

Graft incompatibility in plants: Metabolic changes during formation and establishment of the rootstock/scion union with emphasis on *Prunus* species

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Commercial fruit trees are usually formed by the combination of a rootstock and a scion to broaden the adaptability of scion cultivars to soil and climatic conditions, facilitate agricultural management, and/or increase productivity. In the different cultivated species of the genus *Prunus*, rootstocks having a wide range of uses are scarce, because of rootstock/scion graft incompatibilities that prevent the establishment of a strong and lasting functional union. Graft incompatibility is a problem in cherry, almond, and apricot than in peach or plum. In general, closely related cultivars and species tend to be compatible, but taxonomically distant plants often manifest incompatibility. This review will focus on the knowledge currently available on the metabolic response during the formation and establishment of the stock/scion graft union in order to help the effort for identify future metabolic markers to be used in breeding programs. The physiological, metabolic and molecular mechanisms that cause incompatibility remain unclear and several hypotheses have been proposed to explain it, mostly based on herbaceous species. Few studies are available to explain incompatibility in woody plants. Various phenolic compounds are known to affect cell division, development and differentiation at the graft union. Flavonol (catechins and proanthocyanidins) concentrations increase shortly after grafting and, as a result of the stress induced during the healing response, vacuolar membrane disruption occurs resulting in the escape of phenols from the vacuole into the cytoplasmic matrix, causing dysfunctions in the growth of certain tissues (xylem and phloem), interference with the synthesis of lignin or inducing hormonal imbalances. All these abnormalities result in mechanical weakening of the union, which may manifest during the first year after grafting (translocated incompatibility) or may appear several years later (localized incompatibility), leading to major economic losses. More research is needed to fully understand the mechanism of graft incompatibility, particularly in woody plants. This knowledge is essential to develop molecular markers useful in rootstock breeding programs.

Key words: *Prunus*, interspecific hybrids, localized incompatibility, translocated incompatibility.

INTRODUCTION

Modern fruit growing makes extensive use of selected rootstocks for a variety of purposes, including vigor control, which enable high density planting, disease and pest tolerance/resistance, resistance/tolerance to abiotic stresses like hypoxia, reduce the time to fruiting, increase profit returns, improve fruit quality and yield, improve cold hardiness, cope with the chilling requirement of the scion, and/or to alleviate certain syndromes like replant complex. In addition, modern cultural methods are evolving towards the use of clonal rootstocks as opposed to seeding rootstocks that were commonly used in the

past. The above-mentioned role of rootstocks is the result of active rootstock/scion interactions, most of which are the result of complex processes occurring at the rootstock/scion union, most of which are poorly understood.

The most important of these interactions is compatibility between rootstock and scion. There is rootstock/scion compatibility when a given combination is able to form a solid and durable graft union. Compatibility is difficult to predict, but there is a general consensus that a degree of taxonomic relatedness should exist in order for a particular stock/scion combination to be compatible. The greater the taxonomic distance between stock and scion the smaller the chances of forming a successful graft union. This means that the theoretical success of a particular graft union combination is intraclonal > interclonal > intraspecific > interspecific > intrageneric > intergeneric > intrafamilial (Andrews and Marquez, 1993). Also, different taxa may differ in the degree of relatedness required for the formation of successful graft unions (Rom and Carlson, 1987). Therefore, compatibility is very specific, meaning that a particular rootstock is

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generally not compatible with all commercial varieties of a given species. In modern horticulture this is a limiting factor, particularly in peach and cherry where there is a lack of commercial rootstocks having broad compatibility (Okie, 1987; Zarrouk et al., 2006).

Rootstock/scion graft compatibility is, therefore, a critical issue for orchard performance and longevity. According to Yeoman (1984), compatibility can be regarded as a recognition system in which molecules released from the plasmalemmas combine to form a complex with catalytic activity that subsequently initiates a developmental sequence resulting in the development of a successful graft. To remain functional, the stock/scion union should unify intimately, providing a viable system for the uptake and translocation of minerals, water, assimilates, and hormones throughout the entire lifespan of the plant (Martínez-Ballesta et al., 2010; Gregory et al., 2013; Koepke and Dhingra, 2013). In contrast, graft “incompatibility” leads to unhealthy trees, breakage at the graft union, premature death or failure of the graft combination and incapacity to form a strong and lasting functional union (Zarrouk et al., 2006). In *Prunus* species, incompatibility is a problem in cherry, almond and, especially, apricot, than in peach or plum (Table 1) (Lang and Ophardt, 2000).

Two types of incompatibility are recognized: so called translocated graft incompatibility and localized graft incompatibility (Herrero, 1951; Mosse, 1962). The former is usually expressed during the first year after grafting as growth cessation, defoliation, and leaf discoloration (Herrero, 1951; Mosse, 1962). In peach/plum combinations, this form of incompatibility

has been associated with both biochemical and functional alterations at the graft interface, inducing a carbohydrate blockage at the scion, above the graft union (Moing and Carde, 1988; Moing et al., 1990). In the latter case, incompatibility symptoms occur at a later stage of development (Herrero, 1951; Mosse, 1962), where the presence of some biochemical alterations across the graft union may lead to a slight and delayed incompatibility, as has been described in cherry and peach/plum combinations (Treutter and Feucht, 1991; Salesses and Bonnet, 1992). This type of incompatibility is characterized by anatomical irregularities at the stock/scion union interface, with breaks in vascular and cambial continuity patterns and poor vascular connections that induce mechanical weakness in the union (Errea et al., 2001; Koepke and Dhingra, 2013) that may break out after years of orchard establishment, leading to major economic losses (Herrero, 1951).

Despite the importance of incompatibility for the development of new rootstocks, the physiological and metabolic mechanisms involved in an incompatible response remain unclear (Zarrouk et al., 2006). This lack of understanding is probably due to the lack of adequate methods to analyze the metabolic changes occurring during the development of the stock/scion union. Only a few studies have been made on compatibility in woody plants (Errea et al., 1994; Errea, 1998; Salvatierra et al., 1998; 1999; Pina and Errea, 2005; Koepke and Dhingra, 2013) and the hypotheses that currently explain the causes of incompatibility have been obtained mainly from studies done in herbaceous plants during the early

Table 1. Rootstock/scion compatibility performance in different *Prunus* species and some interspecific hybrids.

Rootstock type	Pedigree	Compatibility performance	References
Japanese plum/peach Fast-growing plums	<i>Prunus salicina</i> × <i>Prunus persica</i>	Very good with plums and apricots, but not with peaches	Zaiger, 1982
	<i>Prunus cerasifera</i> and interspecific hybrids	Performance differs substantially depending on the genotype, ‘Adara’ rootstock confers compatibility even with cherry cultivars when it is used as an interstock	Zarrouk et al., 2006
Peach	<i>Prunus persica</i> × <i>Prunus davidiana</i>	Wide range of compatibility with most peach and nectarine cultivars	Lang and Ophardt, 2000; Zarrouk et al., 2006
Peach/almond	<i>Prunus persica</i> × <i>Prunus dulcis</i>	Wide range of compatibility with most peach and nectarine cultivars	Lang and Ophardt, 2000; Zarrouk et al., 2006
Slow-growing prunes	<i>Prunus domestica</i>	Wide range of compatibility with most peach and nectarine cultivars	Lang and Ophardt, 2000; Zarrouk et al., 2006
Slow-growing prunes	<i>Prunus insititia</i>	Wide range of compatibility with most peach and nectarine cultivars	Lang and Ophardt, 2000; Zarrouk et al., 2006
Cherry	<i>Prunus avium</i> × <i>Prunus pseudocerasus</i>	Wide range of compatibility with most sweet cherry cultivars except ‘Sam’ and ‘Van’	Rom and Carlson, 1987
Cherry	<i>Prunus avium</i>	Incompatible with peach, nectarine and almond but compatible with all sweet cherry cultivars	Beckman and Lang, 2003
Cherry	<i>Prunus mahaleb</i>	Incompatible with several sweet cheery scion cultivars like ‘Lapins’, ‘Chelan’ and ‘Tieton’	Long and Kaiser, 2010
Cherry	<i>Prunus cerasus</i>	Incompatible with several sweet cherry scion cultivars	Long and Kaiser, 2010
Cherry	<i>Prunus cerasus</i> × <i>Prunus canescens</i>	Wide range of compatibility with most sweet cherry cultivars	Long and Kaiser, 2010

stages of development of the graft union (Tiedemann, 1989; Aloni et al., 2008; Koepke and Dhingra, 2013). Also, to study the integrity of the union, destructive methods are necessary in order to carry out anatomical studies to visualize the integrity and functionality of the connections between the stock and the scion.

This review will focus on the knowledge currently available on the metabolic response during the formation and establishment of the stock/scion graft union in order to help the effort for identify future metabolic markers to be used in breeding programs.

Metabolic pathways

Recent data demonstrate that several biochemical pathways are affected during graft union formation (Koepke and Dhingra, 2013). One of these is the metabolism of phenolic compounds (Haslam and Lilley, 1986), which are also involved in the stress and wounding response (Leonardi and Romano, 2004; Cohen et al., 2007). As expected in a normal wound reaction, an intense production of new phenolic compounds has been reported during the establishment of a graft union (Tiedemann, 1989; Hartmann et al., 2002; Kueger et al., 2012). In incompatible heterografts, where stock and scion come from different species, quantitative and qualitative diffusion of these compounds through the union can produce metabolic dysfunctions. It is known that small quantities of phenols can be enough to produce locally limited dysfunctions in the interphase between two or more cells (Treutter and Feucht, 1991; Fiehn et al., 2008). Moreover, studies using callus cultures of *Prunus avium* (L.) L. have shown that the flavin prunin, a typical phenol of *Prunus* species, interferes with the permeability of the tissues, which results in membrane damage (Harborne, 1994). In addition to prunin, other phenols may participate in transport across cell membranes into the extracellular free space (Salvatierra et al., 1999).

Phenolic compounds have also been associated with the different processes involved in cell division, development, and differentiation (Errea et al., 1992; Elstner et al., 1994). In incompatible graft unions, phenols move from the vacuole into the cytoplasm, causing stress that results in growth dysfunction, probably caused by an inhibition of the lignin pathway (Tiedemann, 1989; Elstner et al., 1994; Hartmann et al., 2002). Also, concentrations of flavonols (catechins and proanthocyanidins) increase under stress and grafting is no exception (Errea et al., 1994; Cooman et al., 1996; Errea, 1998).

Hormones are another important class of compounds implicated in the development of the graft union. For instance, auxins are released from vascular strands of both stock and scion to induce the differentiation of vascular tissues (Aloni, 2010; Aloni et al., 2010;

Koepke and Dhingra, 2013). Auxin translocation from the root system has been studied in apple in relation to graft incompatibility, since a supra and basipetal movement of auxin can affect the morphogenetic pattern of the entire plant body (Treutter and Feucht, 1988; Koepke and Dhingra, 2013).

Phenolic compounds and hormones also interact with each other. Lack of compatibility has been associated with a pronounced accumulation of polyphenols above the graft union (Feucht et al., 1992), which are known to affect auxin transport (Errea et al., 1994; Errea, 1998). Other authors have reported that a low indole-3-acetic acid (IAA) content in incompatible combinations may then affect the differentiation of xylem and phloem, as well as lignification (Pina and Errea, 2005; Aloni et al., 2010; Koepke and Dhingra, 2013). This implication of flavonols at work in stressed unions has also been discussed in terms of a delay in senescence during the beginning of the stress phase. The accumulation of flavonols in graft unions and their degradation by oxidases can have important effects on the growth and metabolism of tissues (Pina and Errea, 2005).

Complex physiological disorders are directly related to the presence, absence or interactions of specific combinations of metabolites (Fiehn, 2000). It is often combinations of metabolites, rather than the presence of individual compounds, that are of biological relevance (Hall, 2006; Hardy and Hall, 2012). In particular, a complex disorder such as incompatibility inevitably has a complex biochemical background and depends on specific genetic interactions between the cells of the stock and the scion (Koepke and Dhingra, 2013). Metabolic interactions are difficult to analyze using traditional methods; however, now using a metabolomic approach it is possible to better understand these complex phenomena. Metabolomic technologies now available allow studies of the biochemical composition of biological materials to be made without having prior metabolic knowledge (Hall, 2006; Hardy and Hall, 2012; Kueger et al., 2012).

Formation of the stock/scion union

As mentioned before, the success of grafting depends primarily on the compatibility of the graft union to enable rapid development of vascular connections between the stock and the scion (Olmstead et al., 2006), which, in turn, will allow quick resumption of the growth of both the root and the canopy (Hartmann et al., 2002; Leonardi and Romano, 2004; Cohen et al., 2007). In grafted plants, vascular regeneration is a complex process that includes structural differentiation of the parenchymatous tissue from both sides of the graft union into xylem and phloem (Olmstead et al., 2006; Aloni et al., 2010). The sequence of structural events occurring during the healing of the graft union

in woody and herbaceous plants has been reviewed by several authors (Moing and Carde, 1988; Tiedemann, 1989; Hartmann et al., 2002; Pina and Errea, 2005), but recently Yin et al. (2012) identified six major events: (i) Wound-induced response, (ii) cleanup of cell debris, (iii) cellular communication, (iv) auxin accumulation and responses, (v) cell division and differentiation, and (vi) vascular reconnection. It is now known that some mRNA signals change just 24 h after grafting, and that 48 h after grafting auxins increase at the union and stimulate cell division and differentiation. On the third day, transport was already functioning across the graft union (Yin et al., 2012; Koepke and Dhingra, 2013). This is an indication that new xylem and phloem formation follow the same pattern described by Dengler (2001) in normal shoots and roots.

Phenolic compounds and their metabolic interactions

Phenolic compounds are secondary metabolites present in all higher plants (Harborne, 1994) that are involved in a number of metabolic pathways, interacting with the plant environment and with plant pathogens (Errea, 1998; Pina et al., 2011). It is well known that genes encoding for the enzymes of these pathways, like phenylalanine ammonia-lyase (PAL), polyphenol oxidase (PPO), and peroxidases (POD), are developmentally and tissue-specifically regulated and may be induced by environmental stresses (Pina and Errea, 2008). Phenolic compounds, particularly the ortodiphenols, are susceptible to oxidation by proteins coded by these genes, resulting in the production of quinones and polymeric melanins (Errea, 1998). This process and the reduction of cinnamic acids to their corresponding alcohols to produce lignin is a common phenomenon in the plant kingdom.

There is a group of phenolic compounds, flavonols (catechins and proanthocyanidins), that have important roles in woody plants, including the relationships between rootstock and scion (Treutter and Feucht, 1988; Stafford, 1990; Treutter and Feucht, 1991; Harborne, 1994; Errea, 1998; Salvatierra et al., 1999; Usenik et al., 2006; Martínez-Ballesta et al., 2010). *trans*-Cinnamic acid, a metabolite derived from phenylalanine by the action of PAL, is generally recognized as a marker of environmental stress and a potential site for pathway regulation during the synthesis of flavonoid compounds, xylogenesis, and formation of lignin, one of the main cell wall polymers (Rogers and Campbell, 2004). Pina and Errea (2008) demonstrated for the first time that the level of PAL transcription is enhanced in incompatible *Prunus* unions, resulting in an accumulation of phenol. In incompatible stock/scion combinations, the cellular system is weakened, resulting in loss of structure of the vacuolar membrane, where flavonols are located,

due to lipid peroxidation (Torel et al., 1986). Phenols escape from the vacuole into the cytoplasmic matrix where they are oxidized by peroxidases and phenol oxidases (Hartmann et al., 2002). As a result, ortho-quinones are formed, which may polymerize to become toxic compounds in a number of chemical reactions (Poëssel et al., 1980). In turn, these ortho-quinones can act as crosslinking agents with nucleophilic groups in proteins and other macromolecules, forming irreversible unions that affect the later development of the lignin pathway (Buchloh, 1961; Haslam and Lilley, 1986), impairing a good connection between the stock and scion (Poëssel et al., 1980; Feucht and Treutter, 1991; Pina and Errea, 2005).

The phenylpropanoid pathway has been identified as one of those responsible for physiological failure in stock-scion combinations where *Prunus armeniaca* L. is involved (Pina et al., 2011).

The role of hormones

Endogenous plant hormones are thought to be involved in regulating the complex relationships between rootstock and scion (Sorice et al., 2002; Aloni et al., 2010; Koepke and Dhingra, 2013). In vascular regeneration experiments, when auxins were applied exogenously to stem segments, low concentrations (0.1%, w/w) of indole-3-acetic acid (IAA) stimulated phloem differentiation, whereas higher levels (1.0%, w/w) induced xylem differentiation (Aloni, 1995; 2001; 2010). Likewise, in grafting experiments, an important group of substance involved in the development of compatible graft unions are the auxins released from the vascular strands of the stock and the scion, which induce the differentiation of vascular tissues, thus functioning as morphogenic substances (Aloni, 1987; Mattsson et al., 2003). Auxin translocation from the scion to the stock were found to accelerate the development of a successful graft union in *Cactus* (Shimomura and Fujihara, 1977).

Aloni et al. (2008; 2010) showed that, in grafted *Cucurbita*, exogenous application of high concentrations ($\geq 10^{-7}$ M) of 1-naphthaleneacetic acid (NAA), a synthetic auxin, to the root caused inhibition of root growth. Root growth of incompatible grafts was more affected than that of compatible ones. NAA was also applied directly to the roots of hydroponically grown grafted plants, which caused root decay in incompatible grafts but less harm in compatible ones. The involvement of auxin in the incompatibility mechanism emerged from three additional observations: (i) endogenous IAA analysis revealed that the roots and stems of incompatible combinations contained higher IAA concentrations than the same tissues of compatible ones; (ii) application of the auxin transport inhibitor, 2,3,5-triiodobenzoic acid, to the stems of grafted plants negated root degradation in incompatible combinations, whereas it had only

slight effect on compatible graft combinations; and (iii) root and shoot development of incompatible grafts was normal after blocking basipetal IAA transport by partial stem girdling (Aloni et al., 2008). These results support the hypothesis that auxin produced in the scion is translocated downwards to the root after the graft connection is established, where, after reaching a threshold concentration, auxin triggers degradative processes causing root decay (Aloni, 2010; Aloni et al., 2010).

In *P. tomentosa* (stock) and *P. armeniaca* (scion), a differential accumulation of flavon-3-ols occurring during the early stages of grafting was related to problems in the differentiation of callus (Errea et al., 1994; Salvatierra et al., 1999) and catechin, a flavonol, is known to be a protective agent against IAA oxidation (Lee and Oda, 2003; Parham and Kaustinen, 1977; Salvatierra et al., 1999) and can modulate the IAA oxidase level (Salvatierra et al., 1999). Moreover, the synthesis of prunasin, another flavon-3-ol, can determine incompatibility in *Prunus* (Moing and Carde, 1988; Stafford, 1990) and be stimulated by ABA and GA (Treutter and Feucht, 1991; Salvatierra et al., 1999; Aloni et al., 2010). Similarly, in phloem tissues of heterografts, the increase in prunin above the union indicates that the rootstock influences the normal gradient of phenolic compounds (Salvatierra et al., 1999). Feucht et al. (1988) also noticed that prunin caused less callus growth and inhibited xylogenesis and peroxidase activity. In xylem tissue, prunin and naringenin, which accumulated at the graft union of heterograft trees, could affect wound healing. Although less naringenin than prunin was found, this relatively small amount of naringenin may still be enough to induce dysfunction at the interface between two cells (Errea et al., 1994). Naringenin was found to function as an inhibitor of the growth promoting activity of gibberellin while also stimulating IAA oxidase (Feucht et al., 1988).

CONCLUSIONS

To date, several studies have been performed in order to understand the mechanism of graft incompatibility and yet there are still elements not fully understood. These studies refer to cytological and biochemical responses that occur early in the graft, as well as their consequences for the formation of tissues that lead to vascular connection. However, the vascular connection by itself does not ensure compatibility. Mechanisms such as cell recognition, connections between conductive tissues from rootstocks and scions, the presence of growth regulators and their interactions with others metabolites like phenols or enzymes are still enigmatic. In this way, when compatible and incompatible graft unions in *Prunus*

species are compared, clear differences exist from an early stage of callus differentiation into cambium and vascular tissues, which in compatible unions proceed rapidly. In the other hand, in incompatible unions a large proportion of the callus does not differentiate. The fact that this process could be detected at an early phase of graft development, together with the advantages of new metabolomic approaches to identify metabolic interactions, opens new strategies for the early detection of incompatibility in fruit trees. Moreover, the identification of key genes involved in graft incompatibility, like PAL, offer feasible tools to develop early selection strategies in long term breeding programs. In short, research is continually delivering new insights in this area that will contribute to better explaining the mechanism of graft incompatibility in fruit species, providing new challenges together with new techniques to better understand this phenomenon.

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