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*Review*

# *Agrobacterium*-induced hypersensitive necrotic reaction in plant cells: a resistance response against *Agrobacterium*-mediated DNA transfer

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**High necrosis and poor survival rate of target plant tissues are some of the major factors that affect the efficiency of *Agrobacterium*-mediated T-DNA transfer into plant cells. These factors may be the result of, or linked to, hypersensitive defense reaction in plants to *Agrobacterium* infection, which may involve the recognition of specific signals from the *Agrobacterium* that triggers the burst of reactive oxygen species at the infection site. Evidences of *Agrobacterium*-induced necrosis in target plant tissues and its link to reactive oxygen species are presented. Application of antioxidants, addition of acetosyringone and optimization of pre-culture conditions suppress the *Agrobacterium*-induced hypersensitive necrotic response in target plant tissues, thereby enhancing stable transformation.**

**Key words:** *Agrobacterium*; hypersensitive reaction; necrosis; signal transduction; oxidative burst; transformation.

## INTRODUCTION

Genetic transformation has become an important tool for crop improvement. At present time gene transfer by *Agrobacterium* is the established method of choice for the genetic transformation of most plant species. Compared to direct gene transfer methodologies (particle bombardment, electroporation, etc), *Agrobacterium*-mediated transformation offers several advantages such as the possibility to transfer only one or few copies of DNA fragments carrying the genes of interest at higher efficiencies with lower cost and the transfer of very large DNA fragments with minimal rearrangement (Hiei et al., 1997; Gheysen et al., 1998; Hansen and Wright, 1999; Shibata and Liu, 2000). The most important advantage, however, is the possibility of producing transgenic plants, which are free of marker genes (Komari et al., 1996; Mathews et al., 2001). This has and will continue to have enormous implications with regards to approval by regulatory agencies, public acceptance and marketability of transgenic crops.

Recent advances in molecular biology of *Agrobacterium*-mediated transformation have improved our understanding of the mechanisms of recognition, induction of vir genes and transfer of T -DNA into plant cells by the *Agrobacterium* (Gustavo et al., 1998). However, the efficiency of *Agrobacterium*-mediated T-DNA transfer to plant cells depends not only on the successful recognition and colonization of plant cells by the *Agrobacterium*, but also on the responses of the plant cells to the *Agrobacterium* infection process

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(Zambryski, 1988; Binns, 1990). *Agrobacterium*-mediated transformation involves interaction between two biological systems and is affected by various physiological conditions (Bhalla and Smith, 1998).

Plant cells are known to possess the ability of recognizing invading pathogens and activating defense signal transduction leading to hypersensitive necrotic responses (Lamb et al., 1989; Mehdy, 1994; Dangl et al., 1996; Hammond-Kosack and Jones, 1996; Blumwald et al., 1998; Somssich and Hahlbrock, 1998; Richter and Ronald, 2000). The relationship of *Agrobacterium* to host plants is unique among plant pathogens. Many aspects of the plant-*Agrobacterium* interaction are not yet fully understood. It was earlier reported that *Agrobacterium* does not induce the hypersensitive response in target plants, even though the bacterium introduces several proteins into the host cell (Robinette and Matthysse, 1990). However, there are now several reports of high necrosis and poor survival rate of target plant tissues during the process of *Agrobacterium*-mediated T-DNA transfer (Pu and Goodman, 1992; Deng et al., 1995; Perl et al., 1996; Mercuri et al., 2000; Chakrabarty et al., 2002; Das et al., 2002). This could be the consequence of plant's hypersensitive reaction to *Agrobacterium* infection. Recently, it was demonstrated that plants can modulate their gene expression in response to *Agrobacterium* infection and that *Agrobacterium* can actually trigger the plant defense machinery (Ditt et al., 2001).

Hypersensitive reaction (HR) is known to be one of the plant defense responses and it is generally characterized by a rapid, localized cell death around the infection site and the accumulation of antimicrobial agents (Hammond-Kosack and Jones, 1996; Richter and Ronald, 2000). It is the sequence of events during HR that subsequently lead to necrosis of the collapsed cells (Goodman and Novacky, 1994). In this review article, the mechanisms by which the plant cells perceive and transduce signals from *Agrobacterium* tumefaciens to activate hypersensitive defense responses are suggested. The implications of *Agrobacterium*-induced plant defense responses for stable transformation are discussed and methods to suppress the defense responses proposed.

## *AGROBACTERIUM*-INDUCES NECROSIS AND CELL DEATH IN INVADED PLANT TISSUES

Plant tissue necrosis and cell death is reported to be one of the major factors that reduce the efficiency of *Agrobacterium*-mediated transformation (Gustavo et al., 1998), and is often observed in many crops. Pu and Goodman (1992) and Sangwan et al. (1992) were among the first investigators to report on *Agrobacterium*-induced necrosis in plant tissues. While Pu and Goodman (1992) observed *Agrobacterium*-induced necrosis in tissues of grape explants, Sangwan et al. (1992) reported that *Agrobacterium* infection led to necrosis in target cells of Arabidopsis thaliana. The role of T-DNA genes in the induction of necrosis in host tissues was later demonstrated (Deng et al., 1995) in an experiment with grape plants. Perl et al. (1996), also experimenting with grape tissues, observed that co-cultivation with *Agrobacterium* resulted in host tissue necrosis and mortality. Interestingly, the necrotic response of the grape calli was observed not during co-cultivation, but 48 h after transfer of calli to Agrobacteria-free medium. These observations of *Agrobacterium*-induced necrosis in target plant tissues are gradually generating lots of interest among researchers. Recently, Hansen (2000) worked with maize tissues and observed that co-cultivation with *Agrobacterium* leads to rapid tissue necrosis and cell death. High tissue necrosis was also reported on leaf-discs of grape after co-cultivation with *Agrobacterium* (Das et al., 2002). In this case, the degree of necrotic reaction appears to depend on several transformation parameters, including explant age, pre-culture period, bacterial inoculum density, and infection duration. This confirms the earlier observations of Kumria et al. (2001) that high bacterial density ($A_{600}$ = 0.7 – 1.0 with 10 min infection) or prolonged infection time (15-30 min with the optimal $A_{600}$ = 0.3 – 0.6) adversely affect the growth and regeneration of callus of Indica rice during *Agrobacterium*-mediated transformation. Similarly, Chakrabarty et al. (2002) reported that exposure of cauliflower hypocotyl explants to undiluted culture of *Agrobacterium* ($OD_{600}$ = 0.5) resulted in severe necrosis of the explants whereas diluted culture (1:10 and 1:20 dilution) reduced necrosis to greater extent. Also, the hypocotyls were hypersensitive to *Agrobacterium* infection when no pre-culture was allowed and necrotic reaction was enhanced on explants from 4-day-old seedlings in comparison to 7-day-old seedlings.

It appears, therefore, that exposure of plant tissues to *Agrobacterium* leads to tissue necrosis and cell death, which may invariably affect transformation efficiency. First, necrosis and cell death may occur in the cell layer where T-DNA is transferred. Transgenic cells that are imbedded in such necrotic tissues may be inhibited with regards to regeneration, thus reducing the recovery of transgenic cell clones (Potrykus, 1990). Necrotic tissues are also known to accumulate antimicrobial substances (Goodman and Novacky, 1994) that may inhibit the potential of *Agrobacterium* to colonize plant cells and transfer T-DNA. The active release of chemical signal, which induces the vir genes in *Agrobacterium*, occurs only in living, but not in dead necrotic cells (Shaw et al., 1991). Moreover, dead necrotic cells may also attract opportunistic microorganisms under in vitro conditions, leading to serious contamination that subsequently inhibits plant regeneration. Therefore, necrosis in host plant tissue during *Agrobacterium*-mediated T-DNA transfer drastically reduces transformation efficiency.

The optimization of Agrobacterium-mediated transfor-

systems may therefore require proper understanding of regulatory mechanisms of the *Agrobacterium*-induced necrotic reaction in plants.

## PLANT TISSUE NECROSIS AND CELL DEATH AS DEFENSE MECHANISM AGAINST *AGROBACTERIUM*- MEDIATED GENE TRANSFER

### Plant perception of signals from *Agrobacterium*

An efficient plant defense response usually requires the recognition of specific signal molecules from the invading pathogen (Blumwald et al., 1998; Richter and Ronald, 2000). The presence of chemical signaling between *Agrobacterium* and plant cell has been suggested earlier (Chilton, 1993). Though there are several reports on plant-excreted signals that induce *Agrobacterium* infection (Citovsky et al., 1982; Bolten et al., 1986; Cangelosi et al., 1990; Shaw et al., 1991), we have not come across any report of a concerted study on the signal molecules from the *Agrobacterium* that may elicit defense response in target plant tissues. However, there are reports on genotype-strain specificity during *Agrobacterium*-mediated transformation of plants (Owens and Cress, 1985; Byrne et al., 1987; Hobbs et al., 1989; Fillipone and Penza, 1992), which may indicate the presence of specific signals from specific *Agrobacterium* strain that could be recognized by specific plant genotype. Each *Agrobacterium*-susceptible plant cell (competent cell) has been shown to contain polysaccharide-polysaccharide binding sites recognizable by *Agrobacterium* (Sangwan et al., 1992). It has earlier been shown that the first step in the transfer of T-DNA molecule from *Agrobacterium* to plant is the recognition of a susceptible plant cell (Zambryski, 1988). Therefore, plant cell can be highly susceptible or non-susceptible to *Agrobacterium* infection, depending on the genotype of the host plant and the strain of the *Agrobacterium* (Jordan and Hobbs, 1994).

Non-susceptibility of plant cells to colonization by pathogen is known to be due to successful recognition of the invading pathogen by the plant cell, which generates an internal signal that triggers early defense responses in the plant cells (Somssich and Hahlbrock, 1998). This may also explain the non-susceptibility of plant cells of particular genotypes to infection by particular strain of *Agrobacterium* (Hobbs et al., 1989). The earliest defense reaction observed in non-susceptible plant cells following pathogen attack is oxidative burst (Mehdy, 1994).

### *Agrobacterium*-induced oxidative burst in target plant cells

Oxidative burst is the large and rapid generation of reactive oxygen species (superoxide, hydrogen peroxide, hydroxyl, peroxyl and alkoxyl radicals), which can cause cell damage. It is now widely accepted that the key component of the oxidative burst is hydrogen peroxide. The well- known reactivity of hydrogen peroxide is not due to its reactivity per se, but requires the presence of a metal reductant to form the highly reactive hydroxyl radical, which is the strongest oxidizing agent known and reacts with organic molecules at diffusion-limited rates (Mehdy, 1994; McKersie, 1996). Numerous enzymes use hydrogen peroxide as a substrate in oxidation reactions, but the most prominent among the enzymes is peroxidase. Perl et al. (1996) observed that elevated levels of peroxidase activity in grape tissues correlated with *Agrobacterium*-induced necrosis in the host tissues during *Agrobacterium*- mediated transformation. Interestingly, the increase in peroxidase activity in the target grape tissue was always observed, not before or during co-cultivation with *Agrobacterium*, but 24-36 h after co-cultivation.

Peroxidase is known to mediate oxidative cross-linking of structural proteins in the cell wall (Somssich and Hahlbrock, 1998), and the *Agrobacterium*-induced increase in peroxidase activity in grape tissues could therefore confirm the role of oxidative burst in hypersensitive necrotic responses in plant to *Agrobacterium* infection. The reactive oxygen species (ROS) produced during pathogen-induced oxidative burst could be toxic enough to directly kill the attacking *Agrobacterium* (Wojtaszek, 1997). ROS can also lead to the induction of pathogenesis-related (PR) proteins (Mehdy, 1994), that may inhibit the potential of *Agrobacterium* to colonize and transfer T-DNA to plant cells. Transgene inactivation has been reported as a defense response of plants for expression of foreign DNA (Finnegan and McElroy, 1994), and ROS may play a role in such defense response. This is because the sugar and the base moieties of DNA are susceptible to oxidation by the hydroxyl radical, causing base degradation, single strand breakage, and cross-linking of DNA to protein (McKersie, 1996).

Therefore, it could be speculated that during incompatible plant-*Agrobacterium* interaction, the following sequence of events may occur in the target plant tissues: the first step is perception of specific signal(s) from the invading *Agrobacterium*, followed by the over-production of ROS (oxidative burst) at the site of *Agrobacterium* infection. Then the generated oxygen radicals may lead to plant cell death and necrosis, bacterial cell death, induction of pathogenesis-related genes, followed by the production of antimicrobial substances (phytoalexins, etc) and oxidation of sugar and base moieties of DNA. Therefore, proper understanding of these plant defense signal transduction events could assist in the development of strategies to suppress the *Agrobacterium*-induced defense responses and enhance the efficiency of *Agrobacterium*-mediated transformation, especially in 'recalcitrant' species.

## CONTROLLING PLANT DEFENSE RESPONSES AGAINST *AGROBACTERIUM*-MEDIATED T-DNA TRANSFER

There are reports on successful experiments for optimization of transformation protocols. The strategies employed in such experiments can be grouped into two: 1. Quenching of the *Agrobacterium*-induced oxidative burst; and 2. Reprogramming of an *Agrobacterium*-incompetent plant cell into a competent one.

### Quenching of *Agrobacterium*-induced oxidative burst

The activity of oxidative burst in plant defense responses could be suppressed by the addition of antioxidants such as ascorbic acid, cysteine, citric acid, PVPP, PVP, DTT, and cyclitols (myo-inositol). Some of these compounds are known to scavenge reactive oxygen species, thereby quenching oxidative burst. The application of a mixture of antioxidants has been shown to improve the efficiency of *Agrobacterium*-mediated transformation in some crops (Perl et al., 1996; Das et al., 2002). The combination of PVPP and DTT was found by Perl et al. (1996) to improve the viability of embryogenic grape calli. They observed that tissue necrosis was completely inhibited by these antioxidants while *Agrobacterium* virulence was not affected. In their review of *Agrobacterium*- mediated transformation of plants, Gustavo et al. (1998) reported that efficient transformation of monocotyledonous crops like sugarcane was possible only when a mixture of antinecrotic compounds with remarkable antioxidative activity was added. Recently, Das et al. (2002) applied the double-layer antioxidant method as described by Perl et al. (1996) to control the problem of *Agrobacterium*-induced necrosis during transformation of grape leaf-discs. Therefore, compounds with potential to quench oxidative burst could be used to arrest *Agrobacterium*-induced necrosis in host tissues, thereby improving transformation efficiency.

### Reprogramming a resistant plant cell into a susceptible one

To make plant tissues susceptible to *Agrobacterium* infection, they have to be induced to undergo cellular dedifferentiation (Sangwan et al., 1992). Pathogen recognition may be weakened in dedifferentiating cells probably due to perturbation of membrane structure. Sangwan et al. (1992) explained that pre-culture of explants prior to *Agrobacterium* infection on media containing auxin with or without cytokinins is a good method of inducing cells to undergo dedifferentiation and may serve as rejuvenating treatment to the explant. Juvenile plant cells may be more susceptible to *Agrobacterium* infection than differentiated old cells. Pre-culture treatment has recently been shown to improve the efficiency of *Agrobacterium*- mediated transformation (Chakrabarty et al., 2002; Wu et al., 2003).

Another approach of reprogramming plant cell development is wounding. Wounding is the most effective biological trigger for shifting cells potentially competent for regeneration to the competent state (Potrykus, 1990). Wound healing has earlier been shown to trigger a sequence of reactions at the cellular level that is important for T-DNA- induced transformation (Binns and Thomashow, 1988). The ability of wounded plant cells to enter and carry out one or more cell cycles may be absolutely required for successful transformation (Binns, 1990; Sangwan et al. 1992). Apart from stimulating dedifferentiation, wounding also leads to the excretion of chemical signal that induces *Agrobacterium* infection (Citovsky et al., 1982; Kahl, 1982; Bolten et al., 1986; Potrykus, 1990; Shaw et al., 1991). The wound-exuded chemical signals are phenolic compounds, like acetosyringone. However, monocots do not show the wound response characteristic of the dicot species (Binns, 1990) . Moreover, Escudero and Hohn (1997) demonstrated that the competence of plant cells for *Agrobacterium*-mediated DNA transfer is not necessarily linked to wounding. In this case, exogenous acetosyringone, added in the inoculation and co-cultivation media, replaced the need for wounding.

Apart from inducing vir genes in the *Agrobacterium* (Cangelosi et al., 1990), it is possible that acetosyringone also perturb the *Agrobacterium*-induced defense signal transduction events in plant cells, leading to reprogramming of *Agrobacterium*-incompetent cell to a competent one. In several experiments with monocots and other 'recalcitrant' species, addition of acetosyringone to the inoculation and co-cultivation media improved the efficiency of *Agrobacterium*-mediated transformation (May et al., 1995; Kumria et al., 2001; Mahmoudian et al., 2002; Wu et al., 2003).

It appears, therefore, that with proper optimization of transformation parameters, like duration of pre-culture and addition of acetosyringone (Wu et al., 2003), plant cells of any species could be made competent for *Agrobacterium*-mediated transformation. It was, for example, previously suggested that cereals cannot be transformed by *Agrobacterium* (Potrykus, 1990), but with proper manipulation of transformation parameters, large number of fertile transgenic rice were produced through *Agrobacterium*-mediated DNA transfer system (Hiei et al., 1994; Rashid et al., 1996).

## CONCLUSION

This review shows that *Agrobacterium*-induced necrosis often observed in target plant tissues is linked to

hypersensitive defense reaction in plants to *Agrobacterium* infection. The plant defense mechanisms against *Agrobacterium* involve successful recognition of some sort of signals from the *Agrobacterium* which triggers oxidative burst at the infection site. The HR-inducing factor in *Agrobacterium* is yet to be fully understood. However, Zheng et al. (2003) have recently discovered a gene in *Agrobacterium* vitis that is associated with *Agrobacterium*-induced HR. This gene, aviR, is said to be homologous to luxR, which implies that the *Agrobacterium*-induced HR is regulated by a quorum- sensing mechanism. The *Agrobacterium*-induced HR could lead to rapid and large generation of reactive oxygen radicals in target plant cells, resulting to plant cell death (necrosis), oxidative stress to the invading *Agrobacterium* cells, production of toxic antibacterial substances and the deleterious effects on DNA molecules, especially at the site of oxidative burst. All these factors significantly reduce the efficiency of stable transformation of plants. Therefore, detoxification of the ROS with carefully selected mixture of compounds with high antioxidative activity, and, reprogramming a non-susceptible plant cell into an *Agrobacterium*-competent cell by wounding, addition of acetosyringone and optimization of pre-culture conditions, are possible methods for improving the efficiency of *Agrobacterium*-mediated transformation, especially in 'recalcitrant' crops.

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