Journal of Plant Physiology 176 (2015) 25–35

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph

Physiology

Oxidative stress associated with rootstock–scion interactions in pear/quince combinations during early stages of graft development

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ARTICLE INFO

Article history:
Received 1 July 2014
Received in revised form 10 October 2014
Accepted 12 October 2014
Available online 6 December 2014

Keywords:
Antioxidant enzymes
Callus culture
Graft compatibility
Pear
Quince

ABSTRACT

Exposing a plant to stress situations, such as grafting, generally triggers antioxidant defense systems. In fruit tree grafting, quince (*Cydonia oblonga*) is widely used as a rootstock for pear (*Pyrus communis* L.), but several economically important pear cultivars are incompatible with available quince rootstocks. In this study, grafts were established using an *in vitro* callus graft system mimicking the events taking place in fruit trees. *In vitro* grown callus from pear [*P. communis* L. cv. 'Conference' (Co) and cv. 'William' (Wi)] and quince (*C. oblonga* Mill. clone 'BA29') was used to establish the compatible homografts 'Co/Co', 'Wi/Wi' and 'BA29/BA29', the compatible heterograft 'Co/BA29' and the incompatible heterograft 'Wi/BA29'. The main objective was to determine whether specific isoforms of genes involved in oxidative stress [superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT)] are differentially expressed at the graft interface from compatible and incompatible unions throughout 3 weeks after grafting. Reactive oxygen species (ROS) levels and programmed cell death were also evaluated in the course of graft development. Genes differentially expressed between compatible and incompatible heterografts were identified. Transcript levels of six antioxidant genes (SOD1, SOD3, APX3, APX6, CAT1 and CAT3) were down-regulated 10 days after grafting (DAG) in the incompatible heterograft in comparison to the compatible one. Likewise, SOD enzymatic activities were significantly higher at 1 and 10 days after wounding in the compatible cultivar 'Co' than in the incompatible one 'Wi'. These findings, together with live cell imaging of ROS-specific probes, ultrastructural mitochondrial changes and DNA fragmentation related to apoptotic processes, give indications that within incompatible rootstock/scion interfaces, either the level of ROS is increased or there is a less efficient detoxification system.

Introduction

During the process of grafting, two plant tissues are joined together, and a number of biochemical and structural processes are known to take place in herbaceous and woody grafts (Errea et al., 2001; Aloni et al., 2008; Pina et al., 2009; Wang, 2011; Cookson et al., 2013; Trinchera et al., 2013). These processes include adhesion between grafted partners, callus formation, establishment of new vascular tissue and the formation of a functional vascular system across the graft (Hartmann et al., 2002; Pina and Errea, 2005; Aloni et al., 2010).

The adhesion of the graft partners can be explained by the deposition of cell wall material between the opposing cut surfaces, and the new callus formation is an inevitable response that occurs in compatible and incompatible grafts as a common response to wounding (Moore and Walker, 1981, 1983). Even though the formation of the callus bridge occurs independently of compatibility relationships between the partners, specific responses, *i.e.* phenolic accumulation and symplasmic connectivity affecting the graft success, seem to be triggered already during the earliest interaction steps between callus cells (Moore and Walker, 1983; Moore, 1984; Errea et al., 2001; Pina et al., 2009; Nocito et al., 2010; Trinchera et al., 2013). Additionally, the establishment of functional phloem connections is delayed in non-successful grafts in fruit trees (Ermel et al., 1997; Espen et al., 2005; Pina et al., 2012). Espen et al. (2005) showed that the limited and delayed tracheary element differentiation in incompatible pear/quince grafts

Abbreviations: APX, ascorbate peroxidase; BA29, quince rootstock; CAT, catalase; Co, Conference cultivar; DAG, days after grafting; PBS, phosphate-buffered saline; qPCR, real-time polymerase chain reaction; ROS, reactive oxygen species; SOD, superoxide dismutase; TEM, transmission electron microscopy; Wi, William cultivar.

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http://dx.doi.org/10.1016/j.jplph.2014.10.015
0176-1617/

was associated with a decrease in the activity of programmed cell death (PCD) processes involved in vascular differentiation. Quince is widely used in Europe as a rootstock for pear. However, certain economically important pear cultivars are incompatible with some available quince rootstocks (Musacchi et al., 2000; Hudina et al., 2014). The main characteristics shown by incompatible pear/quince graft combinations are invagination of the cambium and vascular discontinuity at the graft interface (Espen et al., 2005; Ermel et al., 1999).

Although exposing plants to stress situations such as grafting would trigger the antioxidant defense systems, there are indications that, within incompatible rootstock/scion interfaces, either the level of reactive oxygen species (ROS) is increased or there is a less efficient detoxification system. For instance, non-successful grafts show signs of cellular stress such as high accumulation of H_2O_2, superoxide radicals (O_2^-) and the increase in the levels of 2-thiobarbituric acid reactive metabolites (Aloni et al., 2008; Nocito et al., 2010). Environmental stresses, such as wounding, temperature extremes, drought or mineral deficiency, trigger the production of ROS (Miller et al., 2008; Baxter et al., 2014). In other circumstances, plants appear to purposefully generate ROS as signaling molecules to control various normal cellular processes such as photorespiration, β-oxidation of fatty acids, PCD and stomatal behavior (Mahalingam and Fedoroff, 2003; Joo et al., 2005; Lee and An, 2005; Heller and Tudzynski, 2011). A highly complex network of ROS-producing and ROS-detoxifying enzymes acts in diverse cellular processes. In order to regulate the many processes, the biological response to altered ROS levels must therefore be very specific, the specificity depending on the type of ROS, the intensity of the signal, and the sites of ROS production (Gadjev et al., 2008).

ROS are partially reduced forms of atmospheric oxygen and include singlet oxygen (O_2^1), superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and a hydroxyl radical (OH) which are kept under steady state by an antioxidative redox system (Mittler, 2002). The ROS generated in excess due to environmental challenges unbalance the cellular redox system in favor of oxidized forms, resulting in oxidative processes such as membrane lipid peroxidation, protein oxidation, enzyme inhibition and DNA and RNA damage (Mittler, 2002; Wang et al., 2003; Hossain et al., 2009). Antioxidant enzymes inactivate the cytotoxic ROS compounds and minimize their ability to diffuse into the intracellular space. Superoxide dismutase (SOD) is an important antioxidant enzyme and constitutes the first level of defense against superoxide radicals in plants. SOD catalyzes the dismutation of O_2^- to H_2O_2 and O_2. Then, H_2O_2 is removed mainly by the activity of ascorbate peroxidase (APX) and catalase (CAT). Different isoforms of these enzymes are present in the cell compartments, and their expression is under genetic control as well as environmental stimuli (Noctor and Foyer, 1998).

Since the production of ROS is one of the primary and general events following wounding in higher plants (Bi and Felton, 1995; Slesak et al., 2008), coping with oxidative stress could be an important factor in achieving successful grafts. To our knowledge, no studies have been conducted to elucidate the possible involvement of specific antioxidant isoforms in graft (in)-compatibility processes. We hypothesize that either the antioxidant system is less efficient or the ROS level is increased within incompatible rootstock/scion interfaces. We therefore had the following objectives, which will contribute to new insights: (i) identify putative SOD, CAT and APX gene sequences in fruit trees using publicly available plant genome databases; (ii) analyze the gene expression and activity of these enzymes and (iii) monitor ROS formation and localization with bioimaging methods and ultrastructural analysis and determine the level of PCD in different graft union combinations in the course of graft development.

Material and methods

Plant material and growth conditions

Callus tissues from pear (*Pyrus communis* L.) cvs. 'Conference' ('Co', Sawbridgeworth, England) and 'William' (Wi, Aldermaston, England), compatible and incompatible scions, respectively, and callus from the quince rootstock (*Cydonia oblonga* Mill. clone 'BA29', Beaucouzé-Angers, France) were cultured as described in Pina et al. (2009). *In vitro* callus fusions, termed callus grafts, were established by placing two clean-cut callus pieces, one on top of the other, on the agar layer under sterile conditions in the same culture medium and conditions as used for callus initiation. The graft partners were brought together by gently pressing them down on the agar layer. The tissues were used to establish either homografts ('Co/Co', 'Wi/Wi' and 'BA29/BA29') or heterografts ('Co/BA29' and 'Wi/BA29'). Tissue cultures were maintained in a growth chamber at constant temperature 22 ± 2 °C, and a 16-h photoperiod cycle and light was provided by cool white fluorescent tubes. The graft combinations were sampled 0, 1, 3, 5, 7, 10 and 21 days after grafting (DAG). Ungrafted and wounded callus served as controls. Callus samples were frozen in liquid nitrogen and stored at −80 °C until RNA or protein isolation.

Database analysis

Nucleotide sequences of functionally known antioxidant enzymes (SOD, CAT and APX) from different plants were used to conduct tBlastN searches against the NCBI and GDR database, taking advantage of the available NCBI *Rosaceae* ESTs, and the assembled and annotated genomes from apple (Velasco et al., 2010) and peach (Verde et al., 2013). The nucleotide and amino acid sequence similarities were analyzed using the programs provided by NCBI BLAST (http://www.ncbi.nlm.nih.gov/blast/). The deduced amino acid sequences were aligned, and the phylogenetic trees were constructed by the ClustalW method using the neighbor-joining method of the MEGA 4.1 software.

RNA extraction, RT-PCR, sequencing and real-time qPCR

Total RNA was extracted from callus tissue using Trizol reagent (Invitrogen). First-strand cDNA was synthesized from 2.5 μg of RNA using the SuperScript III first-strand synthesis system (Invitrogen), according to the manufacturer's instruction. Gene-specific primers for APX1 (ppa010413m chrom 6), APX2 (ppa010426 chrom 6), APX3 (ppa009582m chrom 7), APX6 (ppa015878m chrom 1), CAT1 (ppa004763 chrom 5), CAT3 (ppa004776m chrom 5), Cu-ZnSOD1 (ppa012845m chrom 6), Cu-ZnSOD2 (ppa009729 chrom 6) and Cu-ZnSOD3 (ppa012725m chrom 7) and amplification in pear and quince were designed based on the sequences already available in the assembled peach and apple genomes. RT-PCRs were carried out with the gene-specific primers (Table 1). The amplified RT-PCR products were separated on 2% agarose gels, cloned into the pGEM-T Vector Systems (Promega) and sequenced to verify gene-specific amplification of the primers. The real-time PCR (qPCR) was performed on a 7500 Real Time PCR Systems (Applied Biosystem). The gene expression values were normalized to the actin gene from peach (GenBank accession number AB046952) and were depicted relative to the values of the corresponding homografts, *i.e.* the Co/Ba29 mRNA level divided by the Co/Co mRNA level and similarly for Wi grafts. Fluorescence values were baseline-corrected and averaged efficiencies for each gene and Ct values were calculated using LinRegPCR program. Gene expression measurements were determined with the Gene Expression Ct Difference (GED) formula (Schefe et al., 2006; Jiménez et al., 2010).