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The tobacco *Ext 1.4* extensin gene family is not regulated by auxins in cells proliferating under hormone control
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Abstract – It was previously shown that the tobacco *Ext 1.4* extensin gene is expressed in cells proliferating under hormone control and during lateral root initiation. Now, the regulation of expression of the *Ext 1.4* gene family is analysed in response to the auxins 2,4-dichlorophenoxyacetic acid (2,4-D) and 1-naphthaleneacetic acid (NAA). On the one hand, it is shown that there is no accumulation of *Ext 1.4* mRNAs after short periods of incubation of roots or leaf strips in the presence of either 2,4-D or NAA. On the other hand, germination of transgenic seeds carrying the (-1331/+46)*Ext 1.4*/β-GUS construct on a 2,4-D containing-medium does not lead to ectopic expression of the chimeric gene. Altogether, these results demonstrate that the genes in *Ext 1.4* family are not early auxin-responsive genes. They may be late auxin-responsive genes or their induction in proliferating cells may be the consequence of a higher requirement of structural proteins for the formation of their particular cell walls.

Keywords: Auxin; Cell wall; Extensin; β-glucuronidase; HRGP; *Nicotiana tabacum*

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; GUS, β-glucuronidase; HRGP, hydroxyproline-rich glycoprotein; IAA, indole acetic acid; MS, Murashige and Skoog; NAA, 1-naphthaleneacetic acid

1. INTRODUCTION

Plant primary cell walls are composed of polysaccharides, ions and proteins that form a dynamic structure. Numerous genes are involved in the synthesis of cell wall components and in cell wall assembly [3]. Different classes of cell wall structural proteins have been defined: hydroxyproline-rich glycoproteins (HRGP) among which are extensins [14], hydroxyproline/proline-rich proteins (H/PRP) [27], and glycine-rich proteins (GRP) [4]. Cell walls may be modified during development. Cell division is tied in with cell wall formation starting with the elaboration of the cell plate [26]. Elongation requires numerous changes in cell wall composition and structure [17, 19]. Auxin is involved in all these processes by triggering a signal transduction cascade leading to changes in gene expression and /or in specific enzymatic activities related to cell wall metabolism [1, 8, 19].

We previously showed that the tobacco *Ext 1.4* extensin gene is expressed in lateral root primordia [24] which development may be stimulated by the auxin indole acetic acid (IAA) [30]. *Ext 1.4* is also expressed in cell proliferating under hormone control either in *Agrobacterium tumefaciens*-induced stem tumours, in roots or in petioles [11]. We also showed that the *Ext 1.4* gene belongs to a small multigene family comprising four members in *Nicotiana tabacum* [10, 24]. Promoters of these genes comprise two to four motifs very similar to the CTAATTGTTTA motif which was shown to bind in vitro the *prha* gene product inducible by 2,4-dichlorophenoxyacetic acid (2,4-D) [22]. These motifs are respectively located at -48 (TAATTGTTTA), -241 (TAATAGTTTA), -454 (TAATTGTTAT) and -618 (TAATTGTTCT) with respect to the transcription initiation site of *Ext 1.4* [Salvà and Jamet, unpublished results]. They were also found in several genes, which are developmentally regulated, pathogen-inducible or auxin-responsive [22]. Altogether, these facts raised the question of the inducibility of *Ext 1.4* genes by auxins. The aim of the present study was to analyze the early regulation of the *Ext 1.4* gene family by auxins in more details.

2. RESULTS

2.1. The *Ext 1.4* gene family is not induced in roots or in leaves in response to auxin

The aim of the first series of experiments was to test the inducibility of *Ext 1.4* genes by auxins (Fig. 1). Two different tissues were used, leaf strips and roots. Auxin treatments were very short as those used for the induction of auxin early-responsive genes [1, 16, 29]. Indeed, analysis of mRNA accumulation was performed after only 2 h of incubation in the presence of either 2,4-D or 1-naphthaleneacetic acid (NAA). Hybridization to a probe specific for the *parA* gene, an auxin early-responsive gene, was used as a control for the efficiency of auxin treatment [29]. Indeed, accumulation of *parA* mRNAs was shown to occur as early as 20 min after addition of 2,4-D and to reach a maximum after 4 h [28]. Control of the amount of mRNAs was performed using a 25S rRNA probe.

As expected, the amount of *parA* mRNAs strongly increased in leaf strips and in roots after auxin treatment performed either with 2,4-D or NAA (Fig. 1). On the contrary, there was no increase in the level of accumulation of *Ext 1.4* mRNAs in response to either auxin treatments (Fig. 1). One kb *Ext 1.4* mRNAs were revealed in leaf strips whereas 1.2 kb *Ext 1.4* mRNAs were found in roots. As previously mentioned *Ext 1.4* belongs to a multigene family comprising four genes in *N. tabacum* *Ext 1.4*, *Ext 1.4B*, *Ext 1.4C* and *Ext 1.4D* [10, Salvà and Frener, unpublished work].

These genes show about 90% identity in their 3' non-coding regions and thus hybridize to the same probe [24]. It means that none of them was induced by auxins in the conditions tested.

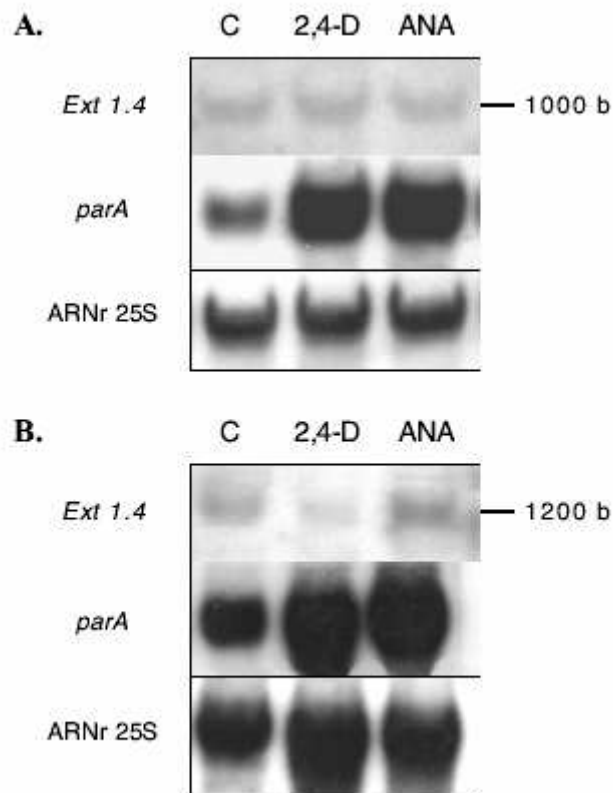


Fig. 1. Accumulation of *Ext 1.4* mRNAs in response to auxin treatment. **A**, leaf strips; **B**, roots. Leaf strips and roots were treated as described in Methods. Total RNAs were extracted from control leaf strips or roots (C), or from leaf strips or roots incubated for 2 h in 10 mg.L^{-1} 2,4-D or NAA. RNA gel blots were performed with 5 μg RNAs per sample. Hybridizations were successively performed on the same membrane with a probe spanning the 3' non-coding region of *Ext 1.4*, a *parA* probe and a 25S rRNA probe. Membranes were over-exposed after hybridization to the *Ext 1.4* probe.

2.2. Expression of the (-1331/+46)*Ext 1.4*/ β -GUS chimeric gene in seeds germinating in the presence of auxin

The *Ext 1.4* gene was chosen for the second series of experiments since its promoter comprises four putative auxin-regulatory elements. Transgenic seeds carrying the (-1331/+46)*Ext 1.4*/ β -GUS chimeric gene were sowed on different media supplemented or not with 2,4-D as previously described [22]. These experiments were performed with 6 independent transgenic lines carrying the (-1331/+46)*Ext 1.4*/ β -GUS construct. Histochemical analyses were performed at different times after beginning of germination. In control plantlets, cotyledons were fully developed after 8 days (Fig. 2B). As expected [23], in plantlets grown on 2,4-D containing-medium, growth was

delayed by about 4 d whereas inhibition of root elongation was observed (compare Fig. 2A-B to Fig. 2C-D).

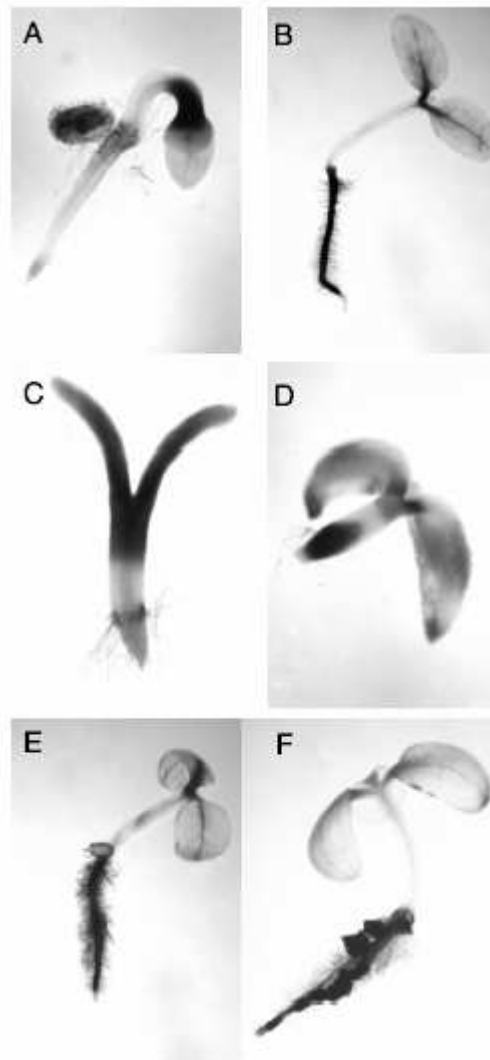


Fig. 2. Influence of 2,4-D on the expression of the chimeric (-1331/+46)*Ext 1.4*/β-GUS gene during germination. **A, B**, control plantlets were analyzed 6 or 8 days respectively after sowing on MS medium modified as described in Methods; **C, D**, plantlets were analyzed 10 or 12 days respectively after sowing on modified MS medium supplemented with 1 mg.L^{-1} 2,4-D. **E, F**, plantlets were grown on modified MS medium for 7 d, and then analyzed 1 or 7 d after transfer on modified MS medium supplemented with 1 mg.L^{-1} 2,4-D. GUS activity was revealed by *in situ* staining.

In control plantlets, after 6 d of germination, the (-1331/+46)*Ext 1.4*/β-GUS chimeric gene was found to be expressed in collar cells and in the hypocotyl hook (Fig. 2A). Eight days after the beginning of germination, GUS staining was observed in the root piliferous zone, at the cotyledon bases and sometimes in cotyledons (Fig. 2B). GUS staining in the root was more precisely located in epidermis and hairs as previously shown [11]. In plantlets grown on 2,4-D containing-medium,

despite the differences observed in morphology, there was no significant difference in the pattern of expression of the (-1331/+46)*Ext 1.4*/β-GUS chimeric gene in root and in hypocotyl (Fig. 2C-D). The expression in cotyledons may have occurred at a slightly higher level.

Finally, plantlets grown on a medium lacking 2,4-D during 7 d were transferred to a 2,4-D containing medium and analyzed after 1 day (Fig. 2E) or 7 d (Fig. 2F). After 1 d, both the number and length of root hairs increased [21]. The pattern of expression of the (-1331/+46)*Ext 1.4*/β-GUS chimeric gene was not modified (compare Fig 2E and Fig. 2B). After 7 d, GUS staining was found in the numerous root primordia induced by 2,4-D (Fig. 1F).

All these experiments show that it was not possible to induce any ectopic expression of the (-1331/+46)*Ext 1.4*/β-GUS chimeric gene in 2,4-D treated-plantlets within a short time. GUS staining was only found in cells induced to proliferate upon auxin treatment.

3. DISCUSSION

Altogether, these results show that *Ext 1.4* genes are not directly inducible by auxins even if they are expressed in cells proliferating upon hormone control and if several putative auxin-regulatory elements are present in their promoters. *Ext 1.4* transcripts do not accumulate in leaf strips or in roots after a short auxin treatment as described in the case of typical auxin-inducible genes [16, 28]. The only modification of the pattern of expression of the (-1331/+46)*Ext 1.4*/β-GUS chimeric gene is found in cotyledons. But, this may be due to the fact that the development of plantlets is delayed after auxin treatment. The time of emergence of the plantlet out of the seed is much longer. This causes additional mechanical constraints that are known to induce the expression of *Ext 1.4* genes [24]. However, the expression of the (-1331/+46)*Ext 1.4*/β-GUS chimeric gene was not found in all plantlet tissues as shown in the case of the *prha* gene, a true auxin early-responsive gene [22]. Thus, *Ext 1.4* genes do not belong to auxin early-responsive genes. In contrast, the *Medicago sativa* MsPRP5 gene encoding a PRP is induced by 2,4-D in cell suspension cultures as early as 20 min after commencing treatment [9]. The *N. tabacum* HRGPnt3 gene encoding a protein with a C-terminal extensin domain is induced in newly-formed root primordia 2 to 6 h after the beginning of IAA treatment [31].

Ext 1.4 genes may belong to late-responsive genes, as may other genes encoding cell wall structural proteins. The carrot *DcPRP1* gene encoding a proline-rich protein was not found to be induced 3 h after IAA, NAA or 2,4-D application, but was expressed at a high level 24 h after one of these treatments [5]. The *Arabidopsis thaliana* proline-rich protein gene *AtPRP3* is expressed in root hairs and its level of expression is greatly induced 48 h after NAA treatment [2].

Alternatively, the expression of *Ext 1.4* genes in cell proliferating under hormone control may be due to the necessity for high level production of extensins. Indeed, usually these cells form calli and they are not organized in tissues. Their cell walls have a particular structure which was shown to have a high content in hydroxyproline [32].

Finally, it seems that there is no general rule for the regulation of expression of genes encoding cell wall structural proteins in response to auxins. Same conclusions could be drawn for other types of regulation of such genes, such as responses to wounding or mechanical constraints [12]. This may be related to the fact that the proteins they encode confer different properties to cell walls.

4. METHODS

4.1. Plant material

Seeds of *Nicotiana tabacum* L. var Samsun were provided by SEITA (Institut Expérimental du Tabac). Plants of *N. tabacum* L. var. Petit Havana SR1 [15] were used for *Agrobacterium tumefaciens*-mediated transformations. Greenhouse culture conditions were a 16 h light/8 h dark cycle at 22±4 °C. *In vitro* culture conditions were a 16 h light (24 °C)/ 8 h dark (20 °C) cycle.

4.2. RNA analysis

Isolation of total RNAs was performed according to [7]. Northern blot analyses were performed as described in [20]. A fragment spanning the 3' non-coding region of the *Ext 1.4* gene was used as a probe specific for the *Ext 1.4* gene family [24]. A probe specific for the *N. tabacum parA* gene was a gift from Prof. T. Nagata [28]. The integrity and the amount of RNAs were systematically checked using a bell pepper 25S rRNA probe kindly provided by Dr. R. Schantz (IBMP-CNRS Strasbourg). All these probes were labeled with [α -³²P]dCTP (3000 Ci/mmol, Amersham Pharmacia Biotech UK Limited) by random priming [6].

4.3. Chimeric gene construct and plant transformation

DNA manipulations were performed according to standard procedures [25]. The (-1331/+46)*Ext 1.4*/ β -glucuronidase (GUS) construct was previously described [11]. Leaf discs of *N. tabacum* L. var Petit Havana SR1 were transformed using *A. tumefaciens* LBA4404 as previously described [11]. Transgenic plantlets were grown *in vitro*.

4.4. Auxin treatments

Leaf strips and roots of *in vitro*-grown plantlets were successively incubated during 4 h in a Murashige and Skoog (MS) medium diluted [18], and during 2 h in fresh MS medium either in the absence of auxin or in the presence of 10 mg.L⁻¹ 2,4-D or 10 mg.L⁻¹ NAA. During the incubation, samples were gently shaken at 24 °C. Seeds of transgenic plants were obtained by self-pollination of primary transformants. They were sown on modified MS medium containing 10 g.L⁻¹ sucrose and 300 mg.L⁻¹ kanamycin. This germination medium was eventually supplemented with 1 mg.L⁻¹ 2,4-D. Histochemical analysis of GUS activity was performed as described in [13].

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