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Anionic peroxidase isoform profiles from calli and barks of pear cultivars and of the quince rootstock EM A

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The anionic peroxidases (E.C. 1.11.1.7.) of three pear *(Pyrus communis* L.) cultivars, representing an increasing scale of graft incompatibility with quince (*Cydonia oblonga* Mill.), and of the quince rootstock EM A, were analyzed by discontinuous non-denaturing polyacrylamide gel electrophoresis. Clear qualitative variations were detected among the electrophoretic patterns of the pear cultivars. These variations were observed at peroxidase isoforms that were slow migrating in the gels and were predominant in the callus extracts instead of the bark extracts. The roles of the electrophoretic methods and of the anionic peroxidase isoforms in the prediction of graft incompatibility are discussed.

Key words*:* graft incompatibility, pear, quince, peroxidase isoforms.

INTRODUCTION

An early and accurate forecast of graft incompatibility is of economic importance, since compatible combinations could be selected before grafting, while incompatible combinations could be avoided. The earliest methods used to predict graft incompatibility were based on the observation of external symptoms or on anatomical investigations. However, these methods are considered to be inadequate, because they require waiting until the symptoms are visible and also because anatomical observations may not correlate with long-term graft survival (Andrews & Marquez, 1993).

Isoforms separated by electrophoresis were one of the earliest *in vitro* methods used for the prediction of graft incompatibility (Copes, 1978). By studying isoperoxidase profiles in *Acer*, *Quercus* and *Castanea*, Santamour (1988) has proposed that when cambial isoperoxidase profiles of stock and scion are similar, a compatible union would occur when they are grafted. This test for the prediction of graft incompatibility was based on observations where adjacent tissues of two individuals of the same species differing only in cambial peroxidase patterns, were unable to reconstitute an operating vascular system.

Aside the work of Santamour, however, little data have been published about isoperoxidase profiles of stocks and scions of incompatible grafting combinations commonly observed, especially in tree fruit production. Isoperoxidase profiles relative to graft incompatibility have been studied in different apricot (*Prunus armeniaca* L.) varieties (Poessel, 1989) and in *Prunus* graftings with varied degree of incompatibility (Schmid & Feucht, 1986), but no clear results have been obtained. Recently, Gulen *et al.* (2002) have surveyed the peroxidase isoform profiles of various quince (*Cydonia oblonga* Mill.) clone rootstocks, and of two pear (*Pyrus communis* L.) cultivars, 'Williams' and 'Beurre Hardy'. They have concluded that electrophoretic detection of compatibility or incompatibility using isoperoxidase analysis could be used in pear-quince graft combinations.

Quince is widely used as a rootstock for pear. Compared with pear rootstocks, quince reduces the size and shortens the time to fruiting of the grafted tree (Lompard & Westwood, 1987). However, the main problem limiting its use, particularly in warmer

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climates, is graft incompatibility that frequently occurs between quince rootstocks and many pear cultivars. The main features shown by incompatible graft combinations are cell necrosis, vascular discontinuity at the graft interface and unlignified cell walls in the wood and bark, which lead to the breakage of the union or the slow decline of the tree (Mosse, 1962; Gur *et al.,* 1978).

It is well-established that the successive structural events which occur at the graft interface during the formation of a compatible union, include the formation of a necrotic layer, the adhesion between the tissues of the two partners, the proliferation of a junction callus, and eventually, cambium differentiation and junction (McCully, 1983; Ermel *et al.*, 1997). Moore (1984; 1986) studying *in vitro* associations of incompatible pear-quince calli and the influence of quince metabolites on pear suspension cultures, has postulated that the incompatibility between pear and quince is not associated with any particular stage of graft development. Moreover, he has suggested that the incompatibility factors probably initiate in the ground tissue (i.e. callus) rather than in a more differentiated tissue and that incompatibility does not require a prior wounding of tissues.

For these reasons, electrophoretic patterns of anionic peroxidase from callus cultures and barks of three pear cultivars and of the quince rootstock EM A were obtained and compared. The results are discussed in the perspective to gain more insight regarding the use of the peroxidase isoform analysis in the prediction and the identification of the possible causes of graft incompatibility between pear cultivars and quince rootstocks.

MATERIALS AND METHODS

Callus production

One-year-old dormant shoots of the pear *cvs* 'Tsakoniki', 'A. Fettel', and 'Williams' and of the quince rootstock 'EM A' were collected in winter and stored at 0°C for approximately 15 days. Then, shoot bases were dipped into tap water for buds to start growing at room temperature. One week later, the new shoots were cut off and surface sterilized by dipping in 70% alcohol, washed for 15 min in 0.4% sodium hypochloride and rinsed three times in sterile distilled water. Shoot tips, 1 cm long, were placed in test tubes (3×10 cm) containing 10 ml medium of Loyd & McCown (1980) supplemented with 20 $g.l^{-1}$ sucrose, 6 $g.l^{-1}$ agar, 0.05 $mg.l^{-1}$ BA and 2 $mg.l^{-1}$ IAA for 'A. Fettel', 0.1 $mg.l^{-1}$ 2,4-D for 'Tsakoniki', 0.02 $mg.l^{-1}$ IAA for 'Williams' and 2 $mg.l^{-1}$ NAA for quince 'EM A'. Calli that were vigorous and yellowish were used for all electrophoretic assays.

Bark sampling

Bark samples of the pear *cvs* 'Tsakoniki', 'A. Fettel' and 'Williams' were obtained from two-year-old pear trees grafted onto quince 'EM A' during the dormant period, (beginning of February) and during the growing season, (approximately 13 weeks after the budburst).

Bark samples of quince 'EM A' were obtained at the same time intervals with the pear bark samples, from annual shoots of ungrafted trees.

Enzymatic extract preparation and native PAGE

All bark samples were homogenized in cold mortar with a pestle in a three-fold volume of 0.1 M sodium phosphate buffer, pH 6.5, containing 10% (w/v) polyvinylpolypyrrolidone (PVPP) and 1 mM phenylmethylsulfonyl fluoride (PMSF). The supernatant obtained after centrifugation (27000 *g*, 20 min, 4°C) was used for all electrophoretic assays. *In vitro* calli were homogenized in the same way, except for that of the tissue: extraction buffer ratio was 2 g: 200 μl.

Discontinuous non-denaturing (2.4% polyacrylamide stacking and 7.5% polyacrylamide resolving gels in the absence of SDS and mercaptoethanol) PAGE was performed with a mini protean II cell (Bio-Rad, Hercules, Calif) according to Laemmli (1970). Active peroxidase bands were visualized by incubating gels in staining solutions containing 100 mM sodium acetate, pH 5.0, 0.5 mM 3-amino-9-ethylcarbazole and 9 mM H_2O_2. Gels remained at staining solutions for about 15-25 min and then they were rinsed in H_2O and fixed in ethanol/acetic acid (40/10, v/v).

RESULTS

The peroxidase isoforms of the pear cultivars and of the quince detected in this study were divided, for comparison purposes, into two groups, according to their relative migration: the slow migrating group (Q_1, Q_2 for the quince and P_1, P_2, P_3 for the pear cultivars) and the fast migrating group (Q_3, Q_4, Q_5 for the quince and P_4, P_5, P_6 for the pear cultivars).

The electrophoretic patterns of the anionic peroxidase isoforms from extracts obtained from bark

samples of the dormant period are shown in Fig. 1. The fast migrating bands of the pear cultivars were stained stronger than the slow migrating ones. The P_4, P_5 and P_6 isoforms were stained at the electrophoretic patterns of the three pear cultivars tested. On the contrary, clear variations between the pear cultivars were observed at the isoforms of the slow migrating group. The P_3 isoform was stained at the electrophoretic patterns of 'A. Fettel' and 'Williams', but it was not stained at the electrophoretic pattern of 'Tsakoniki'. At the electrophoretic pattern of the quince, five (5) isoforms were stained: two (2) at the slow migrating group (Q_1 and Q_2) and three (3) at the fast migrating group (Q_3, Q_4 and Q_5). The electrophoretic pattern of the quince was different from the electrophoretic patterns of the pear cultivars, with the exception of the pair Q_2-P_3 that exhibited the same R_f.

The electrophoretic patterns of the anionic peroxidase from bark extracts obtained from bark samples during the growing season, thirteen (13) weeks after budburst, are shown in Fig. 2. Again, the fast migrating isoforms (P_4, P_5, and P_6) were stained stronger than the slow migrating ones in all of the electrophoretic patterns of the pear cultivars. In advance, the differences observed at the electrophoretic patterns of the bark samples obtained during the dormant season were, also, identified, at the electrophoretic patterns of the bark samples obtained during the growing season. The P_3 isoform was stained at the electrophoretic pattern of 'A. Fettel' and 'Williams', but it was not stained at the electrophoretic pattern of 'Tsakoniki'. The electrophoretic pattern of the quince had the same appearance with the one obtained from the bark extracts of the dormant season.

The electrophoretic patterns of the anionic peroxidase isoforms obtained from the calli extracts are shown in Fig. 3. Contrary to the electrophoretic pat-

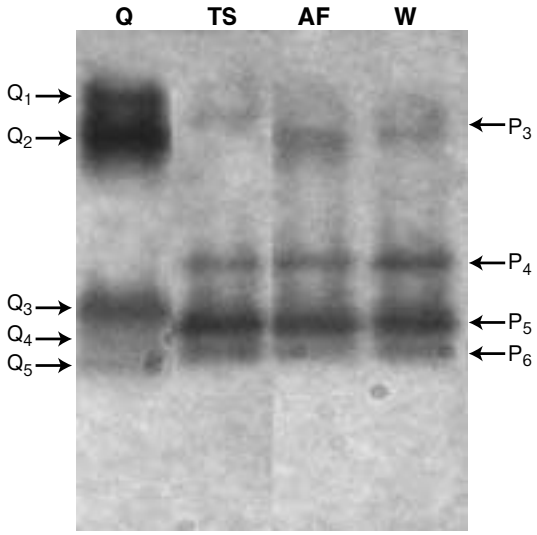

FIG. 2. Anionic peroxidase isozymes from bark extracts obtained 13 weeks after the budburst. Q: quince, EM A, TS: 'Tsakoniki', AF: 'A. Fettel', W: 'Willliams'. Gels were stained using 3-amino-9-ethylcarbazole.

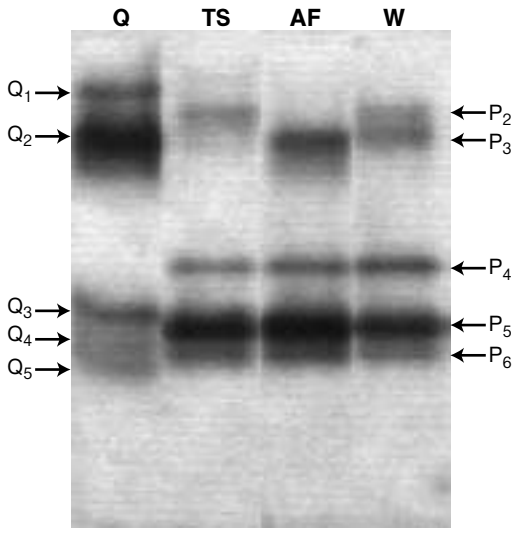

FIG. 1. Anionic peroxidase isozymes from bark extracts obtained during the dormant season. Q: quince, EM A, TS: 'Tsakoniki', AF: 'A. Fettel', W: 'Willliams'. Gels were stained using 3-amino-9-ethylcarbazole.

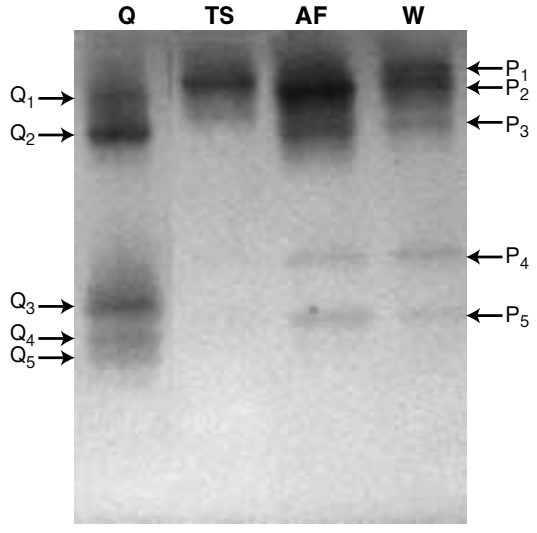

FIG. 3. Anionic peroxidase isozymes from calli extracts of quince EM A (Q), and of the pear cultivars 'Tsakoniki' (TS), 'A. Fettel' (AF), and 'Williams' (W). Gels were stained using 3-amino-9-ethylcarbazole.

terns of the bark extracts, the slow migrating isoforms of the pear cultivars were stained stronger compared to the fast migrating ones. Clear qualitative differences were observed among the electrophoretic patterns of the pear cultivars. These clear differences were revealed due to the strong staining of the slow migrating isoforms. One (1), two (2) and three (3) slow migrating bands were revealed at the electrophoretic patterns of 'Tsakoniki', 'A. Fettel' and 'Williams', respectively. Again, the P_3 isoform was not stained at the electrophoretic pattern of 'Tsakoniki', but it was stained at the electrophoretic patterns of 'A. Fettel' and 'Williams'.

The electrophoretic pattern of the quince callus extract was the same with that obtained from the bark extracts. Again, the only similarity between the electrophoretic patterns of the pear cultivars and the electrophoretic patterns of the quince was the pair Q_2-P_3 that exhibited the same R_f.

DISCUSSION

We analyzed by PAGE the anionic peroxidase isoform profiles of callus and bark extracts of the quince rootstock EM A and of the pear cultivars 'Tsakoniki', 'A. Fettel', and 'Williams'. These pear cultivars were selected because they present an increasing scale of incompatibility with quince, with 'Tsakoniki' being the most compatible one and 'Williams' as the most incompatible one.

The lignification of the cell walls is considered as the main event leading to the formation of a solid union, resulting in a compatible graft (Errea, 1998). Anionic peroxidases have been reported to be more specifically involved in the polymerization of the lignin monomers, based on their affinity for coniferyl alcohol, their location in the cell wall and their expression in lignified tissues (Carpin *et al*., 1999; Christensen *et al.,* 1998). Therefore, and considering the observations by Santamour (1988) that the isoperoxidase profiles of compatible graft partners are more similar than those of incompatible ones, it would be expected that the electrophoretic patterns of 'Tsakoniki' would be more similar to the profile of quince than the electrophoretic patterns of 'A. Fettel' and 'Williams'.

However, that was not the case in our study. The electrophoretic patterns of the three pear cultivars tested were different from the electrophoretic patterns of quince, with the exception of the P_3 isoform. This isoform was the only pear isoform that exhibited the same R_f with a quince isoform (Q_2), but it was stained at the electrophoretic patterns of the moderate incompatible and incompatible with quince pear cultivars ('A. Fettel' and 'Williams', respectively) and not at the electrophoretic patterns of the compatible with quince 'Tsakoniki'. Thus, our results are in agreement with those of Copes (1978) and Hongwen *et al. (*1994) who, working with the conifer *Pseudotsuga menzienzi* (Mirb) Franco and *Castanea* species respectively, found no relationship between the similarity of the peroxidase electrophoretic patterns of the graft partners and the success of the graft union.

Santamour's hypothesis was based on the assumption that peroxidases is the sole class of enzymes involved in the final step of cell wall lignification. However, in recent years, convincing evidence has been presented that obliges the scientific community to consider other oxidases and particularly laccases as enzymes potentially involved in wall lignification (Boudet, 2000). Apart from the lignification process, it has been also proposed that peroxidases are implicated in the graft incompatibility phenomenon through the irreversible oxidation of phenols. As a result, a type of quinones is formed, which might become polymerized becoming toxic compounds for a number of chemical reactions (Errea, 1998). If these oxidations are the main cause of graft incompatibility, then the differences between horticulturally compatible and incompatible, with quince, pear cultivars may be attributed to the presence or absence of particular peroxidase isoforms, resulting in different oxidation rates towards natural phenolic compounds that accumulate at the graft union.

It has been proposed that the behavior of the new callus cells on the callus bridge at the graft interface is of particular importance, as the production of parenchymatic cells from callus tissue and their differentiation into vascular connections will determine the future response of the graft (Hartmann *et al*., 1997; Schoning & Kollman, 1997). Moreover, Ermel *et al.* (1997) have suggested that the first structural event of the incompatibility response in pear-quince grafts is the abnormal differentiation of the new cambial strands (neocambia) originating from the callus bridge. Therefore, the processes that take place at the callus cells, which are at the transient state of differentiation into new vascular connections, are of particular importance relatively to graft incompatibility.

In our study, clear differences between the electrophoretic patterns of 'ground' tissue (calli) extracts and the electrophoretic patterns of more differentiated tissue (bark) extracts were revealed. At the elec-

trophoretic patterns of the pear cultivars, the slow migrating bands were stained stronger than the fast migrating ones, when calli extracts were analyzed, whereas, when bark extracts were analyzed, the fast migrating bands were stained stronger that the slow migrating ones. In advance, the fast migrating isoforms were stained at the electrophoretic patterns of all pear cultivars tested, while clear qualitative differences were revealed at the isoforms of the slow migrating group, especially at the electrophoretic patterns of the calli extracts. Following the above and according to our opinion, this might be a first indication that the isoforms of the slow migrating group might be related to differences between the pear cultivars that may contribute to the expression of graft incompatibility.

In summary, under our experimental conditions of extraction and gel staining, we detected differences among the electrophoretic patterns of the anionic peroxidase of horticulturally compatible and incompatible with quince pear cultivars. These differences were between isoforms that a) were slow migrating in polyacrylamide gels and b) were predominant to callus extracts instead of bark extracts. Further experiments will clarify whether one or more of these peroxidase isoforms play a physiological role associated with the expression of pear-quince graft incompatibility.

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