Characterization of chitinases able to rescue somatic embryos of the temperature-sensitive carrot variant ts11

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Received 15 December 1995; accepted in revised form 1 April 1996

Key words: plant class IV endochitinase (EC 3.2.1.14), class I acidic endochitinase, class II acidic endochitinase, carrot, somatic embryos, ts11

Abstract

To characterize the acidic endochitinase EP3, able to rescue somatic embryos of the carrot cell line ts11, the enzyme was purified from the medium of wild-type suspension cultures. Peptide sequences, deduced amino acid sequences of corresponding PCR-generated cDNA clones, serological relation and biochemical properties showed that there were at least five closely related chitinases, four of which could be identified as class IV EP3 chitinases with an apparent size of 30 kDa. Two other proteins were identified as a serologically related class I acidic chitinase (DcChitI) of 34 kDa, and a serologically unrelated 29 kDa class II acidic chitinase (DcChitII), respectively. Additional cDNA sequences, Western and Southern analysis showed the presence of at least two, but possibly more, highly homologous class IV EP3 genes in the carrot genome. Two class IV EP3 chitinases were tested and found to be able to increase the number of ts11 globular embryos formed under non-permissive conditions. One of the class IV EP3 chitinases as well as the class I chitinase DcChitI promoted the transition from globular to heart-stage ts11 embryos. The class II endochitinase and a heterologous class IV chitinase from sugar-beet were not active on ts11. This suggests that there are differences in the specificity of chitinases in terms of their effect on plant somatic embryos.

Introduction

In carrot (Daucus carota L.) the possibility exists to study the process of embryogenesis in vitro, where it is initiated from cultured suspension cells rather than from the fusion of the male and female gamete as in the developing seed. This process, somatic embryogenesis, has been exploited to select mutants disturbed in the formation of the typical globular, heart and torpedo embryo stages. The carrot ts11 somatic embryo variant cell line, obtained after EMS mutagenesis, was selected on the basis of the arrest in its embryo development at an elevated temperature. The temperature sensitivity of ts11 appeared to be restricted to a relatively short period around the transition from globular to heart-stage embryo. In the same time interval ts11 embryo development at the permissive temperature was sensitive to replacement of the medium by fresh medium. Both temperature- and fresh medium-induced arrest could be overcome by supplementing the medium with secreted proteins from wild-type carrot cells [25].
The causative factor in the rescue of the temperature-impaired somatic embryogenesis in \( ts11 \) was purified from wild-type culture medium, designated extracellular protein 3 (EP3) and identified as an endochitinase (poly(1,4-(N-acetyl-\( \beta \)-D-glucosaminide)) glycanohydrolase; EC 3.2.1.14) with an apparent molecular mass of 32 kDa [11]. Recently, evidence was presented that a transient decrease in the concentration of an otherwise fully functional EP3 endochitinase in the medium of \( ts11 \) embryo cultures grown at the non-permissive temperature causes the arrest in \( ts11 \) embryo development [13]. The EP3 endochitinase was first identified as such by the amino acid sequence of two peptides derived from it [11]. These peptides were from a region sharing extensive homology between class I, II and IV chitinases, and did not allow further classification of the EP3 endochitinase. The aim of the present work was to obtain cDNA clones corresponding to the EP3 protein. To obtain sufficient peptides for amino acid sequencing it was essential to purify the EP3 endochitinase from large-scale suspension cultures of carrot due to the low abundance of the protein. In the course of this work, the presence of multiple EP3 isoenzymes was noted. Two of these were purified, characterized and compared with respect to some of their catalytic, biological and structural properties. The deduced amino acid sequences of the different cDNA clones obtained showed a high homology to each other and were compared with the amino acid sequence of tryptic peptides derived from three of the EP3 endochitinases. From this, the endochitinase originally purified as being capable of rescue of \( ts11 \) could be identified as a class IV chitinase. While both of the EP3 chitinases tested promoted globular embryo formation, only one had the ability to overcome the globular-heart transition. Whereas a heterologous class IV chitinase from sugar-beet was not effective at all, the carrot class I chitinase was also found to be able to promote the globular-heart transition. These results support the notion that certain plant chitinases have a role in plant development besides their well-studied effects in plant-pathogen interactions [10], and that pronounced differences exist between classes and even between individual members of the same class.

Materials and methods

Plant material and cell cultures

Small-scale carrot ‘10’-line (\( D. \) carota cv. Flakkese SG766 Trophy) suspension cultures were grown in liquid B5 medium in the presence of 2 \( \mu \)M 2,4-dichlorophenoxyacetic acid (2,4-D) in 14 days subculture cycles [11]. Large scale suspension cultures were started with a cell density of 4% packed cell volume in 800 ml of medium in 2 l Erlenmeyer flasks on a rotary shaker at 100 rpm, at 25 °C and with a 20 h light period. Alternatively, very large scale cultures (30–100 l) were grown for 14 days at 25 °C in B5 medium at 4 rpm in a pilot scale rotating drum plant cell fermentor. The cultures were started with a cell density of 4% packed cell volume per total volume. Several parameters of embryogenic carrot cell suspension cultures grown in standard 50 ml cultures were compared to 800 ml cultures or the rotating drum fermentor cultures. Growth speed, embryogenic potential and the pattern of proteins accumulated in the medium were all very similar (data not shown). The total protein concentration in the media of suspension cultures increased from 10–20 \( \mu \)g/ml to 20–40 \( \mu \)g/ml between day 7 and day 14 after subculture. Because the protein patterns remained virtually identical, extracellular proteins were purified from cultures grown for 14 days.

The temperature-sensitive variant \( ts11 \) and its parental cell line \( A^+ \) (\( D. \) carota cv. S. Valery), were maintained as described [15]. For the initiation of embryo cultures, suspension cells were sieved through nylon meshes between 50 and 125 \( \mu \)M (‘10’ line) or 70 and 170 \( \mu \)M (\( ts11 \) and \( A^+ \)) respectively and were diluted to 20 000 cells per ml or 2500 clusters per ml respectively in hormone-free B5 medium.

Ts11 embryo rescue assays

Rescue assays were performed with newly initiated \( ts11 \) embryo cultures as described previously [12]. Control experiments were performed in the presence of the buffer in which the chitinases were dissolved. Embryo formation was scored at two or more days after culture initiation.

Chitinase purification

Culture medium from 14 days old cultures was harvested by filtration through 90 and 40 \( \mu \)M nylon mesh and