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Expression of *Agrobacterium rhizogenes* auxin biosynthesis genes in transgenic tobacco plants

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Abstract

Plant oncogenes *aux1* and *aux2* carried by the TR-DNA of *Agrobacterium rhizogenes* strain A4 encode two enzymes involved in the auxin biosynthesis pathway in transformed plant cells. The short divergent promoter region between the two *aux*-coding sequences contains the main regulatory elements. This region was fused to the *uidA* reporter gene and introduced into *Nicotiana tabacum* in order to investigate the regulation and the tissue specificity of these genes. Neither wound nor hormone induction could be detected on transgenic leaf discs. However, phytohormone concentration and auxin/cytokinin balance controlled the expression of the chimaeric genes in transgenic protoplasts. The expression was localised in apical meristems, root tip meristems, lateral root primordia, in cells derived from transgenic protoplasts and in transgenic calli. Histological analysis showed that the expression was located in cells reactivated by *in vitro* culture. Experiments using cell-cycle inhibitors such as hydroxyurea or aphidicolin on transgenic protoplast cultures highly decreased the β-glucuronidase activity of the chimaeric genes. These results as well as the histological approach suggest a correlation between expression of the *aux1* and *aux2* genes and cell division.

Introduction

Auxin and cytokinin are natural phytohormones involved in plant development through processes such as elongation and cell division [13]. The influence of the auxin/cytokinin balance on plant development is exploited by several phytopathogenic bacteria such as *Agrobacterium tumefaciens*, *A. rhizogenes*, *Pseudomonas syringae* pv. *savastanoi*, *Rhodococcus fascians*, *Rhizobium leguminosarum*, *Bradyrhizobium japonicum* and *Xanthomonas campestris* pv. *glycinis*, to create ecological situations favourable to their proliferation through plant growth or exudat stimulations [15, 20, 52]. *A. tumefaciens* and *A. rhizogenes* induce crown gall and hairy root diseases, respectively, resulting from the introduction, integration and expression of bacterial oncogenes in the plant genome (reviewed by Hooykaas and Schilperoort [29]). In this case, two *Agrobacterium* oncogene sets are involved, the first one comprises phytohormone biosynthesis genes, namely the genes encoding the two enzymes of the auxin biosynthesis pathway (*tms1* and *tms2* in *A. tumefaciens*, *aux1* and *aux2* in *A. rhizogenes*) and the gene encoding the isopentenyl transferase in the cytokinin biosyn-

thesis pathway (*ipt* gene); the other comprises genes modulating the phytohormone balance such as the *A. tumefaciens* gene *5* whose product converts tryptophan into indole-3-lactate (an auxin analogue and an antagonist [39]), the gene *6b* of *A. tumefaciens* whose product induces a reduction of cytokinin activity [28, 63] and the *A. rhizogenes rolB* and *rolC* genes presumably responsible for the release of growth factors from conjugated and inactive hormonal forms [18, 19]. However, the *rol* gene functions are still discussed [54, 55].

T-DNA genes display eukaryotic regulation and their promoter regions interact with plant *trans*-acting factors as shown for the octopine synthase (*ocs*) gene and nopaline synthase (*nos*) gene promoters [7, 61, 69]. The T-DNA genes studied so far possess organ and tissue specific expression, developmental regulation or are inducible by different factors. For example, the *nos* gene promoter [5] functions in both vegetative and reproductive organs, and the mannopine synthase (*mas*) promoter functions in leaf and stem tissues [41, 57, 68]. The expression of certain T-DNA genes was shown to be regulated by phytohormones. For example, the *nos* gene is induced by auxin [5], the *mas* gene and the gene *5* are fine-tuned by auxin and cytokinin levels [6, 41, 57], the *rolB* gene is highly induced by auxin [48] whereas *rolC* is reported as weakly auxin-inducible [48].

Organ and tissue specificity of T-DNA genes show common features: they are generally expressed more in root and stem than in leaf tissues [(*nos*) 4, (*rolB*) 11, (*rolC*) 53, 65, 66]; most of them are expressed in meristematic tissues such as *rolB* [1, 48], the octopine synthase gene (*ocs*) [38] or the agropine synthase gene (*ags*) [31]; and some display vascular tissue expression [38 (*ocs*), 46 (*rolC*), 57 (*mas*), 60 (*rolB*)]. The *mas* [41] and *nos* [4] genes have a gradient of expression in the plant, their expression being greater in lower parts while decreasing in the upper parts. This vertical gradient might be correlated with hormonal gradients in the plant. T-DNA genes are also developmentally regulated: the *rolB*, *rolC* genes and gene *5* are expressed during the somatic embryo development [12, 21, 47] while the *nos* and *mas* genes are expressed during flower development [5, 41].

Despite extensive knowledge on the regulation of T-DNA opine genes and some others [16, 64], the regulation of auxin biosynthesis genes from either *A. tumefaciens* or *A. rhizogenes* has not been described. The main regulatory elements of the bidirectional *aux1* and *aux2* auxin biosynthesis gene promoter of *A. rhizogenes* were previously studied by transient expression experiments [22]. The bidirectional organisation and the function of the two *aux* genes involved in the same pathway, suggested a coregulation of the two genes. In this paper, transgenic *Nicotiana tabacum* plants containing the divergent promoter region of these two genes translationally fused with the β-glucuronidase reporter gene were constructed to study tissue specificity and regulation of these oncogenes. The *aux* gene expression was shown to occur in meristematic tissues and to be associated with cell division. Moreover, the auxin/cytokinin balance influences the expression of these oncogenes in cell cultures derived from transgenic protoplasts.

Materials and methods

Plasmid constructions

All DNA recombinant techniques were performed according to standard methods [59]. The construction of the binary plasmids pBiG1 (p*aux1-uidA* fusion) and pBiG2 (p*aux2-uidA* fusion) has been described previously [22]. The 415 bp *Bst* EII-*Bam* HI fragment containing the 394 bp intergenic region of the pRiA4 *aux* genes [10], the first codons of the *aux1* and *aux2* genes was translationally fused, in both orientations, with the *uidA*-coding sequence. This fragment was ligated in frame to the *Bam* HI restricted pBI101.1 plasmid [33] using the *Bst* EII-*Bam* HI *aux* linker [22]. The resulting plasmids were named pBiG1 when the *uidA* sequence was positioned in the same orientation as the *aux1* gene (p*aux1-uidA* fusion) and pBiG2 (p*aux2-uidA* fusion) in the other orientation (Fig. 1).

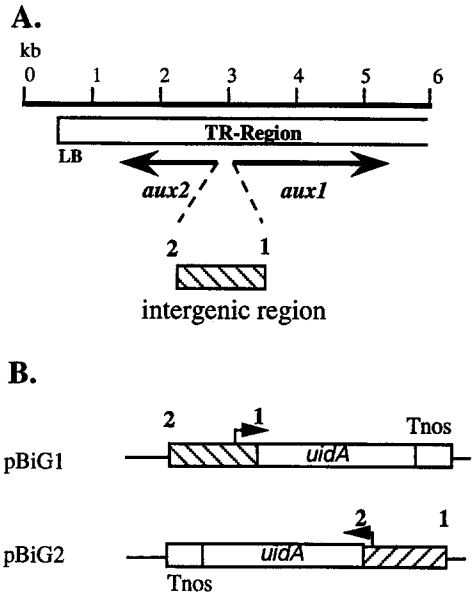

Fig. 1. Description of the promoter region of the *aux* genes and of the constructs used in this study. A. Organisation of the *aux1* and *aux2* auxin biosynthesis genes on the pRiA4 TR-region of *A. rhizogenes*. LB, left border. Arrows indicate the orientation of the transcription. B. Constructions of the p*aux-uidA* fusions. The intergenic region of the *aux* genes was translationally fused to the *uidA*-coding sequence. Tnos: terminator of the *nos* gene.

Plant transformation and regeneration

pBiG1 and pBiG2 binary vectors were transferred into *A. tumefaciens* strain GV3101 (pMP90) [37] as described by Zahm *et al.* [71]. The introduction of the plasmids was verified by Southern blot analysis of total bacterial DNA. Leaf discs of *N. tabacum* cv. Xanthi were infected with *Agrobacterium* strains containing the pBiG plasmids [30] and transferred on MS medium supplemented with 0.1 mg/l naphthaleneacetic acid (NAA), 1 mg/l N-6-benzylaminopurine (BAP), 200 mg/l kanamycin and 500 mg/l cefotaxime. Four to six weeks after infection, shoots were excised and transferred on MS medium containing 200 mg/l kanamycin and 500 mg/l cefotaxime. Kanamycin-resistant tobacco plantlets were propagated *in vitro*, maintained in the greenhouse and analysed by Southern blot experiments.

Histochemical staining

Histochemical staining was performed by incubation of the samples (plantlets, leaf disc, root) in a X-Gluc solution at 37 °C [33]. After staining, they were fixed for 24 to 48 h in 0.2 M phosphate buffer pH 7.2 with 10% paraformaldehyde, 25% glutaraldehyde and 1% caffeine. Dehydration was as follows: 12 h in 70% ethanol, 15 min in 95% ethanol with vacuum infiltration, 30 min in 95% ethanol, 15 min in absolute ethanol with vacuum infiltration, 1 h in absolute ethanol. The embedding was done in LKB historesin as advised by the manufacturer: the samples were pre-embedded in LKB historesin added with LKB activator 15 min with vacuum infiltration, one night, at 4 °C and then oriented and embedded in capsules at room temperature. The 3 μm sections were realised on a Historange LKB microtome and single- or double-stained. Two kinds of staining were performed: a periodic acid-Schiff reaction (PAS), specific for polysaccharides which are stained pink, and a Johansen haematoxylin stain, specific for nucleus, nucleolus and cytoplasm, which appear in grey.

Protoplast preparation

Mesophyll protoplasts were isolated [26] and cultured at 70000 cells/ml in To medium (3 mg/l NAA, 1 mg/l BAP), in To medium supplemented with 100 mM hydroxyurea, in To medium supplemented with aphidicolin (15 or 30 μM) or in To medium with various phytohormone concentrations.

Estimation of DNA synthesis in tobacco protoplasts

Protoplasts were cultured in the presence of [^{3}H] TTP (2 μCi/10^6 protoplasts) in To medium. After different periods of culture, aliquots were collected and DNA synthesis was measured [43].

β-glucuronidase fluorometric assay

After different periods of culture in various media, protoplasts were harvested, washed in 2.5% KCl,

0.2% $CaCl_2$ and 0.07% MES pH 5.8 and β-glucuronidase activity was measured [33]. The protein concentration of extracts was measured according to Bradford [8].

Results

Tissue specificity of the expression of the aux *divergent promoter*

The *aux1* and *aux2* auxin biosynthesis genes of *A. rhizogenes* strain A4 are located on opposite DNA strands of the TR-DNA. The intergenic region (394 bp) between the *aux1* and *aux2* coding sequences, containing the main regulatory elements [22], was translationally fused in both orientations, with the *uidA* reporter gene, leading to two constructions p*aux1-uidA* (pBiG1 plasmid) and p*aux2-uidA* (pBiG2 plasmid) [22] (Fig. 1). Transgenic *N. tabacum* plants were obtained via *A. tumefaciens* transformation and named BG1x plants (p*aux1-uidA* fusion) or BG2x plants (p*aux2-uidA* fusion) (x, plant's name). About ten transgenic plants for each construction were more particularly analysed. Beta-glucuronidase histochemical staining was performed on different tissues of several transgenic tobacco plants and revealed expression only in root meristems, lateral root primordia and apical meristems in both plants (Fig. 2A-D). The root meristems of a same plant were differently stained, some presenting no β-glucuronidase activity, others strong activity. This might correspond to different levels of meristematic activity or to quiescent meristems (data not shown). The specific localisation of expression could explain that the β-glucuronidase activity was almost undetectable on the whole tissues by fluorometric assays.

Inducibility of the aux promoter

To test the wound inducibility of the *aux* divergent promoter, leaf discs from several BG1x or BG2x transgenic plants were wounded by fine scarifications and incubated in water or in MS medium, in sterile conditions. Beta-glucuronidase activity was monitored over four days and was not affected by wounding during the first 24 h (data not shown). Thus, the *aux* intergenic promoter region did not confer wound inducibility. Independently of the wound response test, these results showed that the β-glucuronidase activity of the unwounded discs incubated in MS medium was higher than the activity observed in the corresponding discs, incubated in water. This observation suggested that some elements of the medium could slightly induce the expression. However, this induction was weak and occurred late.

Transgenic leaf discs were also incubated in media containing different concentrations of auxin or cytokinin; no hormonal induction of the *aux* promoter could be detected in mature leaves (data not shown).

Expression in transgenic calli

To address the question whether the *aux* genes are expressed in dividing tissues, analysis was carried out during the sequence of events which lead to callus formation and shoot regeneration. Several leaf explants of different BG1x and BG2x transgenic tobacco plants were placed on regeneration medium, and developing calli were analysed. High expression was detected in proliferating areas by histochemical staining. These areas preferentially appeared close to the wounded areas where cicatrisation and cell division were beginning. Later, calli appeared on the leaf surface, and a high β-glucuronidase activity was detected in the proliferating tissues (Fig. 2E).

Transgenic calli were examined at different times during the regeneration process. After staining with X-Gluc, the samples were fixed, embedded, sectioned at 3 μm and stained. The walls and starch reserves were stained with the periodic acid-Schiff PAS reaction, while the meristematic state of the cells was revealed by the Johansen-haematoxylin staining. There were no morphological modification after two or three days of culture; a weak β-glucuronidase activity was de-

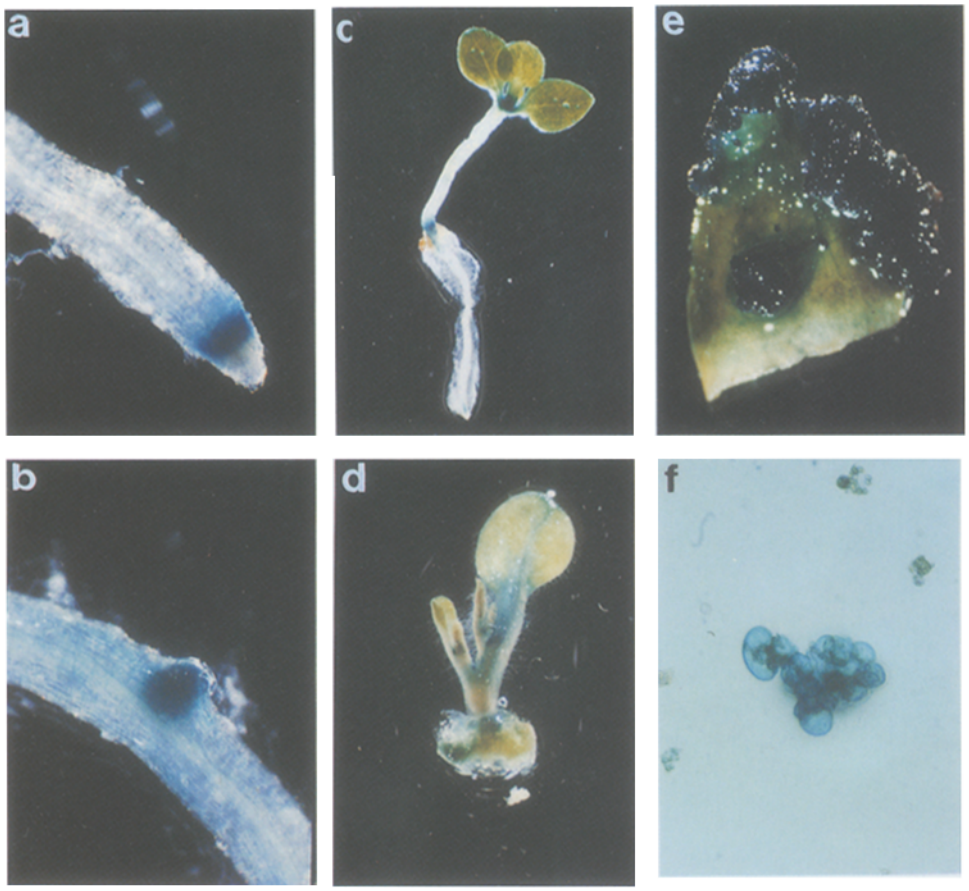

Fig. 2. Tissue specificity of the expression of the *aux* genes. The histo-localisation of the β-glucuronidase activity was performed on different organs and tissues of the transgenic tobacco plants containing the chimaeric p*aux-uidA* fusions. A. Root tip. B. Lateral root primordium. C. Young plantlet. D. Regenerating plantlet from transgenic leaf disc. E. Transgenic leaf disc after two weeks on regeneration medium. Calli are formed on the surface of the disc. F. Cells derived from transgenic mesophyll protoplasts after several days of culture in To medium.

tected around vascular bundles (data not shown). The vascular tissues are presumed to react first and rapidly when regeneration processes are induced. Indeed, they possess partially differentiated cells, able to be engaged quickly in a new dedifferentiation programme and to divide. After one week, calli appeared. Figure 3 presents the location of β-glucuronidase activity on callus transverse sections. Beta-glucuronidase activity was highest in proliferating areas of the calli which are more often localised at the surface (Fig. 3A). In the centre of the callus, more differentiated cells with starch reserves are present and divisions in this central area are less numerous. High expression was localised in areas containing very small cells forming active proliferating centres. In general, enlarged cells with starch reserves, engaged in differentiation process possessed less or no β-glucuronidase activity (Fig. 3A, B). Proliferating calli (Fig. 3A, C) presented a strong blue coloration. However, β-glucuronidase is a stable protein (half-life in mesophyll protoplasts of about 50 h [33]) and some large cells with blue crystals may have had previous meristematic activity and β-glucuronidase expression. Thus, the *aux* genes seemed to be principally expressed in small meristematic cells and in highly proliferating areas of the calli while in the enlarged cells or in cells

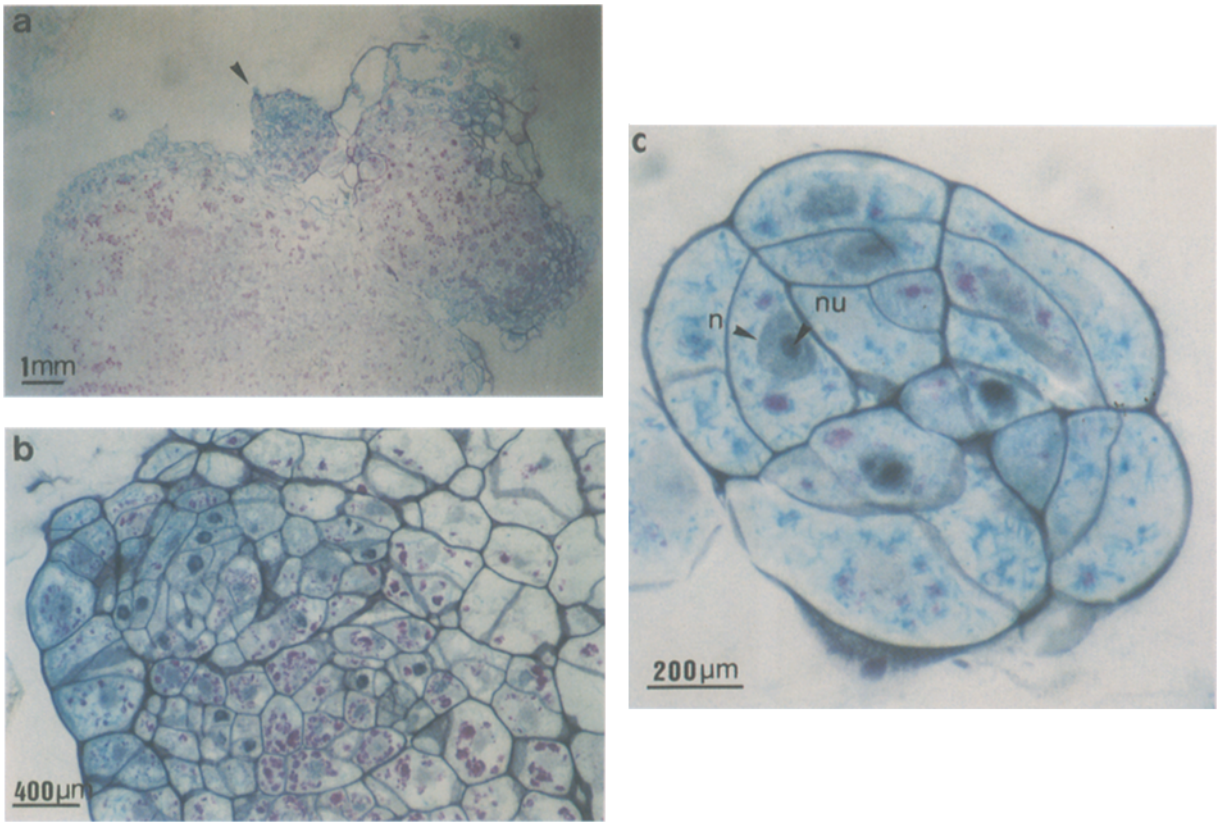

Fig. 3. β-glucuronidase activity during callus formation from transgenic leaf discs. Sections (3 μm) of transgenic calli were performed after β-glucuronidase coloration and different histochemical stainings were realised: after periodic acid-Schiff staining (PAS), starch and polysaccharides are colored in pink; the Johansen haematoxylin staining (H) allowed visualisation of nucleus and nucleolus which are coloured in grey. A. Section in an BG2 transgenic callus on a leaf disc surface. Beta-glucuronidase activity is mainly located in areas at the callus surface. In the centre, cells with starch reserves are present. The arrow indicates a proliferating area on the callus surface (PAS + H, $\times$ 55). B. Area of a meristematic formation. The dividing cells possess nuclei stained in grey; starch reserves are stained pink (PAS + H, $\times$ 215). C. Callus after PAS and H stainings. The nucleus (n) and nucleolus (nu) are well grey stained (PAS + H, $\times$ 625).

engaged in differentiation process, the genes are less or not expressed.

Promoter activity in transgenic protoplasts

Although high transient levels of expression of the p*aux1-uidA* and p*aux2-uidA* fusions were observed in tobacco leaf protoplasts [22], no expression was observed in mature leaves of stably transformed plants. In order to test whether this high activity was due to transient expression or to specific protoplast conditions, protoplasts were isolated from two or three BG1 and BG2 transgenic leaves and cultured in To medium. It is interesting to notice that To medium is optimal to get dividing protoplasts and to allow plant regeneration [26]. Histochemical assays, performed on protoplasts after several days of culture (formation of microcolonies), revealed blue staining (Fig. 2F). Beta-glucuronidase activity was measured at different culture points. During the first 24 h of culture, a low level of β-glucuronidase activity was detected. After 24 h, a significant increase was observed in BG1 and BG2 protoplast cultures. However, a lower activity level was generally observed with BG1 protoplasts compared to BG2 protoplasts (Fig. 4). Several experiments

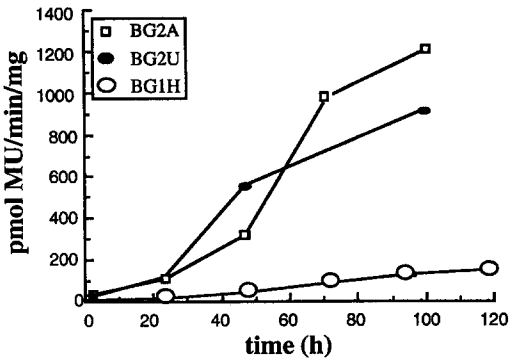

Fig. 4. Beta-glucuronidase activity in transgenic tobacco protoplast cultures. The activities, measured at different time of culture in To medium in protoplasts isolated from one BG1 (BG1H) and two BG2 (BG2A and BG2U) transgenic plants are presented.

on different plants were performed and results were similar, even though relative levels of expression differed according to the protoplast preparations or transgenic plants. Around 90% of the protoplasts were isolated from well differentiated Go mesophyll cells. The first division is considered as relatively synchronised, appearing between 24 and 72 h after isolation (see data below). These results indicated that a high expression was detectable in transgenic mesophyll protoplasts and cells derived from these protoplasts and particularly the 24 h point seems to be important, whereas no expression was observed in leaf tissues.

BG2 protoplasts were isolated and cultured in different media with various hormone concentrations, to test the influence of auxin and cytokinin in protoplast culture medium. As shown in Fig. 5A, both auxin and cytokinin were required to induce maximal expression. In the absence of both hormones or in presence of cytokinin only, no significant expression was detected, whereas an intermediate level of expression was observed with auxin alone. In the presence of standard auxin concentration (3 mg/l NAA) used for protoplast cultures, very low cytokinin concentration (20-fold lower than the standard concentration) is sufficient to induce similar expression than in standard To medium condition (3 mg/l NAA, 1 mg/l BA) (Fig. 5B). In the presence of standard cytokinin concentration (1 mg/l BA), the expression level is correlated with the auxin concentration (Fig. 5C). It is noticeable that auxin had a strong influence on the *aux2* expression in protoplasts, whereas no induction could be detected in transgenic leaf discs. The leaf disc is constituted by well differentiated mesophyll cells which may be less sensitive to hormones, whereas protoplasts are engaged in proliferation process where phytohormones play an important role. Wound inducibility could not be previously revealed and the expression increasing after 24 h, this response could not be a protoplast wound response. Thus, the expression of p*aux2-uidA* fusion was influenced by the balance between the two hormones and the auxin concentration. Low levels of cytokinin were sufficient for expression, this hormone being required to induce maximal expression.

Expression is correlated with cell cycle

Protoplasts of different transgenic BG1x and BG2x plants were cultured in presence of cell cycle inhibitors, to address the question whether a correlation between cell division and high expression of the *aux* promoter in transgenic protoplasts might exist as suggested by the previous results. Aphidicolin, which inhibits the α DNA polymerase and hydroxyurea, a ribonucleoside diphosphate reductase inhibitor interrupting the dNTP synthesis, were used. These biochemical inhibitors arrest plant cells at the G1/S boundary and are used to synchronise plant cell cultures [56, 58]. A cell-cycle arrest can also be obtained when protoplasts are cultured in an auxin and cytokinin depleted medium [51]. Protoplasts were cultured in different media with or without inhibitors. DNA synthesis was controlled and measured by thymidine incorporation to follow the effects of hydroxyurea, aphidicolin, or hormone deficiency, in each experiment. After different times of culture, protoplasts were harvested and washed. DNA synthesis was estimated and β-glucuronidase activity was measured.

In presence of either hydroxyurea, aphidicolin

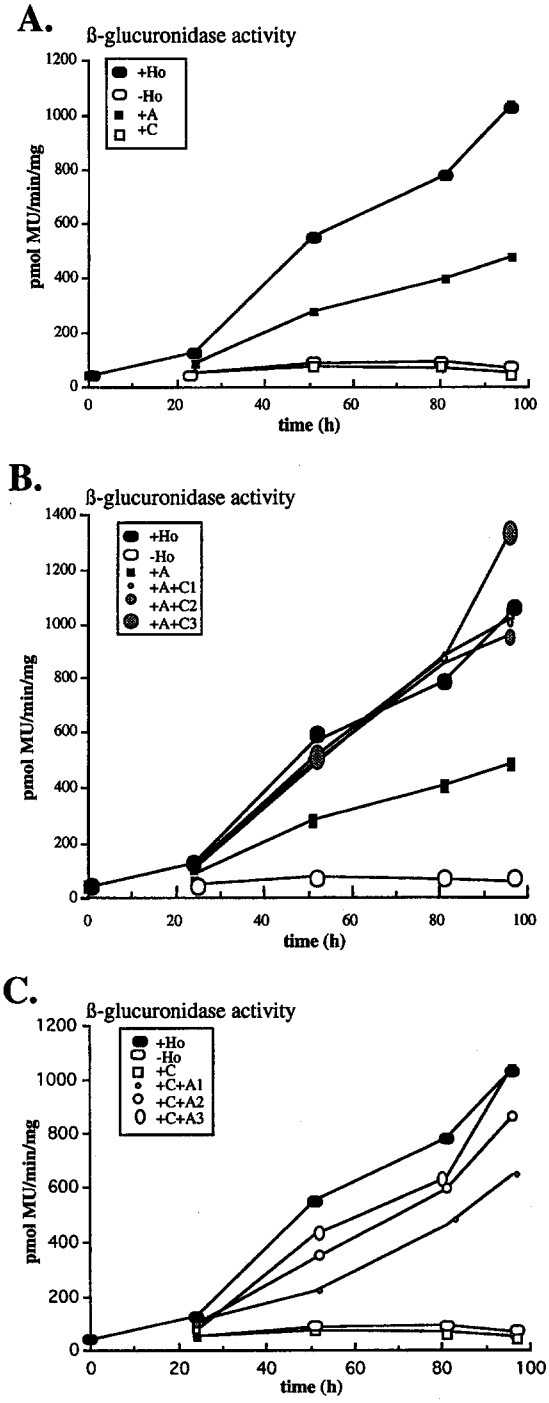

Fig. 5. Kinetics of induction by phytohormones. The β-glucuronidase activity was measured in BG2 transgenic protoplasts after different times of culture. A. Influence of auxin and cytokinin on the expression. + Ho, To medium (3 mg/l NAA and 1 mg/l BA); – Ho, hormone-depleted To medium; + A, To medium with only 3 mg/l NAA; + C, To medium with only 1 mg/l BA. B. Influence of cytokinin concentration on the expression. + A + C1, To medium with 3 mg/l NAA + 0.05 mg/l BA; + A + C2, To medium with 3 mg/l NAA + 0.1 mg/l BA; + A + C3, To medium with 3 mg/l NAA + 0.5 mg/l BA. C. Influence of auxin concentration on expression. + C + A1, To medium with 1 mg/l BA + 0.05 mg/l NAA; + C + A2, To medium with 1 mg/l BA + 0.1 mg/l NAA; + C + A3, To medium with 1 mg/l BA + 0.5 mg/l NAA.

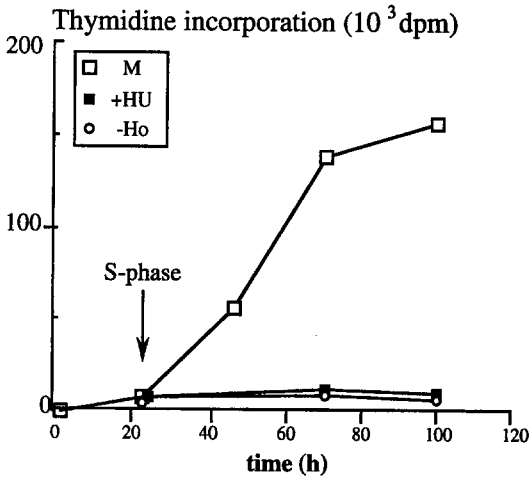

Fig. 6. Quantification of DNA synthesis in protoplasts during culture. The results of one experiment, on one batch of protoplasts are presented; whatever the experiment, the curves presented the same profil. The thymidine incorporation was measured in different conditions and allowed to follow DNA replication in cell culture. The S-phase is beginning approximatively at 24 h, confirming the flow cytometry experiments (data not shown). The different media used were: □ To medium; ■ To medium supplemented with 100 mM hydroxyurea; o hormone-depleted To medium.

or in absence of hormone, DNA synthesis was well inhibited and consequently efficient arrest of the cell cycle was obtained (Fig. 6). In normal To medium culture conditions, DNA synthesis is starting around 20–24 h, corresponding to the beginning of the S-phase. In presence of 100 mM hydroxyurea, β-glucuronidase expression remained low in protoplasts compared to expression level in normal To medium (Fig. 7). The same results were obtained with aphidicolin (data not shown). However, in hormone depleted medium, no expression was detectable compared to the level obtained in medium with hydroxyurea. The modifications of the protoplast growth in presence of hydroxyurea or in absence of hormone

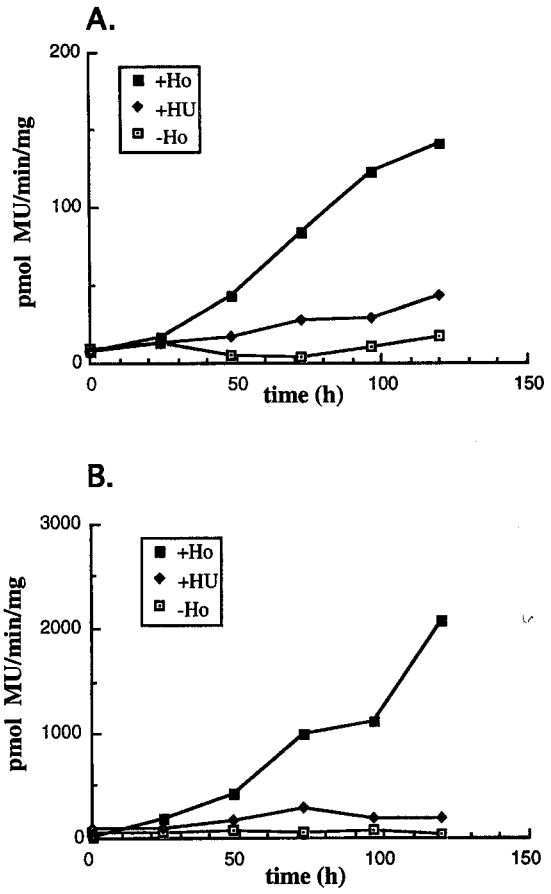

Fig. 7. Two examples of the influence of hydroxyurea on the expression of p*aux-uidA* fusions in cultures of transgenic mesophyll protoplasts. A. Results with one of the BG1 transgenic plant. B. Results with one of the BG2 transgenic plant. The different media used were: + Ho, To medium; + HU, To medium supplemented with 100 mM hydroxyurea; – Ho, hormone-depleted To medium.

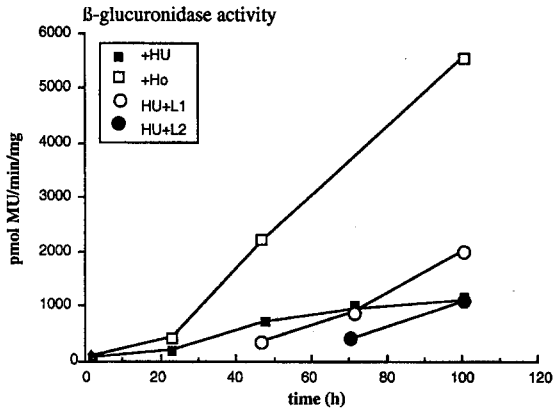

Fig. 8. Reversibility of hydroxyurea inhibition. BG2 transgenic protoplasts were cultured in To medium (+ Ho) or in To medium supplemented with hydroxyurea (+ HU). The samples (HU + L1) and (HU + L2) were cultured in To medium supplemented with 100 mM hydroxyurea during 24 and 48 h respectively, then harvested, washed and cultured in To medium. Batches of protoplasts in the different experiments were periodically harvested (at 24 h, 48 h, 72 h and 102 h), and β-glucuronidase activity was measured.

have not the same consequence on the *aux* gene expression, suggesting a different state of the protoplasts in the two cases (Fig. 6). It suggested two kinds of induction, one sensitive to the presence of an inhibitor arresting cells at the G1/S boundary, the other sensitive to the presence of hormones and to a particular state of the protoplasts.

After one or two days of culture in presence of hydroxyurea, protoplasts were washed and cultured in normal To medium, and β-glucuronidase activity was measured. In tobacco protoplasts, an increase of expression was observed after washing (Fig. 8) suggesting reversibility of hydroxyurea inhibition. The reversibility of the hydroxyurea inhibition was also observed on *Datura innoxia* Mill. cell cultures by Conia *et al.* [14]. Furthermore, a correlation between the p*aux-uidA* expression and the raising of cell cycle inhibition, and thus with cell division, was strongly suggested.

Discussion

Co-regulation and level of expression of the aux *genes*

The *aux1* and *aux2* divergent genes code for the two enzymes of the *de novo* auxin biosynthesis pathway observed in *Agrobacterium*-transformed plant cells. In protoplast transient experiments, the *aux* gene promoter was shown to be a strong promoter in both orientations [22]. In this work, the *aux* intergenic promoter region did not confer a high level of expression in transgenic plants. Furthermore, the *aux1* promoter was weaker than the *aux2* promoter. These results confirmed previous work performed on *A. tumefaciens tms* genes

in transgenic plants [35, 62] or on *A. rhizogenes aux* genes in transgenic roots [2]. The first step of this auxin biosynthesis pathway may act as the limiting one.

The two *aux* genes were shown to be co-expressed, presenting the same tissue specificity. The two T-DNA divergent *mas1'* and *mas2'* genes involved in the mannopine biosynthesis pathway were previously shown to be co-regulated [17, 44, 70]. In petunia, the chloroplast divergent *Cab22R* and *Cab22L* genes encoding chlorophyll *a/b*-binding proteins also show a co-ordinated regulation [24]. The divergent organisation for the two genes might be an element for their co-regulation.

Tissue specificity and induction of the aux *genes*

The *aux* gene promoter activity was localised in meristematic tissues such as root tips, apical meristems, lateral root primordia and lateral meristems. Whereas no activity was detected in mature leaves, the p*aux-uidA* fusions were highly expressed in transgenic mesophyll protoplasts.

High expression of the *aux* promoters was also detected during callus initiation and formation. Other T-DNA genes are also expressed in calli such as *nos* [3], *ags* [31] or *ocs* [42] genes. Previous studies describing the sequence of events in tumour formation [40] or *in vitro* callus formation [27] have suggested some common events between these two processes. In both cases, cell dedifferentiation and unorganised proliferation are induced. This could explain why the genes are strongly expressed in protoplasts, in either transient or stable protoplast assays.

Whereas several T-DNA genes such as *nos* and *mas* genes [5, 41, 57, 68] are wound-inducible, the *aux* intergenic promoter region did not confer wound inducibility.

It was shown that the expression of the *aux* promoter is influenced by the auxin concentration and the balance between auxin and cytokinin. Auxin was necessary for the *aux* gene expression and the level of expression was correlated with its concentration. The auxin induction of the *aux*-genes was late (starting around 20 h) and differed in a significant way from the very rapid auxin induction of the SAUR genes [49]. The auxin-responding elements identified in the SAUR genes [50] were not found in the *aux* intergenic region. Thus, the *aux* gene induction may result from a cascade of events induced by auxin and may resemble more to the auxin induction of the *rolB* gene in transgenic protoplasts [48]. Cytokinins also played a role in the complex regulation of the *aux* genes. According to Meyer and Cooke [51], while auxin is necessary for the beginning of tobacco cell division, cytokinin is required later, just before mitosis, to allow division. Cytokinin contents were shown to increase at the G2/M boundary in tobacco protoplast cultures [56]. In the absence of cytokinin, the expression is not maximal, suggesting that some steps of the cell cycle may not be complete.

Expression and cell cycle

There are several arguments to establish a correlation between expression of the *aux* promoter and cell division. The first one is the localisation of the expression in meristematic tissues, in calli and in protoplast cultures. The second one was provided by the results obtained with inhibitors of the cell cycle on protoplast cultures. Leaf cells are well differentiated towards photosynthetic function and are in a quiescent Go phase. No expression was detected there. During protoplast conversion, these cells progressively become undifferentiated, enter in the G1 phase and divide. Protoplast isolation switches off the leaf programme and starts cell division. The switch occurs around the 24 h, where the S-phase is beginning and may vary a little according to the tobacco variety and the protoplast preparation. This time corresponded to an increase of *aux* gene expression. The inhibition of the DNA replication with aphidicolin or hydroxyurea arrested the cells at the G1/S boundary, in transgenic tobacco cell cultures. This treatment also highly reduced the expression of the p*aux-uidA* fusions. The *aux* gene promoter was consequently under cell-cycle con-

trol. With hormones and an inhibitor, a residual level of expression was detectable compared to the expression in protoplasts cultured in hormone free medium. Some other elements different from a particular step of the cell cycle may also interact with the expression of these genes.

Few data are available on cell-cycle-regulated genes in plants. However, some cDNA clones, isolated from protoplast culture cDNA libraries, were shown to be cell-cycle-regulated. One of the ubiquitin cDNA isolated from *N. sylvestris* protoplast cDNA library is expressed just before the first division and is cell-cycle-related [23]; the *cycO7* cDNA of *Catharanthus roseus* was identified as a S-phase-specific gene [32, 36]; the *par* gene was shown to be expressed during the Go/S transition in tobacco mesophyll protoplasts and is also auxin-regulated [67]. In the same way, few studies mentioned the *cis*-acting elements involved in plant cell-cycle-dependent regulation. Some of these, such as the ACGTCA hexameric sequence, identified in plant histone promoters [34], were searched. An AaGTCA sequence (2932, according to Camilleri and Jouanin [10]) could be a candidate. Two other motifs (ttCGAAAA (3057) and aACGAAA (2836)) showed similarities with the yeast CACGAAA *HO* motif [22]. Transcription of the *HO* gene was shown to be cell-cycle-regulated thanks to the *HO* sequence [9]: the *HO* gene is expressed during late G1 phase. Two motifs (AaGCGT (3001) and ACaGCGT (3178)) sharing similarities with the *Mlu* I ACGCGT sequence were also found. This *Mlu* I element is a common motif found in DNA synthesis genes which are under cell-cycle control, their expression occurring near the G1/S phase boundary. The *Mlu* I sequence, recognised by a specific *trans*-acting factor, was shown to be able to control cell cycle expression of heterogeneous genes [25, 45]. The functionality of these elements in the regulation of the *aux* genes remains to be confirmed by complementary studies.

The expression of the p*aux-uidA* fusions suggested a correlation between cell division and *aux* oncogene expression. This hypothesis raised many questions about the cell cycle step and the required regulatory elements. Since auxins and cytokinins control plant cell division and induce the expression of auxin biosynthesis genes, some regulatory feedback should be necessary for fear that the resulting overexpression would be incompatible with cell life. Some of the negative regulatory elements which were shown in the upstream intergenic region of the genes [22] might play a role in this negative regulation. In our opinion, the *aux* gene system might represent an attractive model to study totipotency, a main characteristic of plants, and to increase our knowledge of gene expression regulation under cell cycle control.

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