

## Deepening into the proteome of maize cells habituated to the cellulose biosynthesis inhibitor dichlobenil

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**C**ellulose biosynthesis inhibitors, such as dichlobenil (DCB), have become a valuable tool for the analysis of structural and compositional plasticity of plant cell walls. By stepwise increasing the concentration of DCB in the culture medium, we obtained maize cells able to cope with DCB through the acquisition of a modified cell wall in which cellulose was partially replaced by a more extensive network of feruloylated arabinoxylans. Recently we demonstrated that the expression of several *Cellulose Synthase* and phenylpropanoid-related genes is altered in DCB-habituated cells. In addition, by using a proteomic approach we identified several proteins induced or repressed in DCB-habituated cells. After a more in-depth analysis, some new proteins induced (two inhibitors TAXI-IV, an  $\alpha$ -1,4-glucan-protein synthase and a pectinesterase inhibitor) or repressed (a chaperonin 60, a fructokinase-1 and a spermidine synthase 1) were identified, and their possible role in the context of DCB-habituation is discussed.

The primary cell wall of grasses and related commelinoid monocots (Type II) is composed of a framework of cellulose microfibrils embedded in a matrix of hemicellulosic polysaccharides (arabinoxylans, xyloglucan and mixed-linked glucans) and smaller amounts of pectins and glycoproteins.<sup>1</sup> Nowadays, there is increasing evidence to suggest that plant cells are capable of monitoring the functional integrity of the primary cell wall.<sup>2-4</sup>

Owing to the key role of cellulose in the cell wall structure, cellulose biosynthesis

inhibitors (CBIs) have become a valuable tool for the analysis of cell wall structure and biogenesis.<sup>5,6</sup> Cell cultures of several species have been habituated to grow in the presence of several CBIs by incremental exposure over many culturing cycles.<sup>7-9</sup> We obtained maize cell lines habituated to lethal concentrations of dichlobenil (2,6-dichlorobenzonitrile, DCB).<sup>10</sup> These cell cultures were enabled to grow through the acquisition of a modified type II cell wall in which cellulose was reduced and partially replaced by a more extensive network of arabinoxylans. Moreover, cell wall hydroxycinnamates, the units linking arabinoxylans, experienced quantitative and qualitative changes.<sup>11</sup> At a molecular level, the expression of several *Cellulose Synthase* and phenylpropanoid-related genes was altered. In addition, by using a proteomic approach, other proteins related to different metabolisms such as carbon, nitrogen and ethylene or detoxification mechanisms were found to be altered.<sup>12</sup>

After a more in-depth analysis of proteomic results, some new proteins with putative relevance for the habituation process were identified. Here, we report those proteins which seem to contribute to the maintenance of cell wall integrity/cell survival following cellulose biosynthesis inhibition by DCB (Fig. 1 and Table 1).

Two maize xylanase inhibitors belonging to TAXI-IV type (*Triticum aestivum* xylanase inhibitor IV) were induced by DCB-habituation, in addition to the putative xylanase inhibitor previously reported.<sup>12</sup> Previous results showed that the architecture of type II cell walls is able to compensate for deficiencies in

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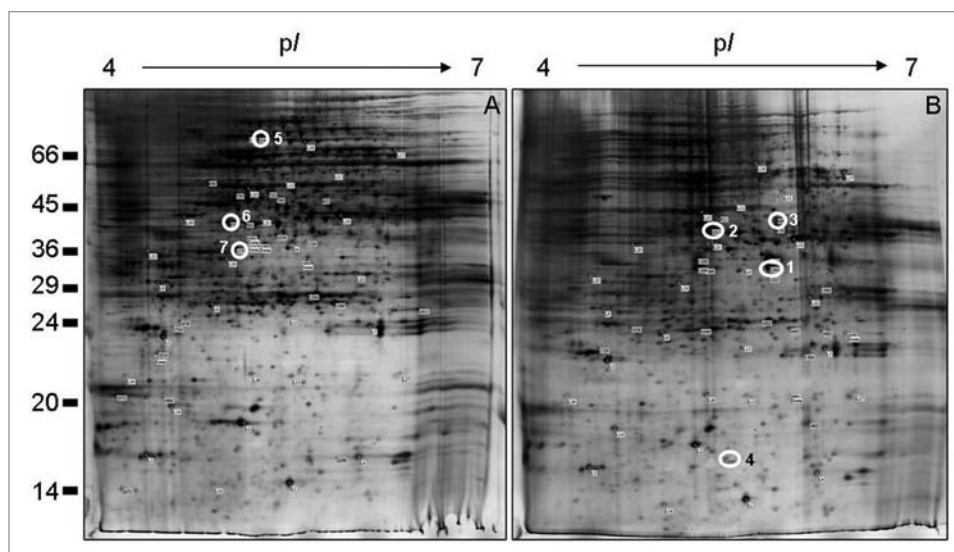
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**Figure 1.** 2-D resolution of the maize cultured cells proteins. Comparison of the total proteins of non-habituated (A) and DCB-habituated (B) maize cells. Numbers on the left indicate the positions of the relative molecular mass standards (X1,000 Da), and the isoelectric point (pI) is given at the top. Gels were loaded with 200  $\mu$ g proteins and visualized by silver staining. The open white circles correspond to the selected spots analyzed by MALDI-TOF/MS or by LC-nanoESI-Q-TOF-MS/MS, which are listed in Table 1.

**Table 1.** Identification of proteins by MALDI-TOF/MS or by LC-nanoESI-Q-TOF-MS/MS after spot excision of 2-D gels (see Fig. 1), with an indication of their theoretical and experimental isoelectric point (pI) and molecular mass (MM (Da))

Protein group	Protein name (accession no.)	(spot no.)	Theoretical pI/MM	Experimental pI/MM
Induced	xylanase inhibitor TAXI-IV (ACG48847)	(1)	7.52/38873	5.70/37018
	alpha-1,4-glucan-protein synthase [UDP-forming] (NP_001105598)	(2)	6.11/41741	7.15/44643
	xylanase inhibitor TAXI-IV (ACG48847)	(3)	7.52/38873	5.74/48052
	pectinesterase inhibitor domain containing protein (NP_001152694)	(4)	5.89/20993	5.37/16927
Repressed	chaperonin 60 (AAA33450)	(5)	5.68/61458	5.25/71522
	fructokinase-1 (ACG44849)	(6)	5.13/34753	5.05/41182
	spermidine synthase 1 (ACG34862)	(7)	5.66/39860	5.09/36000

cellulose content by producing a more extensive network of arabinoxylans. In fact, it has been demonstrated that arabinoxylans from DCB-habituated cell walls were characterized by a marked increase in their relative molecular mass when compared with those obtained from control cells.<sup>10</sup> In the context of DCB-habituation it is tempting to consider that xylanase inhibition plays a role in preventing the depolymerisation of endogenous arabinoxylan.

So far, the relationship between DCB-habituation and TAXI proteins remains unexplained. In a recent paper, it was demonstrated that *TAXI-I* gene expression is specifically induced by the stress phytohormone methyl jasmonate.<sup>13</sup> Therefore, it would be plausible

to consider TAXI induction as a general response to a putative DCB-induced stress. However, TAXI type proteins specifically inhibit bacterial and fungal endo- $\beta$ -1,4-xylanases (G11 group) and therefore it has been suggested that they participate in plant defence (it is the so-called plant defense hypothesis).<sup>14,15</sup> As TAXI type proteins do not seem to be active against plant endoxylanases (to date, all of them identified belonging to G10 group), a role for these proteins in the remodelling of cell wall arabinoxylans is unlikely.<sup>14-16</sup> Nevertheless, as the inhibition specificity for TAXI-IV proteins has not been reported yet,<sup>14</sup> further research will be needed to discard this possibility.

In addition, other cell wall related proteins were induced in DCB-habituated cells, including a pectinesterase inhibitor, which inhibits the action of pectin methylesterases through the formation of a non-covalent complex. Pectin methylesterases have a prominent role in pectin metabolism since they can promote both pectin  $\text{Ca}^{2+}$  binding and pectin susceptibility to specific-degrading enzymes.<sup>17</sup> Although pectins are not major components of type II cell walls, the induction of a pectin methylesterase inhibitor in DCB-habituated cells raises the question of whether this kind of enzyme plays a significant role in the modification of the maize cell wall architecture in this process.

An  $\alpha$ -1,4-glucan-protein synthase (UDP forming; UPTG), a self glycosylation protein, has also been found to be induced in DCB-habituated cells, and it has already been suggested that this has a role in the synthesis of hemicellulosic polysaccharides.<sup>18</sup> Interestingly, a positive correlation between UPTG transcript levels and the active synthesis of cell wall components was found in potato.<sup>19</sup> Moreover, in accordance with the high glycosylation rates of UPTG for xylose and galactose, it has been proposed a role for this protein in xyloglucan biosynthesis,<sup>19</sup> this being the main hemicellulosic polysaccharide in type I cell walls. In our case, it will be necessary to address the possible link between increased UPTG activity and the active synthesis of arabinoxylan during DCB-habituation, as it has been demonstrated that habituated cells replace cellulose by increasing the incorporation of arabinoxylans into the cell wall,<sup>10</sup> and these play a similar role in type II cell walls to that played by xyloglucan in type I.

It has been shown that hexoses may function as stress indicators when cell wall integrity is impaired.<sup>2</sup> In line with this, we have reported three proteins which were altered during DCB-habituation, and which are involved in hexoses metabolism. Here we show that fructokinase-1, which may be directly or indirectly involved in a sugar-sensing pathway,<sup>20,21</sup> was reduced by the habituation. Based on these results, future research is needed to clarify the exact role of hexoses as cell wall stress indicators.

Another interesting scenario would indicate fructokinase-1 as regulating the sucrose metabolism of habituated cells. As fructokinase phosphorylates fructose to fructose-6P, reduced fructokinase activity would inhibit sucrose synthase activity by a fructose accumulation feedback mechanism.<sup>22</sup> Sucrose synthase has a central role in directing carbon flow to sugar metabolism and cell wall synthesis. Therefore, DCB-habituated cells might be expected to have reduced sink strength.

Spermidine synthase 1, involved in the synthesis of the polyamine spermidine, was repressed in DCB-habituated

cells. Although numerous unanswered questions remain regarding polyamines in plants, spermidine is essential, and one of the reasons for this may lay in the fact that spermidine serves as a substrate for post-translational modification of the eukaryotic translation initiation factor 5A, which is essential in all eukaryotic cells.<sup>23</sup> Interestingly, this has been shown to increase in DCB-habituated cells.<sup>12</sup> In this case, it remains to be confirmed whether the increase shown in translation initiation factor 5A is related to the decrease in spermidine.

It has been demonstrated that under stress conditions, the requirement for chaperone function increases, in order to prevent and reverse incorrect interactions of proteins.<sup>24</sup> However, chaperonin 60, another stress-related protein was again found to be repressed in DCB-habituated cells. The repression of these stress-related proteins contrasts with the fact that other stress-related proteins (heat shock protein 70 and peroxidase 52 precursor) were previously reported as having been induced during the habituation to DCB,<sup>12</sup> demonstrating the precise strategy followed by cells habituated to grow in the presence of high DCB concentrations.

In conclusion and on the basis of these and previous results,<sup>10-12</sup> both the modified cell wall architecture and a very precise control of stress responses seem to be responsible for the capacity displayed by DCB-habituated cells to survive in the presence of lethal concentrations of a cellulose biosynthesis inhibitor. These results prompt us to continue investigating the upstream signals controlling all these downstream processes.

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