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# Phloem Cytochemical Modification and Gene Expression Following the Recovery of Apple Plants from Apple Proliferation Disease

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## ABSTRACT

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Recovery of apple trees from apple proliferation was studied by combining ultrastructural, cytochemical, and gene expression analyses to possibly reveal changes linked to recovery-associated resistance. When compared with either healthy or visibly diseased plants, recovered apple trees showed abnormal callose and phloem-protein accumulation in their leaf phloem. Although cytochemical localization detected $Ca^{2+}$ ions in the phloem of all the three plant groups, $Ca^{2+}$ concentration was remarkably higher in the phloem cytosol of recovered trees. The expression patterns of five genes encoding callose synthase and of four genes encoding phloem proteins were analyzed by quantitative real-time reverse transcription-polymerase chain reaction. In comparison to both healthy and diseased plants, four of the above nine genes were remarkably up-regulated in recovered trees. As in infected apple trees, phytoplasma disappear from the crown during winter, but persist in the roots, and it is suggested that callose synthesis/deposition and phloem-protein plugging of the sieve tubes would form physical barriers preventing the recolonization of the crown during the following spring. Since callose deposition and phloem-protein aggregation are both $Ca^{2+}$-dependent processes, the present results suggest that an inward flux of $Ca^{2+}$ across the phloem plasma membrane could act as a signal for activating defense reactions leading to recovery in phytoplasma-infected apple trees.

*Additional keywords*: calcium signaling, *Malus domestica.*

Apple proliferation (AP) is one of the most economically important plant diseases in Europe, requiring quarantine safeguard. '*Candidatus* Phytoplasma mali' ('*Ca*. P. mali') is the disease agent associated with AP; it is a phloem-restricted and wall-less prokaryote, unculturable in vitro and belongs to the class of Mollicutes (21).

Most cultivars of apple (*Malus domestica* Borkh), which is the main host of '*Ca*. P. mali', appear to be susceptible, as well as do wild and ornamental plants of the genus *Malus*. Witches' broom is the most typical symptom of AP and is associated with small and/or bronze reddish leaves, enlarged stipules, leaf-rosette formations, virescent flowers with abnormal number of petals, and low fruit quality (34), which causes significant economic losses.

The spontaneous remission from disease symptoms in apple plants affected by AP is denoted as recovery, which can be permanent under certain circumstances (38). The persistence of symptom remission is affected by the host genotype and by the environment. The recovery potential is related to the colonization behavior of phytoplasmas in AP-infected plants. During winter, '*Ca*. P. mali' is eliminated from the above-ground parts of the tree, due to the degeneration of the sieve tubes in the previous year's phloem (44). In spring, the upper parts of the plant can be recolonized starting from the roots, where the phytoplasma persists throughout the year. In recovered trees, phytoplasmas are no longer able to recolonize the crown, although they persist viable and infectious in the roots (8). What causes recovery is not currently known and its physiological bases are poorly understood. When recovered plants are exposed to a high pressure of disease in the field, the probability of developing symptoms is four times lower if compared with never infected trees (38), which suggests the establishment of a type of induced resistance in the latter.

The intervention of phloem sap proteins, plugging affected sieve elements (SE) and thus avoiding microbe penetration/diffusion and/or loss of nutrients, is a quick and straightforward response in plants undergoing pathogen attack. Phloem protein 1 (PP1) and phloem lectin or phloem protein 2 (PP2), belonging to a well-known class of SE proteins involved in the plugging of sieve pores, were first described in cucurbit phloem sap (42), but are likely present in all dicots. Under oxidative conditions, PP1 monomers and PP2 dimers are covalently cross-linked via disulphide bonds, forming high molecular weight polymers that occlude the sieve pores (42). Such response is normally accompanied by the activation of callose synthase, synthesizing β-1,3-glucan which accumulates on sieve plates as plugs, drops, or plates in close proximity to the invading pathogen (10). The SE closure by protein plugs is a fast and reversible mechanism,

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*The ***e*-X**tra logo stands for "electronic extra" and indicates that the online version contains two articles showing the identification and characterization of cDNAs coding for callose synthases and phloem proteins in apple, a table listing the primer pairs used in qRT-PCR analyses and characteristics of the corresponding amplicons, and two figures showing the relative quantification by qRT-PCR of the expression levels of four or five genes coding for phloem proteins or callose synthase.

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