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Early cell-wall modifications of maize cell cultures during habituation to dichlobenil

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ABSTRACT

Studies involving the habituation of plant cell cultures to cellulose biosynthesis inhibitors have achieved significant progress as regards understanding the structural plasticity of cell walls. However, since habituation studies have typically used high concentrations of inhibitors and long-term habituation periods, information on initial changes associated with habituation has usually been lost. This study focuses on monitoring and characterizing the short-term habituation process of maize (*Zea mays*) cell suspensions to dichlobenil (DCB). Cellulose quantification and FTIR spectroscopy of cell walls from 20 cell lines obtained during an incipient DCB-habituation process showed a reduction in cellulose levels which tended to revert depending on the inhibitor concentration and the length of time that cells were in contact with it. Variations in the cellulose content were concomitant with changes in the expression of several *ZmCesA* genes, mainly involving overexpression of *ZmCesA7* and *ZmCesA8*. In order to explore these changes in more depth, a cell line habituated to 1.5 μM DCB was identified as representative of incipient DCB habituation and selected for further analysis. The cells of this habituated cell line grew more slowly and formed larger clusters. Their cell walls were modified, showing a 33% reduction in cellulose content, that was mainly counteracted by an increase in arabinoxylans, which presented increased extractability. This result was confirmed by immunodot assays graphically plotted by heatmaps, since habituated cell walls had a more extensive presence of epitopes for arabinoxylans and xylans, but also for homogalacturonan with a low degree of esterification and for galactan side chains of rhamnogalacturonan I. Furthermore, a partial shift of xyloglucan epitopes toward more easily extractable fractions was found. However, other epitopes, such as these specific for arabinan side chains of rhamnogalacturonan I or homogalacturonan with a high degree of esterification, seemed to be not affected.

In conclusion, the early modifications occurring in maize cell walls as a consequence of DCB-habituation involved quantitative and qualitative changes of arabinoxylans, but also other polysaccharides. Thereby some of the changes that took place in the cell walls in order to compensate for the lack of cellulose differed according to the DCB-habituation level, and illustrate the ability of plant cells to adopt appropriate coping strategies depending on the herbicide concentration and length of exposure time.

Introduction

Plant cell walls are dynamic structures whose importance resides principally in the key role they play in plant growth and development, enabling cells to adapt to abiotic or biotic stresses (Wolf et al., 2012). Cell walls influence the properties of most plant-based products, including their texture and nutritional value, and condition the processing properties of plant-based foods for human and animal consumption (Doblin et al., 2010). Cell walls are mainly composed of polysaccharides, which are classified as cellulose, hemicelluloses and pectins, and have lower amounts of proteins, phenolics and other minor components. Cell wall composition varies according to the plant type and species (Sarkar et al., 2009), cell type and position within the plant, developmental stage and history of responses to stresses (Doblin et al., 2010). This variability in the composition of cell walls reflects a certain degree of plasticity as regards cell wall structure and composition. One suitable method to study the mechanisms underlying the plasticity of plant cell wall structure and composition consists in habituating cell cultures to grow in the presence of high concentrations of different cellulose biosynthesis inhibitors (CBIs) (for a review see Acebes et al., 2010). CBIs are a structurally heterogeneous group of compounds that affects cellulose synthesis, acting specifically on cellulose coupling or deposition in higher plants. They can induce

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aberrant trajectories in CESA proteins, reduce their velocity or even clear them at the plasma membrane (Acebes et al., 2010; Brabham and DeBolt, 2013). In the last two decades it has been shown that it is possible to habituate undifferentiated cell cultures (calluses and cell suspensions) of various dicotyledonous plants, such as arabidopsis, tobacco, tomato, bean or poplar, to lethal concentrations of diverse cellulose biosynthesis inhibitors and related compounds, such as isoxaben, thaxtomin A, dichlobenil (DCB) or quinclorac, by gradually increasing the concentration in the culture medium. In general terms, in order to cope with the stressful conditions, the cell cultures habituated to these inhibitors modify the characteristic architecture of the type I cell wall (typical of gymnosperms, dicots and most monocots), having reduced levels of cellulose accompanied by a decrease in the hemicellulose content and a significant increase in pectins (Shedletzky et al., 1992; Díaz-Cacho et al., 1999; Encina et al., 2001, 2002; García-Angulo et al., 2006, 2009). The modifications produced in cells habituated to these inhibitors not only depend on the inhibitor concentration but also on the period of time that cells grow in contact with the inhibitor (Alonso-Simón et al., 2004).

There are only two reported studies in which cell cultures from plants with type II cell walls (characteristic of graminaceous plants, together with the other commelinoid monocots) have been habituated to a CBI. These were barley cell suspensions (Shedletzky et al., 1992) and maize callus cultures (Mélida et al., 2009, 2010a,b), habituated to DCB in both cases. The modifications found in these type II cell walls differed considerably from those described for type I cell walls, since the drastic reduction in cellulose content was compensated for an increase in the content of heteroxylans, which had lower extractability and higher relative molecular mass. Furthermore, in the case of maize cell cultures obtained in our laboratory, DCB habituation has implied an enrichment of hydroxycinnamates and dehydroferulates esterified on arabinoxylans. The content of other polymers, such as mixed glucan, xyloglucan, mannan, pectins and proteins, was unchanged or reduced (Mélida et al., 2009). As these characteristics differed from those described for DCB-habituated barley cell cultures, and did not look like any other structure previously described for type II cell walls, we proposed that our DCB-habituated maize cells had a unique structure, which was particularly interesting in order to gain a deeper understanding of the structural plasticity of the type II cell wall (Mélida et al., 2009).

Changes associated with the habituation of cell cultures to CBIs have commonly been analyzed using high concentrations of herbicide and long-term habituation periods. These kinds of study take advantage of the fact that the cell culture characteristics have been "fixed". However, information about the initial changes that take place during the process of habituation is lost, although cells at these stages probably present a huge variability in relation to their cell wall composition, properties and ability to habituate. A previous study conducted in order to monitor cell wall changes during DCB-habituation in bean calluses showed that when they were cultured in 0.5 μM DCB for less than 7 culture cycles no appreciable changes in cell wall FTIR spectra were detected, but when the number of culture cycles growing in DCB presence increased up to 13, the spectra of the cell walls underwent several changes, including an attenuation of the peaks related to cellulose (Alonso-Simón et al., 2004). So, this study established that (a) an initial period would be necessary until cell wall modifications arise and (b) that a set of cell wall modifications, not only a reduction in cellulose content, would be taking place.

Accordingly to these results, the aim of this study was to monitor and characterize the early changes happening during the DCB-habituation of cells with type II walls using maize cell suspensions as a cellular model. First of all, we monitored the changes produced throughout the DCB-habituation process, in order to select a cell line that showed an initial contrastable modification in some cell wall features. Next, we compared the cell growth pattern and cell wall composition of 1.5 μM DCB-habituated and non-habituated cells, paying special attention to the variation in composition of polysaccharides, using a set of techniques such as cell wall fractionation, gas-chromatography, immunodot assays and heatmaps.

Materials and methods

Cell cultures

Maize cell suspension cultures (*Zea mays* L., Black Mexican sweet corn, donated by Dr. S. C. Fry, Institute of Molecular Plant Sciences, University of Edinburgh, UK) from calluses obtained from immature embryo explants were grown in Murashige and Skoog media (Murashige and Skoog, 1962) supplemented with 9 μM 2,4-D and 20 g L^{-1} sucrose, at 25 °C under light and rotary shaken, and routinely subcultured every 15 days.

Habituation to DCB

Maize cell suspensions were cultured in DCB (supplied by Fluka) concentrations ranging from 0.3 to 1.5 μM. DCB was dissolved in dimethyl sulfoxide (DMSO), which did not affect cell growth at this range of concentrations.

Initially, non-habituated maize cells were cultured in media containing three DCB concentrations: 0.3 μM (lower than the I_{50}-concentration of DCB capable of inhibiting dry weight (DW) increase by 50% with respect to the control-value), 0.5 μM (the I_{50} value) and 1 μM (higher than the I_{50} value). After seven subcultures, some of the cells habituated to growth in 1 μM DCB were transferred into media containing 1.5 μM DCB. Habituated suspension cultures are referred to as Shx (n), where '*x*' indicates DCB concentration (μM) and (*n*) number of subcultures in that DCB concentration.

Growth measurements

Growth curves of non-habituated and 1.5 μM DCB-habituated cell lines were obtained by measuring the increase in DW at different culture times. Doubling time (dt) was defined as the time required for a cell to divide or a cell population to double in size in the logarithmic phase of growth, and was estimated by using the equation $\ln Wt = \ln Wo + 0.693t/dt$ (in which Wt: suspension cell culture DW at time '*t*' of the culture cycle; and Wo: suspension cell culture DW at the beginning of the culture cycle). Thus, Wt or Wo logarithmic plotting versus culture time is a straight line where the ordinate at the origin corresponds to the Wt or Wo natural logarithm. Relative growth rate (μ) was determined by the slope of the straight line ($0.693t/dt$). Maximum growth rate was the maximum difference in DW per time unit between two consecutive measurements in growth kinetics.

Cell cluster size was determined after vacuum filtration of cell suspensions that were sequentially passed through different pore-size meshes, and the relative abundance of the clusters was expressed as the percentage of DW retained in each filter.

ZmCesA gene expression analysis: isolation of Total RNA, RT–PCR and PCR

Total RNA was extracted with Trizol Reagent (Invitrogen), and 2 μg of total RNA was reverse-transcribed using the Superscript III first strand synthesis system for RT-PCR (Invitrogen). First-strand cDNA was generated using an oligo(dT)20 primer, and 1 μl of the first-strand cDNA was used as a template in subsequent