Plant Cell Rep (2010) 29:1203–1213
DOI 10.1007/s00299-010-0900-2

REVIEW

Aminoglycoside antibiotics: structure, functions and effects on in vitro plant culture and genetic transformation protocols

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Received: 20 May 2010 / Revised: 6 July 2010 / Accepted: 6 July 2010 / Published online: 20 July 2010
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Abstract Plant transformation protocols generally involve the use of selectable marker genes for the screening of transgenic material. The bacterial gene *nptII*, coding for a neomycin phosphotransferase, and the *hpt* gene, coding for a hygromycin phosphotransferase, are frequently used. These enzymes detoxify aminoglycoside antibiotics by phosphorylation, thereby permitting cell growth in the presence of antibiotics. Nevertheless, the screening for transgenic regenerated shoots is often partial and difficult due to regeneration of escapes and chimeras. These difficulties can be caused, in part, by an incorrect assumption about the mode of action of antibiotics in bacterial and eukaryotic cells and in in vitro tissue culture. The information contained in this review could be useful to establish better selection strategies by taking into account factors such as explant complexity, transformation and selection protocols that allow better accessibility to cells of *Agrobacterium* and antibiotics, and faster regeneration methods that avoid collateral effects of antibiotics on recovered, putative transgenic shoots.

Keywords In vitro · Plant tissue culture · Explants · Selection · Marker genes

Communicated by R. Reski.

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Introduction

Plant transformation protocols generally involve the use of a selectable marker gene for the screening of transgenic material. This gene is often the bacterial gene *nptII*, coding for a neomycin phosphotransferase, or the *hpt* gene, which codes for the hygromycin phosphotransferase. These enzymes detoxify aminoglycoside antibiotics by phosphorylation, thereby permitting cell growth in the presence of antibiotics from the same family, such as kanamycin, paromomycin and gentamicin in the case of *nptII* or hygromycin B in the case of *hpt*. These antibiotics affect plant regeneration negatively, to varying degrees, in non-transgenic material via inhibition of protein synthesis in the chloroplast. Aminoglycosides are therefore commonly used as selective agents in transformation protocols for a wide range of species. Nevertheless, the screening for transgenic regenerated shoots is often partial and difficult due to regeneration of escapes and chimeras. These difficulties can be, in part, due to a wrongful assumption about the mode of action of antibiotics because; in fact, we do not know what aminoglycoside antibiotics do exactly to the plant material and how this affects the regeneration/transformation protocols. In this article, we review the current knowledge about aminoglycosides, their mode of action against bacterial cells, their effects on eukaryotic cells and tissues cultured in vitro to show how a good selection protocol for plant transformation might be established.

What we know about aminoglycoside antibiotics

The aminoglycoside–aminocyclitol antibiotics are a large family of water-soluble, cationic molecules (weak bases) that exhibit broad antimicrobial spectra. Aminoglycosides

fall into different structural classes. The first class, containing a 2-deoxystreptamine ring, includes antibiotics with a 4,6-disubstituted deoxystreptamine ring, such as kanamycin, gentamicin or geneticin (Fig. 1a) or antibiotics with a 4,5-disubstituted deoxystreptamine, as neomycin or paromomycin (Fig. 1b). Other classes include streptomycin that contains a streptidine ring (Fig. 1c) or hygromycin B that contains a hyosamine ring (Fig. 1d). The antibiotic spectinomycin contains only aminocyclitol rings, and for this reason it not considered an aminoglycoside antibiotic. Many of these compounds are natural products, produced primarily by bacteria of the actinomycetes group, but several are semi-synthetic derivatives (e.g., amikacin). The mode of action of aminoglycosides includes interaction with ribosomes, causing a lack of fidelity in the translation process, which seems to be due to the 2-deoxystreptamine and the primed amino sugar structure (Fourmy et al. 1996). Compounds of the streptomycin family are related to 2-deoxystreptamines and have a similar mode of action, but a different binding site (Moazed and Noller 1987). Besides this, aminoglycosides, due to their chemical structure, have quite high affinities for a wide range of compounds and can precipitate a number of these and adsorb to others. Among these are the cell surface, DNA, RNA, serum proteins, phosphatidyl ethanolamine, casein, cellulose and nitrocellulose filters (Hancock 1981a). One of the major advantages of the aminoglycoside–aminocyclitol antibiotics is that they are bactericidal agents causing cell death, with only a few exceptions such as spectinomycin, which is bacteriostatic.

Mode of action of aminoglycoside antibiotics

Aminoglycosides are taken up in three phases by bacterial cells (Bryan and van den Elzen 1975, 1976): an initial ionic binding to cells, followed by two energy-dependent phases (called EDP-I and EDP-II) in which there is an apparent invigorated uptake of aminoglycoside at a slow rate (EDP-I) followed by a very rapid, energy-dependent accumulation

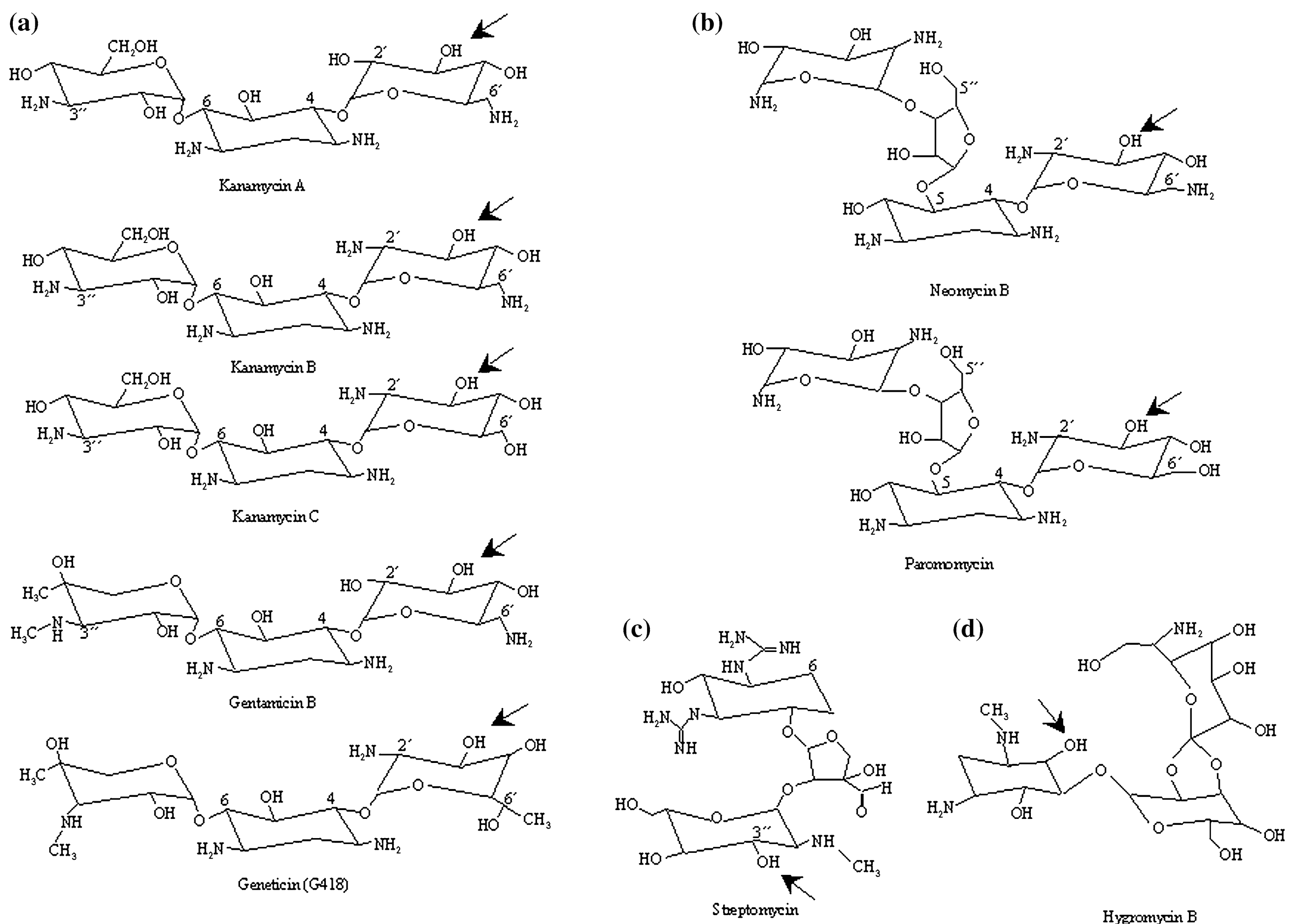

Fig. 1 Classification by chemical structure of the most common aminoglycoside antibiotics used in plant transformation: **a** 4,6-disubstituted deoxystreptamine; **b** 4,5-disubstituted deoxystreptamine; **c** streptidine; **d** hyosamine. The *arrow* indicates the hydroxyl group, which is the site of phosphorylation of the NPTII enzyme or HPT enzyme (hygromycin B)

of the aminoglycoside (EDP-II). Davis (1988) proposed a unified theory for the bactericidal action of aminoglycosides, which suggests that a small amount of aminoglycoside antibiotic penetrates the cell during the EDP-I phase and the beginning of EDP-II and binds to susceptible ribosomes, which results in the misreading of mRNA and the formation of misfolded, non-functional proteins. These proteins can interact with the membrane, resulting in loss of membrane integrity. This causes ion efflux and the formation of pores, precipitating an additional, irreversible influx of antibiotic that saturates all ribosomes in an irreversible fashion, resulting in cell death. This theory has been accepted widely by researchers and its assumptions applied to eukaryotic cells; however, it does not explain either the precise mechanism of bacterial cell death or the many observed aminoglycosides effects on mammalian and plant cells.

Interaction with the cytoplasmic membrane

The uptake of antibiotics into cells is a key element in their functioning, but many gaps exist in our knowledge of the mode of action of aminoglycosides regarding their interaction with the cytoplasmic membrane in both prokaryote and eukaryote cells.

In plant transformation, transgenic cells are selected because they contain the antibiotic resistance gene; however, aminoglycosides affect transgenic and non-transgenic cells, interacting with the cell cytoplasmic membrane in different ways. In mammalian cells, aminoglycoside permeability varies significantly among different cell types. Fluid phase pinocytosis has been shown to be the main mode of uptake of aminoglycoside antibiotics by fibroblasts, although non-specific adsorptive uptake is also probable. It is not clear how aminoglycosides reach their target in the cytosol in sufficient quantities, since aminoglycosides in general are too polar to cross mammalian cell membranes by diffusion. We have not been able to find references about the mode of uptake of aminoglycoside antibiotics by plant cells and, although a situation similar to that of mammalian cells can be assumed, whether aminoglycosides interact with the cell wall is unknown.

In in vitro cultures of mammalian cells, it has been shown that aminoglycosides induce blockage of Ca^{2+} channels (Parsons et al. 1992) and alter the electrogenic transport properties of cultured cells (Todd et al. 1992) and that their effects are calcium receptor mediated (Maldonado-Perez et al. 2006). Aminoglycoside antibiotics bind strongly to phosphoinositides, which seem to be involved in cell proliferation (Ozaki et al. 2000) and affect their membrane distribution and metabolism (Buchanan et al. 1987; Jiang et al. 2006; Morris and Mensa-Wilmot 1997). Furthermore, neomycin and other aminoglycoside antibiotics interact with a range of animal calcium-permeable or cationic channels that are characterized by a wide pore (Nomura et al. 1990; Winegar et al. 1996; Haws et al. 1996; Pichler et al. 1996; Shi et al. 2002; Mead and Williams 2004; Yeiser et al. 2004; Raisinghani and Premkumar 2005). Additionally, pharmacological studies revealed that aminoglycosides could also impinge on the activity of ionotropic glutamate receptors (iGluR) (Harvey and Skolnick 1999; Masuko et al. 1999). The degree of stimulation paralleled the number of amino groups in the aminoglycosides (Masuko et al. 1999).

In plant cells, several studies have provided evidence for the interaction of aminoglycosides with the plasma membrane and/or membrane receptors. Thus, it has been shown that gated Ca^{2+} channels with iGluR-like activity in plants are sensitive to the antibiotic action of aminoglycosides (Dubos et al. 2005). As these iGluRs play an important role in plant development (plant resource allocation to vegetative development, starch and lignin biosynthesis) (Dubos et al. 2003), aminoglycosides could induce changes in the development of the plant by acting through these plant iGluRs underlining the similarities in iGluR agonism between animals and plants. Additionally, it has even been shown that neomycin blocks the slow vacuolar channel in vacuoles from *Arabidopsis thaliana* mesophyll cells in a voltage-dependent manner and shifts the threshold of channel activation to more negative values, while kanamycin blocks these channels less effectively than neomycin (Scholz-Starke et al. 2006). These interactions of aminoglycosides with the cytoplasmic membrane could explain the adverse effects of antibiotics in transgenic lines. Perhaps encapsulation of aminoglycosides in nanostructures, as tested in bacteria in vitro (Saha et al. 2007) and in vivo (Ranjan et al. 2009), would reduce antibiotic toxicity by avoiding these aminoglycosides by cell membrane interactions and result in improved selection. On the other hand, multidrug resistance proteins located in the membrane, which pump antibiotics out of the cell, have been described in bacterial (De Rossi et al. 2006) and mammalian (Notenboom et al. 2006) cells. In plants, Mentewab and Stewart (2005) showed that an *Arabidopsis* kanamycin resistance gene encodes an ATP binding cassette (ABC) transporter, which could prevent ribosome inactivation by translocating kanamycin into the vacuole.

Effect on ribosomes

In bacterial cells, once aminoglycoside antibiotics reach the cytosol, the ribosome is the primary target via direct interaction with ribosomal RNA. Binding of the antibiotic modifies the shape of the ribosome when a tRNA is present at the A site (Jerinic and Joseph 2000). As a result, the error rate in amino acid incorporation is increased and

translocation of the tRNA–mRNA complex is inhibited (Noller 1991; Spahn and Prescott 1996) in vitro (Davies et al. 1965) and in vivo (Edelmann and Gallant 1977). However, aminoglycoside antibiotics interact with a great variety of RNA molecules and any biological function involving RNA is a potential target (Walter et al. 1999). Additionally, the entry of aminoglycosides into the cell has pleiotropic effects: K^+ efflux, transient stimulation of RNA synthesis, putrescine excretion, mistranslation of mRNA to yield aberrant proteins, depletion of polyribosomes, loss of cell membrane integrity resulting in increased permeability, inhibition of DNA replication and blockade of the initiation of DNA replication by disruption of ori-C-membrane interaction (Hancock 1981b; Wright et al. 1998). It has been suggested that the influence of aminoglycosides on cyclic AMP and guanosine pentaphosphate levels could explain some of these pleiotropic effects (Hancock 1981a), which do not necessarily constitute a lethal event (Hancock 1981b).

Aminoglycosides affect mitochondrial ribosomes of mammalian cells, and mitochondrial and chloroplastic ribosomes in plants, by suppression of translation termination and protein misreading (Kurtz 1974; Brummett and Fox 1989; Howard et al. 1996; Keeling et al. 2001; Mingeot-Leclercq and Tulkens 1999; Tiboni and Chinali 1997), but they may also affect protein synthesis at higher concentrations probably through non-specific binding to eukaryotic ribosomes and/or nucleic acids of target cells. Molecules with a hydroxyl function at C-6′ in place of an amino function, such as kanamycin C, gentamicin A and geneticin, are more effective inhibitors of eukaryotic protein synthesis (Fig. 1). Geneticin was shown to bind directly to the eukaryotic 80S ribosomal complex, while misreading in the cytoplasmic (80S) ribosome of plant cells has also been observed in a wheat embryo system in vitro (Wilhem et al. 1978). In this system, misreading occurred with certain antibiotics, which had clear structural similarities, whereas others had much lower activity. In mammalian cells, protein synthesis has also been shown to be affected to differing extents depending on the antibiotic used (Buchanan et al. 1987). Besides these effects on ribosomes, aminoglycosides can also induce reduction in the relative expression of mRNA in mammalian tissue (Stacey and McLean 2000), increase production of hydrogen peroxide, increase the rate of phospholipidosis, inhibit Na/K-ATPase, induce injury to organelles such as mitochondria and lysosomes (Ali 1995) and, for plant tissue, interfere not only with photosystem I, but also with ATP production in the mitochondria and with DNA structure and integrity (Teixeira da Silva and Fukai 2003), induce chlorophyll deficiency in seedlings, inhibit plastid translation and inhibit expression of hemoA photosynthetic genes (Yaronskaya et al. 2007). A unifying feature of most of these effects is membrane damage, which parallels the findings in bacteria.

Finally, resistance to antibiotics, very common in bacteria, has been associated also with plants. A point mutation in the 16S plastid rRNA conferred aminoglycosides resistance to *Nicotiana plumbaginifolia* (Yeh et al. 1994) and alfalfa (Rosellini et al. 2004). The same mutation was found in several kanamycin-resistant events in both species. The reason this mutation occurred repeatedly and consistently is unknown.

Interactions of aminoglycosides and other antibiotics with in vitro culture and regeneration methods

Species, genotype and explant nature

The effects of aminoglycosides on mammalian cells are well documented, with a huge amount of publications on the use of antibiotics in humans and animals. However, in plants there is little information, and aminoglycoside effects on plant cells are only known as a consequence of investigations dealing with plant genetic transformation in which these effects are not studied in depth. In mammals, kidney cells, hair cells from the inner ear and fibroblasts are the main cell types affected, while other cell types, such as human melanocytes, are not affected by relatively high concentrations of aminoglycosides (Halaban and Alfano 1984). A similar situation in plants with certain cell types more sensitive to antibiotics than others is to be expected, although there are no published studies.

Plant susceptibility to antibiotics seems to change broadly among species, genotypes and plant tissues. The effect of aminoglycosides during the regeneration process consists of inhibition of both cell proliferation and differentiation. It has been suggested that this depends on the in vitro experimental conditions during explant culture and regeneration (Lin et al. 1995; Joersbo and Okkels 1996a; Chauvin et al. 1999). The differing sensitivities of cotyledon, hypocotyl, leaf explants and callus to the same antibiotic have been reported widely and, at the same time, we have to expect that aminoglycosides will affect explants in different ways just on the basis of their different chemical structures. For example, the application of paromomycin instead of kanamycin ensures successful regeneration of some kanamycin-sensitive plant species (Mihaljevic et al. 2001). In apricot leaf regeneration, paromomycin allowed regeneration of buds, but these buds appeared ‘mummified’ during several subcultures, neither growing nor dying; kanamycin bleached the leaves and tissues and later killed the buds, whereas gentamicin killed the leaf explant and did not allow it to regenerate (Petri et al. 2005). Likewise, Joersbo and Okkels (1996b), working with sugar beet

cotyledons, found that hygromycin was 20- and 30 times more toxic than kanamycin and neomycin, respectively. Additionally, kanamycin affects callus proliferation from leaf explants in such a way that Jordan and McHughen (1988) designed an assay to recognize transgenic shoots from escapes using this principle. Schmitt et al. (1997) claimed that the incapacity of the callus to become morphogenetic and regenerate shoots could be attributed to an increase in the methylated DNA in response to antibiotic. An exogenously applied dose of antibiotics would mimic a pathogen attack, thereby inducing a defense reaction and hypermethylation, with subsequent cell death and decreased regeneration capacity. More interesting is the research described by Zhang et al. (2001) into the effect of kanamycin on tissue culture and somatic embryogenesis in cotton. These authors found that cotton embryogenic callus was less sensitive than non-embryogenic callus and that kanamycin reduced or even inhibited the initiation and development of somatic embryos. Thus, there was a reduction in the numbers of globular, heart-shaped and torpedo-shaped embryos, but not for the cotyledonary embryos that increased at all kanamycin concentrations up to 125 mg/l. They concluded that selection of transformed embryos was more difficult in mature embryos, which highlights the importance of explant complexity in antibiotic-based selection. This differential sensitivity to antibiotics has been observed also in olive embryogenic callus versus embryos (Perez-Barranco et al. 2009). One reason for this differential sensitivity to antibiotics among non-embryogenic tissues, embryogenic tissues and embryos could be due to changes in cell wall or plasma membrane composition when cells gain complexity. Endress et al. (2009) analyzed the cell wall polysaccharide composition of embryogenic and non-embryogenic calli from different explants of *Medicago arborea* and found significant differences, with higher levels of pectic polysaccharides and glucans on cell walls in embryogenic calli.

These results seem to be in agreement with those from assays of the phytotoxicity of agrochemical compounds (Zilkah and Gressel 1977; Zilkah et al. 1977), although in these investigations aminoglycoside antibiotics were not tested. Zilkah et al. (1977) studied the phytotoxic effects of a range of compounds using three systems: cell suspension, calli and seedlings of several species. They found significant differences in phytotoxicity between the three systems due to selective mechanisms that can occur at many levels; penetration, permeation, translocation, in vivo toxification or detoxification, differential existence of alternative metabolic pathways, lack of target, etc. Not all of these selective mechanisms are available in all systems, with cell suspension and calli being the most sensitive ones. Therefore, the greater the structure and complexity of a given explant, the more difficult it is to determine the phytotoxicity of a compound, since different selective mechanisms will prevent the compound reaching all the cells. Hence, and in relation to aminoglycoside transport in in vitro systems, Weide et al. (1989) observed that kanamycin transport through tissues of in vitro-germinated seedlings occurred over rather short distances and for this reason apical meristems did not die.

Another factor that could make the selection of transgenic shoots difficult is the presence of endogenous, non-specific kanamycin phosphotransferase activity in the tissues of some species (Dandekar 1992), which is supported by the many similarities found between APH(3′)-IIIa and eukaryotic proteins (Hon et al. 1997). The literature on transformation experiments describes species (i.e., monocots and legumes) that are very tolerant of or highly resistant to antibiotics, especially kanamycin (Hauptmann et al. 1988). However, it was shown later that these species could be transformed by using antibiotic selection with a different explant or different regeneration/transformation protocol (Oreifig et al. 2004; Scott et al. 1998; Grant et al. 1998; Polowick et al. 2000). These apparent contradictions indicate that generalization of the results obtained in a particular regeneration/transformation system in relation to aminoglycosides has to be done carefully, since many factors affect in vitro culture and the selection process. For instance, in tobacco plastid transformation, the seemingly trivial step of initiating plants from nodal cuttings rather than directly from seeds was necessary to generate leaves that were transformable; for example, plants grown in vitro from seeds developed slowly, with thin stems and shortened internodes, produced only spontaneous spectinomycin-resistant shoots and no transformants, while the bombardment of leaves taken from vigorously growing plants resulted in the regeneration of spectinomycin-resistant shoots (McCabe et al. 2008).

Interaction with components of the medium

In bacterial cells, aminoglycoside uptake is rate limiting and is blocked or inhibited by antagonists such as divalent cations (Mg^{2+}, Ca^{2+}), anions (nitrate, chloride, lactate, phosphate, tartrate, citrate, sulfate), salts ($MgCl_2$, $CaCl_2$, KCl, NaCl, sodium phosphate), polyamines (putrescine, cadaverine, spermidine), hyperosmolarity, low pH and anaerobiosis (Hancock 1981a). On the other hand, it has been demonstrated that EDTA, a chelator of divalent cations, and aminoglycosides act synergically against bacterial cells (Davis and Iannetta 1972). Many of these compounds are present in animal and plant tissue culture media and probably could interact with antibiotics, thus interfering with the selection process. In this way, calcium is implicated in a variety of processes in plant growth and development. Among other properties, Ca^{2+} ions have the

ability to interfere with the uptake of extracellular solutes such as Na^+ and K^+ (Epstein 1961). It has been suggested that this effect is due to decreased membrane permeability (Colmer and Fan 1994). In plant tissue culture, as observed in animal cell culture (Crawford 1972), calcium may interact with organic ions such as aminoglycosides (Joersbo and Okkels 1996b). Thus, the actual concentration of Ca^{2+} in the growth medium during selection is critical for the outcome of transformation experiments using aminoglycoside antibiotics because it has been shown that Ca^{2+} can alleviate and even abolish the toxic effects of aminoglycosides. The interaction between Ca^{2+} and different aminoglycoside antibiotics seems to be competitive, apparently specific and takes place at the level of the plasma membrane, where Ca^{2+} may be able to reduce binding and subsequent uptake of aminoglycosides. Regarding this effect of calcium in the medium, different authors found that explant sensitivity to kanamycin changed depending on the gelling agent added to the culture medium (Chauvin et al. 1999; Laine et al. 2000). They argued that differences in the uptake of the antibiotic resulting from competition for membrane transporters between Ca^{2+} ions and aminoglycosides could explain their results.

Positive effects of antibiotics on plant tissue

In contrast to the biocidal effects of antibiotics on bacteria and eukaryotic cells, certain antibiotics have been shown to stimulate plant growth and development, particularly at low concentrations (Holford and Newbury 1992); high concentrations are generally inhibitory. The first such report (De Ropp 1949) showed stimulated growth and induction of root production in bacteria-free cultures of *Helianthus annuus* and *Vinca rosea* due to additions of penicillin, penicillin G or streptomycin. Similarly, the aminoglycosides kanamycin and streptomycin enhanced shoot differentiation from leaf sections of tobacco while kanamycin also stimulated shoot production from carrot callus (Owens 1979) and improved regeneration from apricot leaves (Burgos and Alburquerque 2003) at low levels. Antibiotics used to control *Agrobacterium* growth in the medium also have similar behavior; for example, cefotaxime, alone or in combination with timentin or vancomycin, and vancomycin alone stimulated shoot organogenesis in apricot (Burgos and Alburquerque 2003). Cefotaxime also increased callus growth and organogenesis from wheat, barley and corn calli (Mathias and Boyd 1986; Mathias and Mukasa 1987; Danilova and Dolgikh 2004). In plum, timentin increased the regeneration percentage from hypocotyl explants by 25% (Padilla et al. 2003), but it was detrimental to regeneration from 'Helena' apricot leaves (Burgos and Alburquerque 2003). It has been suggested that some antibiotics or their parts mimic plant hormones. Holford and Newbury (1992) showed that concentrations of carbenicillin and penicillin G commonly used in plant tissue culture break down to give physiologically active levels of the auxin phenylacetic acid. This offers a mechanism for the stimulation of growth caused by these two antibiotics. However, the chemical structure of cefotaxime does not readily suggest a breakdown product with auxin-like properties, nor do the structures of the aminoglycosides and a further mode of action may have to be sought for these compounds. The plant hormone-like effects of antibiotics may complicate the hormone ratio in culture media and influence the *Agrobacterium*-mediated transformation efficiency by reducing the efficiency of regeneration (Lin et al. 1995).

Interactions with the regeneration/transformation system

Selection of transformants has met with little success in many species due to the lack of efficient protocols for regeneration of adventitious shoot buds from the transformed tissues, with many species described in the literature as antibiotic resistant or difficult to transform being recalcitrant to regeneration. This is still common in many species, not only woody, and has more impact on economically important cultivars. This has prompted some workers to adopt non-tissue culture-based approaches not dependent on the regeneration of adventitious shoot buds for generating transgenic plants (Rohini and Rao 2000; Youssef et al. 2007). On the other hand, in many cases, a good degree of regeneration protocol success in plant transformation is not sufficient because the regeneration and selection methods must be compatible. Many regeneration protocols based on callus regeneration have the handicap that they work better under dark conditions, but sometimes calli are less sensitive to antibiotics when grown in darkness (Lee et al. 2006), making the selection process difficult. A similar situation has been described for plastid transformation experiments based on regeneration of dark-grown calli, as transcription and translation levels have been shown to be lower in proplastids than in chloroplasts (Silhavy and Maliga 1998). Additionally, in dark conditions, the development of chloroplasts is inhibited, which results in lower levels of expression of selection marker genes that confer resistance to selective chemicals (Carrer et al. 1993). Lee et al. 2006 have pointed out that suitable selection markers and promoters that accumulate high transcript levels in both proplastids and chloroplasts, free from light and developmental regulation, are necessary for plastid transformation. The constitutive promoter Prrn has recently been used in

plastid transformation, with success in spectinomycin-based selection (McCabe et al. 2008).

Besides the fact that aminoglycoside interaction with medium components could produce a reduction/increase in antibiotic activity, it is clear that antibiotics lose their activity during the selection period. Although studies on antibiotic stability in culture media during regeneration and selection are rare and information ambiguous, it has been shown that the extent of antibiotic inactivation depends on the extracellular pH, the temperature and light, but the precise rate of inactivation is unknown resulting in antibiotic concentrations used for selection being higher than what would be required if they did not lose their activity over time. Additionally, developing a selection protocol using antibiotics always involves establishing an inhibitory dose, but to obtain this inhibitory effect in regeneration the antibiotic is added to the medium from the first day. However, in the transformation protocol, the antibiotic is added 2–10 days later and sometimes the medium is not the same, or more than one medium is used during the whole process of regeneration/transformation, so its effect is different. It would be advisable to establish the inhibitory doses in each medium and in each situation of the protocol. Failure to do so could be one of the factors responsible for the escape of shoots commonly observed in the transformation protocols published for many species. The application of selective agents a few days after the co-culture with *Agrobacterium* lets tissues grow and develop, so they are more resistant to antibiotics and most of the developed buds escape, as has been demonstrated in plum hypocotyl transformation, where an early kanamycin application reduced the number of escapes without affecting transformation efficiency (Padilla et al. 2003). For rice, Lee et al. (2006), working in plastid transformation, found that the application of selection pressure once the transformed callus had regenerated into a plantlet was not an effective method for the achievement of homoplastomy. However, alternative selection strategies including ‘delayed selection’ have been successful in obtaining transgenic plants in kanamycin-sensitive species such as apple (Yao et al. 1995; Yepes and Aldwinckle 1994), apricot (Machado et al. 1992) and almond cultivars ‘Boa Casta’ (Miguel and Oliveira 1992) and ‘Ne Plus Ultra’ (Ramesh et al. 2006). Obviously, these delayed selections produced many escapes and probably chimeras. Additionally, it has been suggested that antibiotic depletion in the vicinity of transgenic cells could permit regeneration of non-transgenic cells (Rosellini et al. 2007) and that escape shoots may be generated because of detoxification by adjacent, transformed cells (Jordan and McHughen 1988). Furthermore, it has been suggested that, as aminoglycosides are inactivated by phosphorylation reactions and the energy source and the phosphate donor of this reaction are the ATP reservoir of the transformed cell, part of the accumulated chemical energy is utilized to inactivate the inhibitor instead of promoting essential metabolic and morphogenetic processes (Oreifig et al. 2004).

What seems to be clear is that in most transformation systems, the generation of a number of escapes is expected. Regeneration of escapes and chimerical shoots at high frequencies has been reported in some species, although their importance and frequency have been underestimated. The problem of chimerism seems to be more frequent than originally thought and it has been reported in several herbaceous species, including tobacco (Schmülling and Schell 1993), soybean (Christou 1990), potato (Rakosy-Tican et al. 2007), rice (Christou and Ford 1995), flax (Dong and McHughen 1993) and strawberry (Mathews et al. 1995). Chimeras were also recovered frequently from woody fruit trees such as kiwifruit (Li et al. 2003) and apple (Flachowsky et al. 2008). Very high frequencies have been reported in *Citrus*, for which escapes and chimeras account for 90% of regenerated lines (Costa et al. 2002; Domínguez et al. 2004). Although a method to detect and estimate the level of chimeras in transgenic tobacco and apricot plants has been described recently (Faize et al. 2010), problems with aminoglycoside selection continue.

Conclusions

The mode of action of aminoglycosides on prokaryote and eukaryote cells is very complex and has many facets; interactions with different compounds in the medium according to their chemical structure, with cell membranes at many levels and, inside the cell, with RNA, organellar membranes, etc. Additionally, the severity of the effects seems dependent in all cases on the nature of the aminoglycoside structure. Aminoglycoside antibiotics have been used as selective agents for more than 20 years and their usefulness in plant transformation is clear, although some unexpected results are probably due to erroneous assumptions about their mode of action and interactions with transformation–regeneration protocols. These unknown effects of antibiotics together with regeneration problems have made it difficult to transform many agronomically important species, mainly woody ones. The information contained in this review could be useful for establishing better strategies when developing transformation and selection protocols.

Nevertheless, widespread public concern over the use of marker genes conferring antibiotic resistance, in addition to the problems described with antibiotic selection, has led to the development of alternative selection systems for transformed tissues. Herbicide resistance genes have been used as selection marker genes for plant genetic transformation (Miki and McHugh 2004). Selection systems based

on the replacement of sucrose as a carbon source in the growth medium with a sugar that the plant cells are unable to utilize, such as xylose, mannose, galactose or arabitol, have been employed successfully in some species (Haldrup et al. 1998; Joersbo et al. 1998; LaFayette et al. 2005). Selection systems employing genes that confer resistance to phytotoxic substances, such as 4-methyl tryptophan (Goddijn et al. 1993), cyanamide (Weeks et al. 2000), betaine aldehyde (Daniell et al. 2001), 2-deoxyglucose (Kunze et al. 2001), galactose (Joersbo et al. 2003), D-alanine and D-serine (Erikson et al. 2004), S-aminoethyl l-cysteine (Perl et al. 1993), methotrexate (Herrera-Estrella et al. 1983) and gabaculine (Gough et al. 2001; Rosellini et al. 2007), have been used with success in some species. However, although some of them have been compared with kanamycin as the selection agent and higher transformation efficiencies (Joersbo and Okkels 1996a; Ramesh et al. 2006; Rosellini et al. 2007; Ballester et al. 2008) and lower numbers of escapes have been reported (Rosellini et al. 2007), the shift from antibiotic selection to any of these systems is slow and they have not been tested in many species, including difficult-to-transform fruit tree species (Petri and Burgos 2005). Nevertheless, all of them continue to produce escapes and maybe chimeras. Probably, in the future, aminoglycosides will be eliminated as selection agents from plant transformation due to the discovery of new and better selection agents or new transformation methods. Until that happens, it will be important to take into account the structure, function and effects on cell and explant development of aminoglycosides to establish good selection strategies.

Acknowledgments This work was partially supported by BIOC-ARM BIO-AGR07/04-0011 and Séneca 08665/PI/08. IMGP thanks the Spanish Council of Scientific Research (CSIC) for a I3P post-doctoral contract. The authors thank Prof. Fernando Pliego-Alfaro for the critical review of the manuscript and Dr. David J. Walker for English language editing.

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