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A negative selection scheme for tobacco protoplast-derived cells expressing the T-DNA gene *2*

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ABSTRACT

The amido hydrolase encoded by the T-DNA gene 2 catalyzes the conversion of indole-acetamide, α-naphthalene acetamide, and other substrate analogues into the corresponding auxins. As a result, only gene 2-expressing protoplast-derived tobacco cells can grow in medium containing low concentrations (0.2–1 μM) of α-naphthalene acetamide as auxin precursor. However, in a mixture of SR1 and SR1,gene 2^+ protoplast-derived cells, cross-feeding occurs and consequently no positive selection for gene 2 is obtained. A 100-times higher concentration of α-naphthalene acetamide (between 30 and 300 μM) provides a negative selection scheme. Only the tobacco cells expressing gene 2 are sensitive to the high naphthalene acetamide concentration and cannot grow to colonies, while cells lacking the gene 2 product regenerate calli even in mixed gene 2^+ and gene 2^- cell populations. Thus, gene 2 might provide a unique biochemically defined marker to investigate mutations and gene inactivation.

INTRODUCTION

There are several pathways by which plants can convert tryptophan to the naturally occurring auxin indole-3-acetic acid (for a review, see Marumo, 1986). However, in micro-organisms such as Pseudomonas savastanoi and in plants transformed with the T-DNA of Agrobacterium tumefaciens, another pathway has been found, in which tryptophan is converted into indole-3-acetamide (IAM) and then to indole-3-acetic acid (IAA) . This pathway is believed to be foreign to plants since IAM has been found only in conjunction with high concentrations of IAA in plant seedlings and citrus fruits.

Oncogenic T-DNAs encode enzymes that produce auxin and cytokinin so that infected tissues can proliferate without the need of exogenous hormones (for a review, see Inzé et al., 1987). Gene 4 codes for an isopentenyl transferase involved in the production of the cytokinin isopentenyl adenosine. An amidohydrolase encoded by gene 2 hydrolyzes the intermediate indole-3-acetamide (IAM), formed by the product of gene 1, into indole-3-acetic acid (IAA) (Thomashow et al., 1986; Van Onckelen et al., 1986).

Because the gene 2 product also catalyzes the conversion of α-naphthalene acetamide (NAM) into the biologically active auxin α-naphthalene acetic acid (NAA) (Inzé et al., 1984), it was postulated that plant cells containing the amidohydrolase gene 2 product should be able to grow on NAM as the only auxin supplement. On the other hand, high concentrations of NAM would not be toxic for normal plant cells but could be deleterious for cells capable of converting NAM to NAA. On this basis, it has been shown that NAM in the medium allows negative selection of gene 2-expressing seedlings (Budar et al., 1986). Also, it has been demonstrated that gene 2-expressing petunia callus is strongly inhibited or killed while wild-type callus grows normally in the presence of 30 μM NAM (Klee et al., 1987).

The auxin requirements and auxin cytotoxicities for tobacco protoplasts and protoplast-derived cells have been studied in detail (Caboche, 1980; Caboche et al., 1984). Exogeneously applied NAA efficiently stimulates mitotic activity and growth of protoplast-derived cells only in the concentration range between 1 μM and 20 μM at high cell density and in the concentration range between 0.05 μM and 1 μM at low cell density. Based on these observations, we postulated that different NAM concentrations would be differentially growth-promoting or toxic depending upon whether cells were derived from the tobacco plant SR1 or a transgenic SR1 plant transformed with gene 2 (Budar et al., 1986). The results demonstrate that SR1 protoplast-derived cells only initiate cell division and grow to colonies when the medium is supplemented with 5 up to 250 μM NAM. On the other hand, gene 2-expressing protoplast-derived cells can be stimumated to grow by concentrations between 0.5 and 10 μM NAM but are sensitive to concentrations higher than 30 μM. These results allowed the development of a negative selection scheme for gene 2 expression which, together with the availability of the gene 2 probe, might become especially valuable for fundamental studies on gene inactivation, mutagenesis, and T-DNA stability.

MATERIALS AND METHODS

Plant Material

All studies were performed on Nicotiana tabacum cv. Petit Havana SR1 and on the transformed SR1 plant 2441-7 (Budar et al., 1986). Plant 2441-7 was obtained after leaf disc transformation by Agrobacterium C58C1Rif^R(pGV2441), selection for kanamycin resistance, and screening for the expression of gene 2. Genetic analysis showed the presence of only one functional gene 2 locus (Budar et al., 1986).

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Protoplast Regeneration and Tissue Culture

Protoplasts from sterile-grown SR1 or 2441-7 plants were isolated in K3 medium deprived of hormones and regenerated as described (Depicker et al., 1985). After protoplast isolation and two successive washings, the protoplasts were diluted to a final concentration of 10^5 protoplasts/ml. At that moment, the cytokinin BAP was added and the auxin NAA, normally added up to a concentration of 0.1 mg/l was omitted or substituted by different concentrations of NAM (Sigma). After 3 days an equal volume of medium was added and after 7 days the protoplast-derived cells were embedded in agarose discs (Shillito et al., 1983), which gives an average plating density of 2×10^4 cells/ml. The surrounding liquid medium was refreshed every week while gradually lowering the sucrose concentration by 0.1 M. Cell division was followed under the microscope and colony formation was scored 5 weeks after protoplast isolation.

Microcalli or leaf fragments were grown on Murashige and Skoog medium containing 0.7% agar supplemented with different concentrations of BAP, NAA, or NAM as indicated (Results).

RESULTS

Physiological Concentrations of NAM as Auxin for Tobacco SR1 Protoplast-derived Cells

For the regeneration of tobacco protoplasts, NAA is commonly used as the required auxin in the medium. Physiological concentrations of NAA lie between 1 and 20 µM (0.1 and 3 mg/l); lower concentrations fail to induce mitotic activity while higher concentrations are toxic for sustained growth and colony formation (Caboche et al., 1984). First, we determined whether NAM could similarly promote growth of protoplast-derived cells and thus substitute for NAA in the medium of regenerating SR1 tobacco protoplasts. Freshly isolated protoplasts prepared from SR1 were resuspended in K3 medium containing 1.3 µM (0.3 mg/l) BAP and 0–0.05–0.1–0.25–0.5–1–2–5–10–20–50 mg/l NAM, respectively (Figure 1).

In all media tested, SR1 protoplasts regenerated cell walls in 48 h. These protoplast-derived cells enlarged but did not divide in medium without NAA or containing up to 2 µM (0.25 mg/l) NAM, while SR1 cells in medium containing 3 to 250 µM (0.5 mg/l up to 50 mg/l) NAM showed mitotic activity and colony growth (Figure 1, triangles; Figure 2A). The speed with which divisions were apparent was related to the supplemented concentration of NAM. In 5 µM NAM it takes 2 weeks before mitotic activity is visible, while on 25, 50, and 250 µM NAM, cell divisions are apparent the third or fourth day after protoplast isolation. The retardation of growth on low NAM concentrations disappears however during the fourth week of colony formation and large greenish calli are formed, which sometimes initiate shoot formation. This difference in callus phenotype at low NAM concentrations is probably related to qualitative differences in the auxin effects of NAM compared to NAA. Concentrations of NAM between 25 and 250 µM yield high plating efficiencies with white, compact calli. This led to the conclusions that NAM can promote growth in auxin-free medium, and that unlike NAA, it is not toxic for plant cells in any tested concentration.

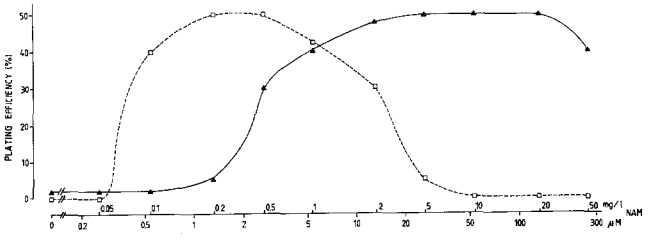

Figure 1. Plating efficiency of protoplast-derived cells of tobacco SR1 (▲) and SR1,2441-expressing gene 2 (□) as a function of different NAM concentrations. Protoplasts were isolated in the absence of hormones and plated at a density of 10^4 cells/ml in K3 medium supplemented with BAP (1.3 µM) and various concentrations of NAM. The plating efficiency was measured 5 weeks after protoplast isolation by scoring the number of colonies in respect to the initial number of cells. Different growth rates were not taken into account because this work aimed at the development of a negative selection scheme. The scale on top of the abscis line indicates the NAM concentrations in mg/l, while the subdivisions under the abscissa give the molar NAM concentrations.

Growth-promoting Activity of Different NAM Concentrations for Tobacco Protoplast-derived Cells Expressing Gene 2

After determining the concentration range in which NAM allows the regeneration of SR1 tobacco protoplasts to microcalli, we asked whether the conversion of NAM into NAA by the amidohydrolase in gene 2-transformed cells would alter the concentration range promoting their regeneration into calli. SR1,gene 2-transformed protoplast cells should be able to convert NAM to the more potent auxin NAA so that the first cell divisions can be induced by lower concentrations of NAM than for control SR1 cells. Concomitantly, the conversion of high concentrations of NAM into NAA might result in an inhibitory or toxic NAA concentration for gene 2-expressing protoplast cells, while untransformed cells could continue to proliferate. In order to test this possible clonal stimulation or inhibition of growth for cells expressing gene 2, protoplasts prepared from a plant transformed with gene 2 (SR1 2441-7; Budar et al., 1986) were washed and incubated in media supplemented with different concentrations of NAM.

The results are summarized in Figure 1 (squares) and shown in Figure 2B. Amidohydrolase expressing protoplast-derived cells (gene 2^+) regenerate efficiently into white microcalli in NAM concentrations between 0.5 and 10 µM. A NAM concentration of 25 µM still allows the initiation of the first cell divisions but strongly inhibits colony formation by stopping growth at a size of 5 to 20 cells. NAM concentrations of 50 to 250 µM block the first cell divisions of protoplast-derived cells and consequently do not allow colony formation. This sensitivity for high NAM concentrations of gene 2-transformed tobacco protoplast cells resembles the sensitivity of normal tobacco protoplast cells for high NAA concentrations (Caboche et al., 1984; Muller et al., 1985).

High NAM Concentrations Specifically Counterselect Gene 2-Expressing Protoplast-derived Cells

The results, summarized in Figure 1, clearly suggest that low and high NAM concentrations would promote differential growth of gene 2^- versus gene 2^+ protoplast-derived cells. In order to test whether a low and high NAM concentration could be used to select for or against gene 2-expressing cells in a mixed population, reconstruction experiments were set up. Non-transformed SR1 and gene 2^+-transformed protoplasts were mixed in different ratios (100/1, 10/1, 1/1, 1/10, and 1/100) after isolation and allowed to regenerate.

In low NAM concentrations (0.5 µM), both gene 2^- and gene 2^+ protoplast-derived cells grew and, unex-

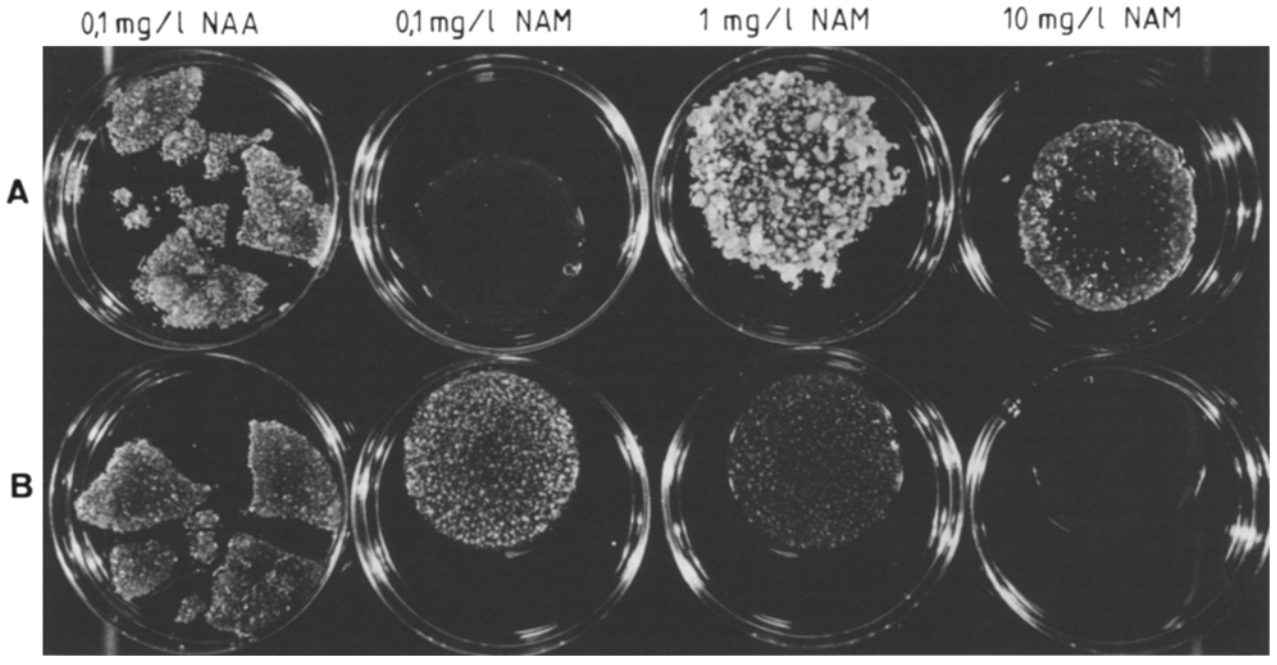

Figure 2. Differential growth-promoting activity of NAM on normal SR1 (A, top row) and on gene 2-expressing SR1,2441 (B, bottom row) protoplast-derived cells. The colony formation of both type of cells under normal hormone conditions is shown on the left. If 0.5 µM (0.1 mg/l) NAM is supplied, only gene 2-expressing cells can grow (positive selection). On the other hand, 50 µM (10 mg/l) NAM in the medium causes cell death only for gene 2-expressing cells (negative selection).

pectedly, no enrichment was obtained for gene 2^+ colonies. This can be explained by assuming that NAM is converted into NAA by the gene 2^+ cells and then released into the medium. Probably, cross-feeding of NAA (or another NAM metabolite) occurs so that positive selection for gene 2^+ cells in a mixture with gene 2^- cells is not possible. Therefore, the gene 2 marker cannot be used as an alternative to the antibiotic resistance gene markers.

On the other hand, in high NAM concentrations (> 50 µM), only SR1 protoplast-derived cells gave good plating efficiencies while the growth of SR1,gene 2^+ cells was efficiently counterselected. This means that the conversion of high concentrations of NAM into NAA is only toxic for the cells converting the NAM and not for the SR1 cells in the mixture. However, some escapes of gene 2^+ cells forming colonies were noticed especially in the reconstruction mixtures with few gene 2^+ cells (Breyne et al., in preparation). This type of background is presumably unimportant so long as the major use of this negative selection scheme is to select for gene 2^- mutant cells in populations of gene 2^+ cells.

Analysis of Gene 2 Inactivation in Regenerating leaf protoplasts

In order to determine the efficiency of the NAM counterselection for the expression of gene 2, we measured whether plant cells would escape the selection. Approximately 10^7 protoplasts were prepared from SR1,2441-7 leaves and incubated in medium supplemented with 50 µM NAM. Only one colony was obtained indicating a stringent counter selection for gene 2-expressing cells. At the moment, we are determining whether this colony is an escape or a mutant modified at the gene 2 locus or elsewhere.

In contrast to our observations on single cells, we noted that when the regenerating protoplasts were first grown to a critical size (approximately 50 cells) in medium containing 5 µM NAA, addition of up to 250 µM NAM could not stop colony formation. Thus, high auxin concentrations block the first divisions of protoplast cells but cannot block cell division in microcalli (> 20 cells). This parallels the selection conditions to obtain auxin resistance (Muller et al., 1985) : sensitivity to high auxin concentrations is only apparent when applied to protoplast-derived cells.

Morphology of Callus and Leaf explant Growth on Different Hormone Concentrations

After we established the conditions for positive and negative selection for gene 2 expressing tobacco cells, we determined whether they also were applicable on callus tissue. SR1 and SR1,2441-7 microcalli, regenerated from protoplasts and approximately 6 weeks old, were transferred onto solidified LS medium containing 1.3 µM BAP, and 0.5, 5, and 50 µM NAM, respectively. SR1 calli on the two lowest concentrations of NAM are green, form shoots, and can be distinguished from SR1,2441-7 calli which remain amorphic, compact, and white. On medium supplemented with 50 µM NAM both SR1 or SR1,gene 2-expressing calli grow as a very watery mass, although SR1,gene 2^+ calli are more inhibited. The cut edges of leaf explants react similarly to microcalli on this medium : gene 2-expressing plants develop white amorphous callus, while normal tobacco plants induce green callus and shoots.

When BAP is omitted from the medium and NAM is added in a concentration of 0.5 to 5 µM, the cut edges of SR1 leaves remain unresponsive though many branched roots can develop. However, the cut edges of leaves expressing gene 2 react with the growth of a rim of callus consisting of very large cells. The roots which develop from the leaf discs are thick, short, and not branched. Moreover, many are covered with root hairs and not positively geotropic.

These results show that in contrast to gene 2$^+$ protoplast-derived cells, gene 2-expressing calli cannot be positively or negatively selected, but calli and leaves with or without gene 2 can be distinguished morphologically by growth on different concentrations of NAM.

DISCUSSION

Tobacco protoplast cells do not show cell division in auxin-free medium, but the cells increase in size and remain metabolically active for at least a week. Increasing NAA concentrations between 0.5 and 15 µM can be correlated with an increasing number of dividing SR1 cells during the first week; however, this retardation of cell division disappears after 3 weeks and high plating efficiencies can be obtained at low auxin concentrations. High NAA concentrations (above 30 µM) inhibit colony formation from protoplast-derived cells or small cell clumps, especially at low cell density, but not when the microcalli obtain a critical cell size (more than 20 cells) (Caboche et al., 1984; Muller et al, 1985). Apparently cell clumps obtain a degree of autonomy in the regulation of the intracellular auxin concentration. The basis of auxin toxicity is not yet clear. NAA conjugates can be formed, presumably as detoxification by a specifically induced enzymatic pathway and it has been suggested that the auxin sensitivity is related to this NAA conjugation processes at low cell density (Caboche et al., 1984). These NAA conjugates are stored and although the enzyme activity is as yet not demonstrated, there is no doubt about the hydrolyzation of auxin amide conjugates. Our results show that NAM can stimulate cell divisions and promote growth of protoplasts-derived cells. NAM is taken up by tobacco protoplasts and exerts auxin effects, presumably after modification at a controlled rate. SR1 protoplast cells require ten times more NAM than NAA to initiate the mitotic activity of mesophyll protoplast-derived cells. However, while 25 µM NAA inhibited colony formation of SR1 cells, no upper inhibitory limit for NAM was found. SR1 cells expressing the amidohydrolase encoded by gene 2 showed the same dose-response curve for NAA as for NAM. Therefore, the observation that NAM at concentrations higher than 30 µM blocks cell division can be explained by the unregulated conversion of NAM to NAA in gene 2-expressing cells.

A clearly different physiological response dependent on the presence of the gene 2 product is seen with leaf explants on media supplemented with 1.3 µM BAP and 5 µM NAM : SR1 wounded leaves grow green shoot-forming callus while cut edges of SR1 gene 2-expressing leaves primarily grow compact, white callus. On medium with 5 µM NAM and without cytokinin, callus growth is stimulated primarily on the edges of gene 2-expressing leaf discs and abnormal, thick, and non-branched roots lacking positive geotropism are induced; in contrast, wild-type SR1 leaf discs remain unresponsive or produce normal roots into the medium.

Budar et al. (1986) described the screening of gene 2-expressing transgenic plants and demonstrated a negative selection scheme for gene 2-expressing seedlings. Here we report selection schemes applied to a large population of protoplast-derived cells. The sensitivity for high auxin concentrations is primarily apparent during the first cell divisions of protoplast-derived cells and during root development. Indeed, the counter selection of gene 2 on media with a high NAM concentration is similar to the selection conditions used to isolate auxin-resistant tobacco cells (Muller et al., 1985).

This method to counterselect tobacco cells expressing gene 2 may be very useful since most of the markers used for forward mutagenesis suffer from the disadvantage that the molecular basis is as yet unknown. The gene 2$^+$ → gene 2$^-$ selection scheme based on growth in medium with 50 µM NAM is stringent and allows subsequent analysis of the forward mutations at the DNA level by cloning the mutated locus. It is possible to score directly for gene 2 mutation frequencies in vivo or in vitro, either at spontaneous rates or induced by somaclonal variation in tissue culture or by mutagens (Breyne et al., in preparation). In addition, specific constructions can be designed to select for deletions or chromosomal rearrangements and for transposon insertions (De Greve et al., in preparation). Therefore, the selection scheme against gene 2 expression might become the counterpart of the selection schemes for HPRT mammalian cells (Stout and Caskey, 1985).

In summary, the gene-2-based reversible positive and negative growth conditions might become very valuable to assess gene activation versus inactivation, to determine gene mutation frequencies, and to probe for T-DNA stability in somatic and meiotic cells.

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