Research Article

AtchitIV gene expression is stimulated under abiotic stresses and is spatially and temporally regulated during embryo development

Liliane B. de A. Gerhardt[1], Cláudia Magioli[1], Ana B.U.C.M. Perez[1], Rogério Margis[1,2],
Gilberto Sachetto-Martins[1] and Márcia Margis-Pinheiro[1]

[1]*Universidade Federal do Rio de Janeiro, Departamento de Genética, Laboratório de Genética Molecular Vegetal, Rio de Janeiro, RJ, Brazil.*
[2]*Universidade Federal do Rio de Janeiro, Instituto de Química, Departamento de Bioquímica, Rio de Janeiro, RJ, Brazil.*

Abstract

The expression of *AtchitIV* gene was analysed in *Arabidopsis* plants submitted to abiotic stresses. Transcript accumulation was detected in leaves in response to UV light exposure, exogenous salicylic acid administration and wounding. Transgenic *Arabidopsis* plants carrying *AtchitIV* promoter::*gus* fusion also showed differential expression of the reporter gene in response to these treatments. The *AtchitIV* expression was also analysed during *Arabidopsis* embryo development. GUS assay demonstrated *AtchitIV* promoter activation in zygotic embryos from torpedo stage up to full maturation. Promoter deletion analysis indicated that all the 5' *cis*-acting elements responsible for the specific tissue expression are located in a region of 1083 bp, adjacent to the start of transcription. A negative regulatory region located between portions -1083 and -600 was also observed.

Key words: abiotic stress, *Chia4 Arabidopsis thaliana* endochitinase, embryogenesis, gene expression, promoter.

Introduction

Plants respond to environmental changes by controlling the expression of a large number of genes. Endochitinases are constitutively expressed in several organs of healthy plants (Samac *et al.*, 1990; Beerhues and Kombrink, 1994; Robinson *et al.*, 1997; Yeboah *et al.*, 1998). However, the modulation of chitinase expression was observed in response to Nod factors during the nodulation process (Staehelin *et al.* 1994 and 1995; Goormachtig *et al.*, 1998), as well as in adverse conditions, suggesting that these enzymes are involved in plant defence responses (Samac and Shah, 1991; Margis-Pinheiro *et al.*, 1993; Beerhues and Kombrink, 1994; Büchter *et al.*, 1997; Busam *et al.*, 1997; Dong and Dunstan, 1997; Yu *et al.*, 1998; Ancillo *et al.*, 1999). It has also been demonstrated that plant endochitinases expression is spatially and temporally regulated during plant development processes such as somatic embryogeneses (de Jong *et al.*, 1992 and 1993). In carrots, the presence of EP3 chitinase increased the number of globular embryos of ts11 mutants in non-permissive temperature conditions and also promoted the transition from the globular to heart-shape stage (de Jong *et al.*, 1992 and 1993). In *Picea glauca*, a chitinase gene is also activated during maturation of somatic embryos (Dong and Dunstan, 1997).

We have previously described the isolation and characterisation of *A. thaliana Chia4* chitinase, the *AtchitIV* gene, which accumulated very rapidly in *Arabidopsis* leaves challenged with *Xanthomonas campestris* bacteria (Gerhardt *et al*., 1997). Recently, the expression pattern of the *AtEP3* chitinase from *Arabidopsis* was also described. Sequence analysis revealed that *AtEP3* and *AtchitIV* correspond to the same gene (Passarinho *et al*., 2001). In the same study, *in situ* hybridization, revealed that *AtchitIV/AtEP3* is expressed in "nursing" cells surrounding the embryos during the development of somatic embryos. In plants, *AtchitIV/AtEP3* expression was detected in mature pollen and in growing pollen tubes until they enter the receptive synergid, but no activity was detected in embryos. *AtchitIV/AtEP3* expression was also detected in hydathodes, stipules, root epidermis and emerging root hair. Based on *AtchitIV/AtEP3* gene expression pattern, Passarinho and co-workers proposed that this *Arabidopsis* chitinase is involved in programmed cell death (PCD).

Send correspondence to Márcia Margis-Pinheiro. Universidade Federal do Rio de Janeiro, Departamento de Genética, Laboratório de Genética Molecular Vegetal, Caixa Postal 68011, 21941-970, Rio de Janeiro, RJ, Brazil. E-mail: margism@biologia.ufrj.br.

In this paper, analyses of induction and temporal accumulation of *AtchitIV* transcripts in response to abiotic stresses were conducted. In order to identify the location of possible cis acting elements involved in these responses, chimeric constructs harbouring the *gus* coding region fused to several regions of the putative *AtchitIV* promoter, consisting of the intact 1923 bp and its deletions, were used to obtain *A. thaliana* transgenic plants. Analyses of tissue expression pattern of the different promoter deletions during plant development were also carried out.

Materials and Methods

Plant material and abiotic stress treatments

Arabidopsis thaliana plants ecotype Columbia-0 (Lehle seeds) were grown under controlled conditions in a growth chamber at 22 °C, with 75 % relative humidity and at daily period of 16 h. Five to six week-old plants were submitted to treatments and rosette leaves were collected at different times after the onset of the stresses. For salicylic acid (SA) treatment, plants were sprayed with a 2 mM salicylic acid solution (pH 6.5), and harvested at 0,5; 1; 3; 5; 6 and 20 h after treatment. For UV irradiation treatment, plants were irradiated twice for 15 min with 254 nm UV light at an intensity of 0,8 W/m^2, and harvested at 0; 16; 23; 30; 48 and 58 h after exposure. For wounding stress, leaves were squeezed twice across their surface with tweezers, and harvested at 0,5; 1 and 4 h after wounding. Plants were submitted to heat shock treatment with a temperature of 34 °C for 4 h. The 5 °C cold shock treatment was also performed for 48 h. Transgenic plants were submitted to the same treatments.

RNA-blot hybridisation

RNA was isolated from *Arabidopsis* plants as described by Ragueh *et al.* (1989) and about 20 µg of total RNA were used for northern-blot experiments. Membrane hybridisations were performed according to Sambrook *et al.* (1989) using the [^{32}P]αdCTP *AtchitIV* cDNA labelled fragment at 42 °C. Probes were prepared using the random priming method (Feinberg and Vogelstein, 1983). Densitometry analyses were performed using the public domain NIH ImageJ program from the National Institute of Health, US (available at http://rsb.info.nih.gov/ij/).

Construction of chimeric genes

Fusion of the 1923 bp of the *AtchitIV* 5' flanking region and its deletions (Δ1500, Δ1083, Δ600 and Δ300) to the *gus* coding region were obtained by introducing a *Nco*I site at the start codon ATG of the chitinase gene by site directed mutagenesis (Ho *et al.*, 1989; Higushi *et al.*, 1988). To amplify the Δ1923, Δ1500, Δ1083, Δ600 and Δ300 fragments of the *AtchitIV* promoter by PCR technique, different forward primers (5'cat cgt cga cga att ctt taa act aat gga aac aag ttt c3'), (5'cat cgt cga cga att caa cgg agt att tct tta cc3'),

(5'cat cct gca gaa ttc cct ggt gac ta3'), (5'cat cct gca gga att cac ttt aga ttt ggt tcg act tta ttt a3') and (5'cat cct gca gga att ccg atc ata agt cat aat tca gaa aat t3') were used with a general reverse primer (5'ggg agt cac cat ggt gat gtt gtt ga3'), respectively. The PCR reactions contained 100 ng of genomic DNA, 1XPCR buffer (Perkin Elmer), 2 mM MgCl$_2$, 0.2 mM dNTPs, 5 µM primer and 1 unit of Taq polymerase (Perkin Elmer). The reactions were heated to 92 °C for 5 min followed by 30 cycles of amplification consisting of 30 s at 92 °C, 30 s at 60 °C, and 1 to 2 min at 72 °C, depending on the size to be amplified. The amplified fragments were cloned into pGUS1, a plasmid with a GUS promoterless expression cassette and the 3' octopine synthase termination signal (Plant Genetic System N.V., Belgium). These fusions were cloned into the plant transformation vector pDE1001 (Plant Genetic System N.V., Belgium) using the *EcoR*I and *Hind*III restriction sites. The pDE1001 T-DNA contains the neomycin phosphotransferase gene (*nptII*), under the control of the NOS promoter, as plant-selectable marker providing resistance to kanamicin. The 1923 bp of the 5' promoter region of the *AtchitIV* chitinase appears in the EMBL data bank under accession number Y14590.

### Plant transformation and histochemical GUS assay

The plant transformation vectors were introduced into the disarmed *Agrobacterium tumefaciens* strain C58C1(PMP90) by triparental mating (Bevan, 1984). *Arabidopsis* plants obtained from seeds of ecotype C-24 (Lehle seeds) were transformed by the root explants method via *A. tumefaciens* as described by Valvekens *et al.* (1988) and Clarke *et al.* (1992). Histochemical GUS assay was performed according to Jefferson (1987) with minor modifications described by Sachetto-Martins (1995), using 1.0 mM potassium ferri- and 0.5 mM ferrocyanide and staining for 16 h. Embryos were removed from seeds immersed in a 100 mM phosphate buffer (pH 7,0) containing 1.8 mM cycloheximide, and then submitted to histochemical analysis. Transgenic plants challenged with abiotic stresses were assayed for GUS 16 h after the onset of the stresses.

Results and Discussion

In this study we investigated the activation of the *AtchitIV* chitinase gene in response to abiotic stresses and during plant development. The induction of *AtchitIV* chitinase transcripts accumulation in leaves in response to abiotic stresses was examined by RNA blot hybridization. Five to six week-old *A. thaliana* plants were challenged with UV, salicylic acid, wounding, heat and cold shock. According to the results, accumulation of *AtchitIV* mRNA was induced by UV irradiation, salicylic acid and wounding (Figure 1). UV induction was evident very shortly after treatment. Maximal transcript accumulation occurred after

1 h and was maintained at high levels until 6 h after the onset of the stress. Despite the lower level, after 58 h, the mRNA detected was still higher than that found in non-stressed plants (Figure 1A). Salicylic acid induced *AtchitIV* transcripts accumulation in *A. thaliana* leaves at low level, as revealed by densitometry, to be around 1,5 fold after 3,5 h of treatment (Figure 1B). The *AtchitIV* induction by salicylic acid, an intermediate compound of the signal transduction pathway of pathogen infection, in addition to its induction by bacteria infection (Gerhardt *et al.*, 1997), reinforces the role of this chitinase in plant defence. In addition to UV light irradiation and SA treatment, wounding also produced *AtchitIV* transcript accumulation

in leaves. In stressed leaves, maximal response occurred 1 h after wounding and transcript levels decreased rapidly afterwards (Figure 1C). In contrast to these treatments, heat and cold shock were not able to induce *AtchitIV* transcripts (data not shown). The *PvChi4*, a *Phasealus vulgaris Chia4* chitinase gene that has high identity with *AtchitIV* (70,2%), is also induced by UV irradiation (Margis-Pinheiro *et al.*, 1993). The non-activation of the *AtchitIV* transcript accumulation in response to heat stress contrasted to that observed on *PvChi4* bean chitinase gene, which is highly induced by higher temperatures (Margis-*Pinheiro et al.*, 1994).
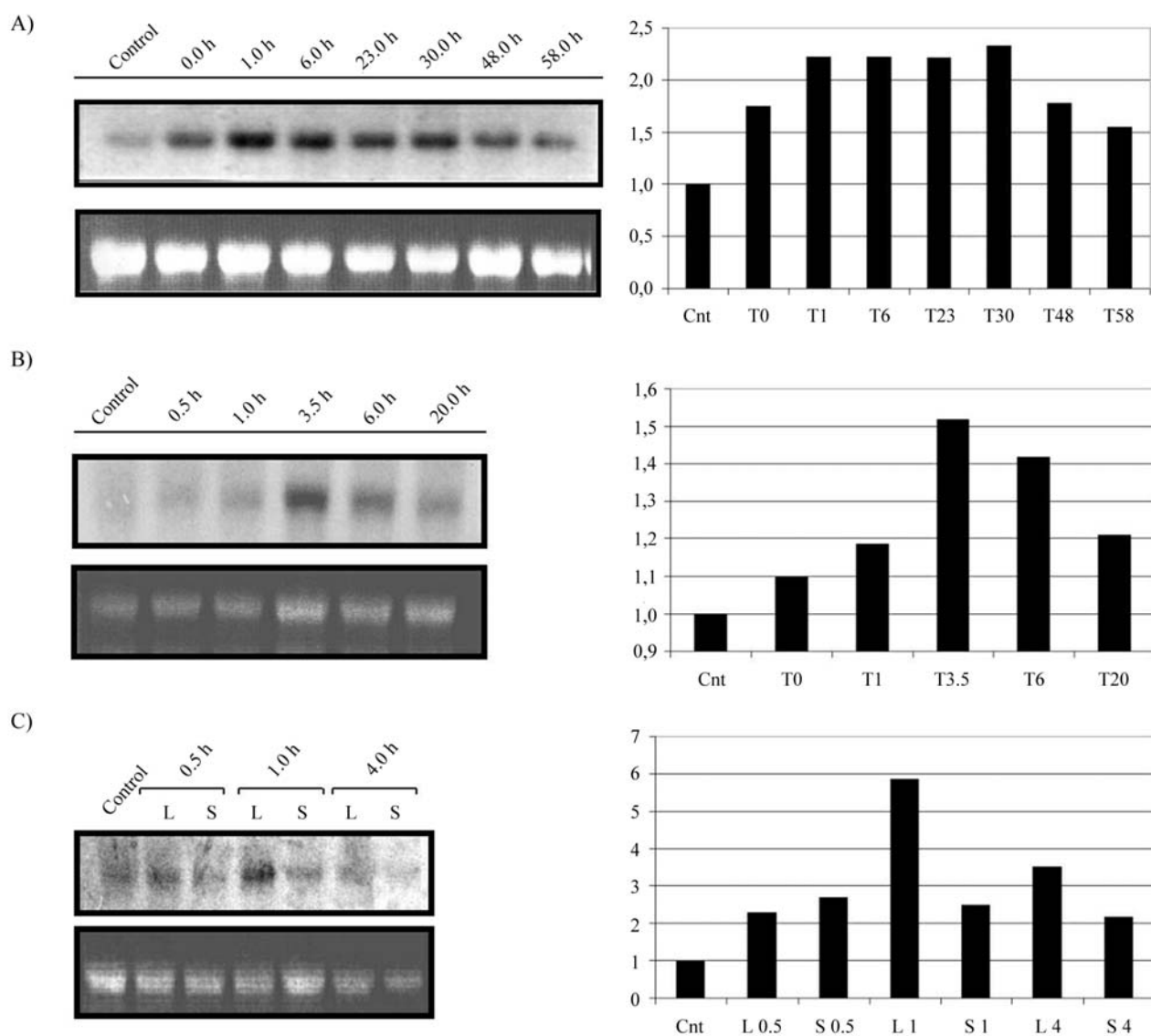

Figure 1 - *AtchitIV* transcripts accumulation in leaves in response to abiotic stress analysed by RNA blot hybridization. RNA from leaves of stressed plants collected at different time points, shown in hours, after the onset of treatments and RNA from leaves of control plants cultivated under normal conditions. A) UV irradiation (the 0.0 point represents the transcript levels just after the end of treatment); B) Salicylic acid treatment; C) Wounding treatment: (L) and (S) represent the local and systemic wounding leaves. The *AtchitIV* cDNA was used as a probe. Histograms correspond to the quantitative ratio between the autoradiographic signal and the ethidium bromide staining of RNA samples (bottom panels).

The expression pattern of *AtchitIV* promoter was analysed during plant development using at least five independent GUS positive transgenic lines of each construct. The general expression pattern of the *AtchitIV* gene has been reported previously (Passarinho *et al*., 2001). In that study, the tissue expression pattern of the *AtchitIV/AtEP3* gene was analysed using 1100 bp of the promoter region fused to the *gus* coding region. Transgenic plants carrying this construct presented GUS activity in the meristematic region, hydathodes, root epidermis, pollen and in stigma during the fertilisation process (Passarinho *et al.*, 2001). In order to identify possible promoter regions involved in the control of *AtchitIV* expression, in the present study we analysed the tissue expression pattern of transgenic plants containing promoter deletions at the positions -1923, -1500, -1083, -600 and -300. The GUS accumulation pattern obtained was identical for the transgenic plants lines containing the $\Delta1923$, $\Delta1500$ and $\Delta1083$ chimerical constructs, indicating that all regulatory regions needed for *AtchitIV* tissue expression are present within the 1083 bp of the promoter. During seed germination, GUS staining was observed in seedling cotyledons and weakly in root, for all analysed constructs. GUS activity was also detected in the meristematic region just before the emergence of the first leaves and remained during plant development (Figure 2A). In seedlings, GUS staining was also observed in stipules, hydathodes and root epidermis (Figures 2A and 2B). In leaves of flowering plants, GUS activity was detected in hydathodes at the end of veins and along leaf margins as well (Figure 2C), confirming the constitutive expression of the *AtchitIV* gene in unstressed leaves of control plants (Figure 1). In flowers, GUS staining was observed in pollen, in a stage-dependent manner, and in the stigma during the fertilisation process (data not shown). These results confirm a previous study in which the expression of transgenic *Arabidopsis* plants containing the *AtchitIV/AtEP3* 1100 bp promoter was analysed (Passarinho *et al.*, 2001). In addition, this paper reports that GUS activity was also verified in the floral receptacle, during floral senescence and at the beginning of silique development (Figure 2D). The *AtchitIV* activation during the senescence process of the floral receptacle corroborates the role of this chitinase in PCD proposed by Passarinho *et al.* (2001). During seed development, GUS activity was detected in the micropyle region (Figure 2G) and in the endosperm (data not shown), next to the radicule pole, whose tissues are addressed to senescence and degradation, respectively.

In reproductive organs, transgenic plants harbouring the $\Delta600$ and $\Delta300$ constructs presented the same GUS activity pattern observed for the $\Delta1923$, $\Delta1500$ and $\Delta1083$ constructs. Different from the previous constructs, plants carrying the deletions $\Delta600$ and $\Delta300$ expressed GUS in the whole leaves and seedlings (Figures 2E and 2F). These results suggest the presence of a restrictive regulatory region between the positions -1083 and -600.
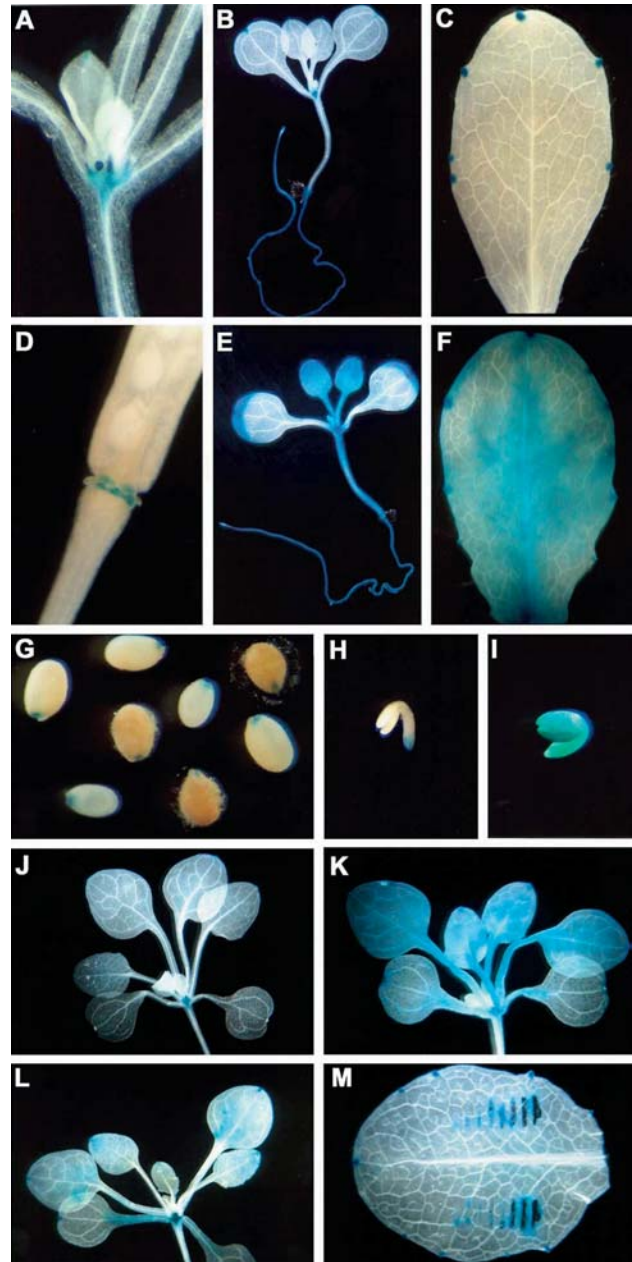
Figure 2 - Expression pattern of *AtchitIV* promoter in *A. thaliana* plants. A-D and G-M show representative GUS activity in transgenic plants harbouring promoter constructs at positions -1923, -1500, -1083. E and F show representative GUS activity in transgenic plants harbouring promoter constructs at positions -600 and -300. Histochemical localisation of GUS activity in *Arabidopsis* transgenic plants: A) Seedling - detail of the meristematic region (40X); B) Ten day-old seedling (6,5X); C) Leaf (10X); D) Silique - detail of the floral receptacle (35X); E) Ten day-old seedling ($\Delta300$ promoter) (6,5X); F) Leaf ($\Delta300$ promoter) (10X); G) Seed from different developmental stages (globular - mature stage) (40X); H) Embryo - late torpedo stage (40X); I) Embryo - mature stage (40X); J) Seventeen days-old control plant (6,5X); K) Seventeen day-old UV irradiated plant (6,5X); L) Seventeen day-old salicylic acid sprayed plant 6,5X); M) Wounded leaf (10X).

Despite some differences in the staining intensity for the $\Delta600$ and $\Delta300$ constructions, all five promoter::*gus* fusions showed a similar tissue expression pattern during seed development. Histochemical analyses of zygotic em-

bryos at different stages of development were performed demonstrating that the reporter gene was not expressed at the globular and heart stages (data not shown). The *AtchitIV* promoter activity started in the radicule pole at torpedo to late torpedo stage (Figure 2H). The same expression pattern could be observed in late torpedo embryos (data not shown). In mature embryos GUS activity was detected in the whole embryo (Figure 2I). The expression pattern observed in zygotic embryos was identical for all *AtchitIV* promoter deletions studied, indicating that all necessary elements to control the embryo tissue expression are present within the 300 bp. However, a more intense activity was verified for embryos containing the Δ300 bp construct, when compared to the Δ1923, Δ1500, Δ1083 and Δ600 constructs, indicating that a second regulatory negative element could be located between the positions -600 and -300. The regulation of *Chia4* chitinases during embryogenesis has been demonstrated. *Picea glauca* basic chitinase is involved in embryogenesis (Dong and Dunstan, 1997). Transcripts of this chitinase were highly abundant in embryogenic tissues even in cotylledonary embryos and in plantlets. Nevertheless, the carrot *EP3*, another *Chia4* chitinase, was not detected in zygotic embryos nor in somatic embryos, although its expression had been demonstrated during seed development and somatic embryogenesis process. The *EP3* expression suggests a "nursing" function during zygotic and somatic embryogenesis (Van Hengel *et al.*, 1998). During somatic embryogenesis, the *EP3* expression was verified in embryogenic and non-embryogenic cultures (Van Hengel *et al.*, 1998). During somatic embryogenesis, Passarinho and co-workers (2001) found that *AtchitIV/AtEP3* expression was restricted to embryogenic cultures in cells close to the developing embryos, but not in the embryos themselves. In contrast to our results, the expression of *gus* was not seen during zygotic embryo development (Passarinho *et al.*, 2001). However, Passarinho and co-workers (2001) used the ecotype Wassilewskija to obtain *Arabidopsis* transgenic plants carrying *AtchitIV/AtEP3* promoter-*gus* fusion, whereas in this study the C24 ecotype was employed for plant transformation. Differences in expression pattern between parental ecotypes of *A. thaliana* may explain the contradictory data obtained in this study and by Passarinho and co-workers concerning the *AtchitIV/AtEP3* expression during embryo development.

In order to map regulatory regions in the *AtchitIV* promoter accounting for the response to abiotic stresses, seventeen day-old transgenic plants containing the different promoter deletions were submitted to UV irradiation and salicylic acid treatment. Both treatments were able to induce GUS staining in transgenic plants containing the Δ1923, Δ1500 and Δ1083 chimeric constructions (compare Figure 2J with Figures 2K and 2L). Since the basal GUS activity in leaves of plants containing the Δ600 and Δ300 deletions was already very high, it was not possible to show further accumulation of GUS activity in response to different treatments. These results suggest that the regulatory *cis*-elements necessary for UV light and salicylic acid response are present within the 1083 bp of the 5' flanking region.

Wound response observed by RNA-blot hybridization (Figure1C), was also demonstrated in the promoter-*gus* transgenic lines (Figure 2M). All constructs analysed were able to mediate wound activation of the *gus* gene, indicating that all *cis*-acting elements necessary for this response are present within the 300 bp *AtchitIV* proximal promoter region.

Together these results indicate a combination of positive and negative regulatory elements for the tissue-expression pattern of the *AtchitIV* gene. Negative regulatory elements seem to be located between -1083 and -600 bp. These elements are responsible for expression mediated by the *AtchitIV* promoter to stipules, hydathodes and roots. The presence of a second negative regulatory region between -600 and -300 positions can also be suggested since a more intense widespread GUS activity was observed in Δ300 plants. Furthermore, the results presented here indicate that all 5' regulatory elements, needed for tissue expression pattern of *AtchiIV* gene are located within 300 bp upstream of the translational start codon. Activation of *AtchitIV* promoter during stress treatments indicate that UV and salicylic acid regulatory regions are located downstream to 1083 and wounding-activating elements lie between -300 and +1.

The induction of *AtchitIV* transcript accumulation in the early response to several abiotic stresses support the involvement of this chitinase in plant defence mechanism. The induction of *AtchitIV* transcript accumulation by salicylic acid, an intermediate in the signal transduction pathway during pathogen infection, corroborates the earlier results where this chitinase was triggered by bacteria attack (Gerhardt *et al.*, 1997). In conclusion, we propose that *AtchitIV* chitinase is involved in the early responses to pathogen attacks such as the establishment of hypersensitive reaction. Besides the involvement of *AtchitIV* chitinase in plant defence, its expression pattern suggests an additional role in different aspects of plant development such as embryogenesis processes.

Acknowledgments

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