior was in agreement with the results of gene expression analyses, although we cannot exclude the idea that other members of both the *ACS* and *ACO* mutagenic families may be involved in differential ethylene production.

Taken as a whole, physiological and molecular data strongly suggest that undifferentiated cells belonging to a pear graft-incompatible quince clone (EMC) may induce an early senescence-like status in a closely co-cultured pear callus of the B clone. Such an effect was not detected, at least in a 7-day period, when combining homologous undifferentiated cells (B/B) in a similar growing system. Causes of the early senescence-like status in the mixed system may be traced back to both ROS production and ethylene evolution, since a strong interplay between these factors has been hypothesized to act along the signal pathways leading to senescence and cell death (Van Breusegem and Dat 2006). In fact, environmental and developmental changes promoting ROS production through various organelles and enzymes may initiate signal transduction cascades, involving cross-talk with ethylene and other phytohormones, leading to senescence and cell death-related gene activation (Van Breusegem and Dat 2006). The expression patterns of SAGs (Figure 4) and ethylene evolution (Figure 2D) analyses seem to suggest this possibility, whereas the data concerning TBA-reactive metabolite levels (Figure 2C) clearly indicate an increase in ROS production in the B callus grown in the heterologous combination. Notwithstanding this, the factors eliciting these responses still need to be investigated. Here, we can only speculate about the existence of as-yet unidentified incompatibility factors which somehow act on mitochondrial activities involved in both cytochrome c oxidase- and AOX-dependent respiratory chains, as indicated by the negative effect exerted by the presence of heterologous cells on both AEC and AOX protein levels in the B callus. AOX may function to prevent the formation of reactive oxygen species by diverting reductants in excess of cytochrome pathway capacity down the alternative pathway (Purvis and Shewfelt 1993, Maxwell et al. 1999, Arnholdt-Schmitt et al. 2006). It has been shown that the downregulation of the cytochrome pathway in tobacco is accompanied by an increase in AOX expression and then in the mitochondrial capacity to prevent ROS evolution. Interestingly, the impairment of this response may lead to ROS-induced cell death, indicating the existence of a coordinated regulation of the two mitochondrial electron transport pathways (Vanlerberghe et al. 2002). This control is thought to be essential not only to satisfy cell metabolic energy demands but also to modulate the initiation of a cell death pathway responsive to mitochondrial respiratory status. Comparing the AEC values of B calli grown in the two combinations (Table 1), we can reasonably suppose that a downregulation of the cytochrome pathway may occur in response to the presence of heterologous cells. Such a response could be due to a cyanide overproduction through ACO activity along the ethylene biosynthetic pathway or, as previously suggested, through the hydrolytic activity of glycosidases on the cyanogenic glycoside prunasin (Gur et al. 1968, Moore 1986).

In conclusion, results presented in this work show that the physiological status of the B callus can be dramatically influenced by the presence of spatially separated heterologous undifferentiated cells in its neighboring environment. Such behavior seems to be independent of direct physical contact between the cells and thus allows us to speculate about the existence of species- or cultivar-specific intrinsic diffusible factors which could play a role in determining the success of an actual graft union. However, to verify and extend this hypothesis to a graft interface, further investigations should be carried out, since cell cultures used in this study do not physically interact and therefore do not exactly represent the cell types involved in forming a graft union in trees because of the artificial phytohormone and nutrient supply.

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