Botrytis cinerea is the causal agent of grey mould disease and a non-host necrotrophic pathogen of maritime pine (Pinus pinaster). Recent evidence suggests that pathogen challenge can alter carbon uptake in plant cells; however, little is known on how elicitor-derived signalling pathways control sugar transport activity. P. pinaster suspended cells are able to absorb D-[14C]glucose with high affinity, have an H+-dependent transport system (\(K_m = 0.07 \text{ mM}\); \(V_{\text{max}} = 1.5 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ DW}\)), are specific for D-glucose, D-fructose, D-galactose and D-xylene, and are subject to glucose repression. When elicited by B. cinera spores, suspended cells exhibit calcium-dependent biphasic reactive oxygen species (ROS) production, the second burst also being dependent on NADPH oxidase, mitogen-activated protein kinase (MAPK), and de novo transcription and protein synthesis. Challenging suspended cells incubated in sugar-free medium resulted in an up to 3-fold increase in glucose transport capacity over non-elicited cultures 24 h after elicitation, and a 14-fold increase over elicited cells incubated with 2% glucose. Enhanced glucose uptake depended on NADPH oxidase and calcium influx, but not MAPK. In contrast, the increase of glucose transport activity induced by sugar starvation was dependent on the activation of MAPK but not NADPH oxidase. Both responses appeared to be dependent on de novo transcription and protein synthesis.

**Keywords:** *Botrytis cinerea* — Monosaccharide transporter — Non-host resistance — Oxidative burst — *Pinus pinaster* — Sugar uptake.

Introduction

Co-evolution of plants and pathogens led to the development of a complex array of plant defence mechanisms. Increasing knowledge has revealed that the essentials for resistance lie at the cellular level, and rest upon two principles: prompt recognition of the pathogen, and subsequent triggering of the defence repertoire. Recognition of the pathogen usually culminates in a programmed cell death event of both infected and adjoining plant cells, a process known as the hypersensitive response (HR) (Lamb and Dixon 1997).

The importance of sugar in the regulation of various aspects of plant physiology other than carbon metabolism has been well documented (Koch 1996). The not so obvious role of carbon metabolism in the overall defence response has nonetheless been substantiated by studying enzymes such as extra-cellular invertase and sugar transporters (STPs) (Truernit et al. 1996, Ehness et al. 1997, Bourque et al. 2002), suggesting that sugar mobilization and uptake might be crucial for the HR and the activation of defence mechanisms. During challenge, both plant and pathogen cells compete for the same nutrients, and the importance of controlling carbon availability has been highlighted previously (Clark and Hall 1998, Sutton et al. 1999). For infected plants, coping with the induction of several defence responses also requires energy, as was demonstrated by the increase in mitochondrial activity in challenged sweet potato roots (Greksak et al. 1972).

Sucrose produced by photosynthesis in source tissues represents the main form of reduced carbon transported in the sieve elements of the phloem, although some plant species can also translocate sucrose derivatives (Büttner and Sauer 2000). In sink tissues, unloaded sucrose can be imported from the apoplast directly via sucrose transporters or, alternatively, sucrose can be hydrolysed to glucose and fructose by cell wall-bound invertases and taken up via monosaccharide transporters (MSTs) (Williams et al. 2000). The MST family consists of at least 26 genes in *Arabidopsis thaliana*, and multiple genes have been identified in other plant species. The kinetic properties of the encoded proteins have been studied mainly by heterologous expression in yeasts or *Xenopus* oocytes, and all the transporters characterized so far are energy-dependent H+ symporters (Büttner and Sauer 2000). Some genes such as *MST1* in tobacco show sink tissue specificity (Sauer and Stadler 1993). Conserved motifs in MST promoter regions suggest that these genes may be differentially regulated by various transduction signals, including stress-responsive pathways. Despite indications that plant MSTs are highly regulated in response to wounding and pathogen attack (Truernit et al. 1996, Lalonde et al. 1999, Delrot et al. 2000, Delrot et al. 2001), there is little...
B. cinerea enhances glucose transport in P. pinaster

knowledge on how this regulation occurs and how it coordinates with sink metabolism signalling.

Maritime pine (Pinus pinaster Ait.) is a two-needle pine distributed throughout the west Mediterranean basin. P. pinaster is a pioneer species, and its adaptability and phenotypic characteristics make it an economically vital forest species, particularly for the pulp and timber industries, being considered one of the three most important pine species in Europe. Botrytis cinerea, the agent of grey mould disease, is a non-host necrotrophic fungus (Bi et al. 1999) capable of infecting >200 hosts (Govrin and Levine 2002). Here we report the characterization of glucose transport in P. pinaster suspension-cultured cells and its involvement in the defence response to B. cinerea. Evidence for the involvement of an H⁺-dependent monosaccharide transport system subject to glucose repression and elicitor-specific regulation is provided.

Results

Effect of B. cinerea elicitation on ROS production by P. pinaster suspension-cultured cells

Reactive oxygen species (ROS) generation in maritime pine suspended cells was evaluated during the time course of B. cinerea elicitation by fungal spores or mycelium extract. The intracellular production of superoxide radical (O₂⁻) was monitored through the cumulative reduction of the tetrazolium dye 3′(1-[phenylamino-carbonyl]-3,4-tetrazolium)-bis(4-methoxy-6-nitro) benzene-sulfonic acid hydrate (XTT) (Fig. 1). The results showed that B. cinerea spore challenge was able to induce two bursts of O₂⁻ (phase I and II oxidative bursts). The first burst occurred almost immediately after challenging and lasted for 5 h, while the second burst, at least 5-fold higher than the first, started 9 h after elicitation. No oxidative bursts were observed upon challenge with mycelium extract (Fig. 1A, insert). Further elicitation experiments were carried out only with B. cinerea spores. The dependency of B. cinerea-induced oxidative bursts on components of signal transduction pathways, as well as on de novo transcription and protein synthesis, was also studied. For this purpose, staurosporine [a mitogen-activated protein kinase (MAPK) inhibitor], diphenyleneiodonium (DPI; an NADPH oxidase inhibitor), LaCl₃ (a calcium channel inhibitor), α-amanitin (a transcription inhibitor) or cycloheximide (a protein synthesis inhibitor) were added prior to elicitation. As can be seen in Fig. 1B, of all the inhibitors tested, only LaCl₃ is able to suppress the phase I oxidative burst. Phase II also requires transcription and de novo protein synthesis to occur, and depends on MAPK activation and NADPH oxidase activity.

Transport of monosaccharides by P. pinaster suspension-cultured cells

Pinus pinaster suspended cells were maintained in liquid medium containing 3% glucose as the sole carbon and energy source. Uptake of glucose was studied over a concentration range of 0.02–0.5 mM, at pH 5.0 to impose a transmembrane electrochemical proton gradient. The results showed that in cells harvested at the late exponential growth phase, when glucose had fallen to around 0.02%, initial uptake rates of D-
B. cinerea enhances glucose transport in P. pinaster

Enhancement by B. cinerea

Glucose uptake followed simple Michaelis–Menten kinetics (Fig. 2A), suggesting the presence of carrier-mediated transport. Application of computer-assisted non-linear regression analysis yielded the following kinetic parameters: \( K_m \), 70 \( \pm \) 15 \( \mu \)M glucose; \( V_{max} \), 1.48 \( \pm \) 0.17 nmol glucose min\(^{-1}\) mg\(^{-1}\) DW.

To ascertain the involvement of a proton-dependent monosaccharide transport system, the initial uptake rates of 0.02–0.2 mM \( D-\left[^{14}C\right]\)glucose were measured in the presence of the protonophore carbonyl cyanide \( m \)-chlorophenylhydrazone (CCCP). CCCP inhibited up to 50% of sugar transport (Fig. 2B), pointing towards a glucose : proton symport mechanism. The energetics of sugar transport were evaluated further in the presence of the lipophilic cation tetraphenylphosphonium (TPP\(^+\)), which dissipates the transmembrane electric potential, thus interfering with the net influx of positive charges into the cells. The initial uptake rates of \( D-\left[^{14}C\right]\)glucose were reduced by about 30%, (Fig. 2B), indicating that the transmembrane electrical gradient contributes significantly to the driving force for sugar uptake. To evaluate the accumulative capacity of the transport system, the non-metabolizable glucose analogue 3-O-methyl-\( D-\left[^{14}C\right]\)glucose (3-O-MG) was used. As shown in Fig. 2C, 3-O-MG was concentrated in the intracellular compartment to levels of about 50-fold, and the protonophore CCCP prevented 3-O-MG accumulation.

The specificity of the glucose transport system was evaluated by estimating the initial uptake rates of 0.02–0.2 mM \( D-\left[^{14}C\right]\)glucose in the presence of other sugars (Fig. 2D). Eadie–Hofstee plots indicated that \( D \)-fructose, \( D \)-xylose, 2-deoxy-\( D \)-glucose, 3-O-MG and \( D \)-galactose (not shown) behaved as competitive inhibitors, suggesting that they share the same transport system. \( D \)-Mannitol, as well as \( D \)-arabinose (not shown), had no effect on glucose transport.

Regulation of P. pinaster monosaccharide : \( H^+ \) symport system

In cells cultivated with an initial glucose concentration of 3%, harvested at early exponential growth phase when glucose had fallen to around 2%, the initial uptake rates of 0.02–0.5 mM \( D-\left[^{14}C\right]\)glucose were significantly lower than those measured in cells collected at the end of the exponential growth phase (Fig. 2A), and depended linearly on the external sugar concentration up to 100 mM. At this concentration, corre-
B. cinerea enhances glucose transport in P. pinaster

Responding to about 2% glucose in the medium, an uptake rate of 21 nmol glucose min⁻¹ mg⁻¹ DW was measured. Taken together, the results suggest that cells are able to take up D-glucose by a saturating transport system with high affinity and a non-saturating component, with the activity of the monosaccharide : H⁺ symport system appearing to be repressed by high sugar levels in the culture medium.

To study the induction of glucose transport activity in response to the decrease of glucose concentration, the accurate dependence of the permease activity on sugar levels in the medium was evaluated. For this purpose, cells were grown in a medium with an initial glucose concentration of 1.2% and the uptake of D-[¹⁴C]glucose was measured in cell aliquots harvested from the culture at the time periods indicated in Fig. 3A. Growth of cell suspensions and sugar consumption are also depicted. Glucose disappeared from the medium within 13 d, and the arrest of cell growth was associated with the decline of sugar content. In addition, once the external levels of glucose had fallen to around 0.7% (day 9), the activity of the monosaccharide transport system was significantly increased from basal levels. The maximal activity of the permease was observed at day 13, when glucose was completely exhausted from the culture medium. At day 14, extensive loss of cell viability and subsequent decrease in uptake activity were observed (data not shown).

The regulation of the P. pinaster monosaccharide : H⁺ symport system by glucose was characterized further by study-

---

**Fig. 3** Regulation of the P. pinaster glucose transport system by sugar levels. (A) Biomass (open squares), glucose concentration (filled circles) and Vₘₐₓ of the monosaccharide : H⁺ symport system (filled diamonds) in maritime pine suspension-cultured cells grown with an initial glucose concentration of 1.2% (w/v). (B) DPI, staurosporine, LaCl₃, cycloheximide and α-amanitin were added to the culture medium at day 12, and D-[¹⁴C]glucose transport was measured in cell aliquots at day 13.

---

**Fig. 4** Effect of the elicitation of P. pinaster suspension-cultured cells with B. cinerea spores on the activity of the monosaccharide : H⁺ symport system. (A) Pine cells were collected and washed after 9 d in culture, as indicated in Fig. 3A, and D-[¹⁴C]glucose uptake was determined in cells resuspended in mineral medium without sugar, in the absence (open triangles) and presence of 2×10⁵ spores ml⁻¹ (filled triangles) and 6×10⁴ spores ml⁻¹ (open squares). D-[¹⁴C]glucose uptake by cells resuspended in a medium containing 2% glucose and 6×10⁴ spores ml⁻¹ (closed squares). (B) Vₘₐₓ of glucose transport 24 h after elicitation with B. cinerea (6×10⁴ spores ml⁻¹) in the presence of DPI, staurosporine, LaCl₃, cycloheximide or α-amanitin.
ing the effect of staurosporine, DPI, LaCl₃, α-amanitin and cycloheximide on the increase of transport activity mediated by glucose depletion. For this purpose, cells were collected at day 12 in the conditions described in Fig. 3A and incubated in the presence of the inhibitors for 24 h. As can be seen in Fig. 3B, of all the compounds tested, only DPI did not inhibit carrier derepression.

**Effect of B. cinerea spore elicitation on the activity of the P. pinaster monosaccharide : H⁺ symport system**

To evaluate the role of B. cinerea elicitation on the regulation of P. pinaster glucose transport, cell suspensions were incubated in the presence of different spore concentrations, up to 6×10⁴ spores ml⁻¹. Elicitation experiments were performed in pine cells collected at day 9, in the conditions described in Fig. 3A, when glucose in the medium had fallen to around 0.7% (w/v). Cells were washed and resuspended in media with or without sugar, in the presence or absence of spores (Fig. 4A). When elicitation was performed in cells resuspended in a medium with 2% glucose, B. cinerea failed to induce MST activity. In contrast, when B. cinerea spores (6×10⁴ spores ml⁻¹) were added to cells incubated in sugar-free medium, there was an up to 3-fold increase in glucose uptake activity compared with non-elicited cultures 24 h after elicitation, and a 14-fold increase compared with elicited cells resuspended with 2% glucose. Transport experiments were also performed in spore suspensions to investigate the possible contribution of B. cinerea spores to overall glucose uptake. No glucose uptake was evidenced up to 24 h after inoculation (data not shown).

To compare the mechanisms underlying the increase of monosaccharide : H⁺ symport activity mediated by fungal elicitation and by glucose depletion, the effects of staurosporine, DPI, LaCl₃, α-amanitin and cycloheximide were also evaluated in glucose-starved cells elicited with B. cinerea (Fig. 4B). From all the compounds tested, only staurosporine did not affect the increase of glucose uptake mediated by fungal challenge. By comparing the results depicted in Figs. 3B and 4B, both responses appeared to be dependent on transcription and protein synthesis, because they were strongly inhibited by α-amanitin and cycloheximide, respectively. Also, calcium influx seems to be involved in the increase of glucose uptake promoted either by glucose depletion or by fungal elicitation, as LaCl₃ markedly inhibited both responses.

**Discussion**

P. pinaster suspension-cultured cells respond to B. cinerea spore elicitation with a biphasic ROS burst

An intricate network of defence mechanisms allows most plants to resist a wide variety of pathogens, such as viruses, bacteria, fungi or even animals (Dangl and Jones 2001). Non-host resistance is responsible for most of the plants defence capability, by providing broad-spectrum resistance to numerous pathogens (Heath 2000). Recently, Mysore and Ryu (2004) reported two types of non-host resistance, distinguishable by the ability to produce ROS, triggering the HR during the defence process. The HR-symptomless response is considered to be type I resistance. In type II resistance, non-host pathogens are capable of inducing ROS generation and trigger programmed cell death, in a process highly similar to gene-for-gene resistance (Lamb and Dixon 1997). In the present work, challenging maritime pine cells with B. cinerea spores resulted in the induction of two intracellular ROS bursts. In contrast, B. cinerea mycelium was incapable of inducing ROS production, suggesting the absence of elicitor motifs susceptible to recognition. ROS production by pine cells in response to B. cinerea elicitation is strongly consistent with the involvement of a type II non-host resistance.

When the dependency of B. cinerea-induced oxidative bursts on components of signal transduction pathways was studied, it was shown that in P. pinaster elicited cells, phase I ROS production was dependent on calcium influx but not on...
the activation of MAPKs and NADPH oxidase. These results point to the involvement of early calcium signalling in the induction of the defence response, as shown before (Knight et al. 1991, Pugin et al. 1997, Blume et al. 2000, Lecourieux et al. 2002). Alternative mechanisms of ROS production independent of NADPH oxidase may include lipooxygenase, and apo-

thetic enzymes, such as copper amine oxidase, flavin polyamine oxidases, oxalate oxidase or a secretory peroxidase (reviewed by De Gara et al. 2003, Apel and Hirt 2004). However, activation of MAPKs and NADPH oxidase was shown to be involved in phase I ROS production in some plant–pathogen interactions (Tenhaken et al. 1995, Kottum et al. 2000, Suzuki 2002). In addition to calcium influx, MAPKs and NADPH oxidase seem to play a role in the activation of the phase II oxidative burst. Moreover, the results show the involvement of de novo transcription and protein synthesis during the phase II but not the phase I oxidative burst. Taken together, the results strongly support the involvement of different mechanisms of ROS generation during phase I and phase II oxidative bursts.

P. pinaster suspension-cultured cells display activity for an H⁺-dependent monosaccharide transport system regulated by glucose levels

The utilization of glucose by heterotrophic plant cells requires its transport across the plasma membrane (PM), which constitutes an important step for plant growth and development. The kinetic studies reported herein pointed towards the involvement of a high-affinity glucose transport system in P. pinaster suspended cells cultivated with a low sugar supply. The observed $K_m$ value is of the same order of magnitude as that described for Daucus carota (Krook et al. 2000), Nicotiana tabacum (Verstappen et al. 1991), Olea europaea (Oliveira et al. 2002) and Pisum sativum (Ritte et al. 1999) sugar carriers. These results, together with those regarding energetics and specificity, suggest that saturating monosaccharide transport in P. pinaster suspended cells is mediated by carriers of the MST family, which comprise proton-dependent carriers with a broad specificity for hexoses and pentoses, and $K_m$ values for the preferred substrate typically between 10 and 100 μM (Blüttner and Sauer 2000).

Proton-coupled STP activity can be regulated in two major ways: (i) indirectly, by regulating H⁺-ATPase activity; or (ii) more specifically, by controlling the expression of STPs at the transcriptional and post-transcriptional levels (Lalonde et al. 1999). It is believed that genes encoding MSTs are differently regulated by sugars in higher plants. For instance, in Vitis vinifera berry flesh cells, the Vvht1 gene (Vitis vinifera hexose transporter 1) is up-regulated by sugar (Fillon et al. 1999), whereas H⁺-dependent glucose transport activity in O. europaea suspended cells is repressed by sugar (Oliveira et al. 2002). In contrast, hexose transporter genes of photoautotrophic Chenopodium rubrum suspensions are constitutively expressed and are not regulated by sugars (Roitsch and Tanner 1994). The results presented in this work indicate that P. pinaster suspended cells seem to adapt the glucose transport capacity to the sugar concentration in the culture, a high-affinity H⁺-dependent monosaccharide symport system being induced when growth was carried out in low sugar. In contrast, pine cells grown with high sugar levels apparently absorb glucose by a ‘diffusion like’, non-saturating mechanism. Transport that depends linearly on the external sugar concentration has been described in other plant cells and tissues (Delrot et al. 2000, Krook et al. 2000, Oliveira et al. 2002), but the underlying mechanisms are still poorly understood. Recently, Etcheberria et al. (2005) reported that a sucrose-inducible endo-
cytic process is involved in the uptake and accumulation of external solutes by heterotrophic sycamore suspended cells.

Elicitation by B. cinerea spores induces the activity of the P. pinaster monosaccharide : H⁺ symport system

Abiotic and biotic stresses can severely impact carbon partitioning and source–sink relationships. Yet, knowledge is still scarce on how elicitor-derived signalling pathways influence the regulation of sink genes. During plant–microbe interaction, the pathogen becomes an additional sink tissue, and a competition for solutes is set up in the apoplast. The use and control of host carbon sources has been shown to be dependent on the pathogen (Clark and Hall 1998, Sutton et al. 1999). However, despite some knowledge on the mechanisms of solute transport into the pathogen, very little is known about the mechanisms of sugar sequestration by host cells during plant–pathogen interactions. An increase of sugar uptake by host cells mediated by pathogen elicitation has been related to the activation and fuelling of the defence response to infection, the decrease of sugar availability to the pathogen and the recovery of leaked nutrients from dead cells (Bourque et al. 2002). According to our results, the activity of the P. pinaster monosaccharide : H⁺ symport system is induced upon elicitation by B. cinerea spores in a dose-dependent manner. Induction of glucose uptake was observed 12 h after challenge, reaching a maximum after 24 h, a time period during which P. pinaster suspended cells evidenced ROS generation. These results are consistent with a role for high affinity sugar carriers in the fuelling of defence mechanisms, as suggested by Williams et al. (2000). A correlation between sink metabolism and defence responses has already been reported. In C. rubrum cells, both glucose and the fungal elicitor chitosan resulted in a coordinated mechanism regulating sink-specific (invertase) and defence-specific (Pal) genes (Ehness et al. 1997). However, evidence on the role of STPs in the defence response is contra-
dictory. The expression of Arabidopsis STP4, a gene encoding a high affinity monosaccharide : H⁺ symporter with transcript specificity for heterotrophic tissues, is rapidly induced in the presence of fungal or bacterial elicitors, as well as after wound-
ing (Truernt et al. 1996, Fotopoulos et al. 2003). In contrast, Bourque et al. (2002) showed that challenging tobacco sus-
pended cells with the fungal elicitor cryptogein blocks glucose transport in the first minutes of treatment. According to these
authors, during challenge, pathogenic fungi can respond by releasing toxins and proteinaceous elicitors that directly or indirectly alter the PM H⁺-ATPase activity and cause PM depolarization. As a consequence, PM permeability and H⁺-dependent co-transporters can be affected at the beginning of infection. These authors demonstrated, however, that blocking of the glucose transporter was accompanied by phosphorylation of the carrier and took place prior to PM depolarization, but it is still unclear whether the proteinaceous elicitor cryptogein may have other toxic effects not related to PM depolarization. It was also suggested that this might be a host mechanism to halt the cell metabolism, triggering the HR cell death.

Glucose and B. cinerea elicitation differentially regulate the induction of the P. pinaster monosaccharide : H⁺ symport system

Low sugar and B. cinerea elicitation were shown to increase glucose uptake in P. pinaster suspended cells, depending on de novo protein synthesis. Our results pointed to the existence of distinct signal transduction pathways regulating the induction of the maritime pine monosaccharide : H⁺ symport system by sink-specific and defence-specific stimuli, supported by the differential involvement of MAPK and NADPH oxidase (Fig. 5). The results also suggest that calcium influx is necessary for the transduction of both signals, as LaCl₃, a calcium channel blocker, was able to inhibit the increase of glucose uptake promoted by either glucose depletion or fungal elicitation. Calcium, an important component of most signal transduction cascades, has been associated with both source–sink regulation (Barker et al. 1998, Iwata et al. 1998) and biotic stress (Knight et al. 1991, Pugin et al. 1997, Blume et al. 2000, Lecourieux et al. 2002), and was shown to be important for ROS production during the P. pinaster–B. cinerea interaction.

In contrast to calcium, NADPH oxidase and MAPK are differentially involved in the regulation of glucose transport in P. pinaster by glucose depletion and by B. cinerea challenge. The activity of the ROS-generating PM NADPH oxidase was required for the elicitation-dependent increase of glucose uptake, which is consistent with a role for this enzyme in the early signal transduction of the defence signal (Lamb and Dixon 1997, De Gara et al. 2003). The conserved MAPK cascades have been shown to be activated upon a vast array of signals (Hirt 1997), including sugar (Ehness et al. 1997), and biotic and abiotic stresses (Suzuki et al. 1995, Agrawal et al. 2003). Ehness et al. (1997) showed that different MAPKs were activated during the sugar and defence responses in C. rubrum suspension-cultured cells, due to the differential effect of stauroporine on invertase and Pal gene expression.

The present results suggest that low sugar levels and B. cinerea elicitation correspond to two distinct signals that can be integrated, regulating glucose uptake upon activation of possibly different MST genes (Fig. 5). In fact, analysis of the expression profile of STPs in B. cinerea-challenged Arabidopsis plants, made available through the Affymetrix microarray service run by NASC (Craigon et al. 2004), points to the induction of pathogen-specific STP genes. This information is helpful in providing new insights into the involvement of host sugar transport capacity as a strategy of defence against non-host pathogens.

Materials and Methods

P. pinaster suspension-cultured cell maintenance and characterization

Heterotrophic P. pinaster Ait. suspended cells were regularly maintained in Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 3% glucose, 5 mg liter⁻¹ dithiothreitol, 0.1 g liter⁻¹ myoinositol, 1 mg liter⁻¹ benzyl adenine and 2 mg liter⁻¹ 2,4-D, at pH 6. For growth characterization, cells were cultivated with 1.2% glucose and aliquots (1–5 ml) were harvested from the culture, with the supernatant being used for glucose quantification. Biomass was determined by dry weight measurement, after filtration using pre-weighted GF/C filters (Whatman, Clifton, NJ, USA), and oven drying at 60°C for 24 h. Glucose levels were determined by the glucose oxidase method (GOD, Roche Applied Science, Indianapolis, IN, USA).

Quantification of intracellular superoxide radical by B. cinerea-elicited P. pinaster suspension-cultured cells

Botrytis cinerea was grown in potato dextrose agar (PDA) plates for 20–30 d until extensive sporulation occurred. Spores were recovered by adding 10 ml of 0.03% Tween-20 to each plate. The suspension was filtered through gauze to remove contaminating hyphae. The spore concentration was determined using a haemocytometer. Alternatively, B. cinerea was cultivated in potato dextrose (PD) liquid medium, at 25°C with agitation (150 rpm). Mycelia were harvested from 12 d cultures, by centrifugation at 5,000×g for 5 min, followed by resuspension in sterile water. Mycelia were lyophilized for 48 h in a Christ Alpha RVC Lyophilizer (B-Braun, Melsungen, Germany) and ground with a mortar and pestle to a fine powder.

For elicitation experiments, P. pinaster suspended cells in mid-exponential growth phase were harvested, centrifuged at 5,000×g for 5 min and resuspended in MS medium, at a final density of 0.1 g FW ml⁻¹. B. cinerea spores or mycelia extract were added to final concentrations of 6×10⁴ spores ml⁻¹ and 2 mg ml⁻¹, respectively.

Intracellular levels of superoxide radical (O₂⁻) were quantified by the reduction of XTT (Invitrogen-Molecular Probes, Eugene, OR, USA) to a soluble formazan (Able et al. 1998). Immediately before elicitation with B. cinerea, 0.5 mM XTT was added to the cell suspension, followed by incubation in the dark, at room temperature with agitation. Experiments were performed in the absence and presence of stauroporine on invertase and Pal gene expression.

The present results suggest that low sugar levels and B. cinerea elicitation correspond to two distinct signals that can be integrated, regulating glucose uptake upon activation of possibly different MST genes (Fig. 5). In fact, analysis of the expression profile of STPs in B. cinerea-challenged Arabidopsis plants, made available through the Affymetrix microarray service run by NASC (Craigon et al. 2004), points to the induction of pathogen-specific STP genes. This information is helpful in providing new insights into the involvement of host sugar transport capacity as a strategy of defence against non-host pathogens.

Measurement of sugar uptake by pine suspension-cultured cells

Estimation of d-[¹⁴C]glucose uptake rates. P. pinaster suspension-cultured cells were harvested, washed twice by centrifugation at 5,000×g for 5 min and resuspended in sugar-free MS medium, at pH 5.0 and 4°C, at a final concentration of 4 mg ml⁻¹ DW. To estimate initial uptake rates of d-[¹⁴C]glucose (Radiochemical Centre, Amersham, UK), 1 ml aliquots were transferred to 10 ml flasks, with agitation (100 rpm) at 25°C. After 3 min incubation, the reaction was started by the addition of 40 µl of an aqueous solution of radiolabelled
sugar (500 dpm nmol⁻¹). Sampling times were 0, 180, 360 and 540 s, time periods during which the uptake was linear. The reaction was stopped by adding 5 ml of ice-cold sugar-free MS medium and the mixtures were immediately filtered through GF/C filters (Whatman, Clifton, NJ, USA). The filters were washed with an additional 10 ml of the same medium and transferred to vials containing scintillation fluid (OptiPhase Hisafe II; LKB Wallac, Turku, Finland). The radioactivity was quantified using a Packard Tri-Carb 2200 CA liquid scintillation counter (Packard Instruments Co., Inc., Rockville, MD, USA). The results were corrected for non-specific binding of labelled sugars to the filters and/or the cells, by diluting the cells with 5 ml of ice-cold MS medium without sugar, before the addition of labelled sugar.

**Determination of substrate specificity.** The specificity of the glucose transport system was determined by measuring 0.02–0.2 mM D-[¹⁴C]glucose uptake in the presence of non-labelled substrates. The final cold substrate concentration was at least 10-fold higher than the Kᵥ value estimated for the glucose transport system.

**Accumulation studies.** Aliquots of 10 ml of maritime pine suspensions were collected at the final exponential growth phase, when glucose in the medium had fallen to 0.02%, and incubated for 3 min, at 25°C, under agitation (100 rpm). The reaction was started by the addition of an aqueous solution of radiolabelled 3-O-MG (specific activity of 3,000 dpm nmol⁻¹) at a final concentration of 0.1 mM. One ml aliquots were taken from the reaction medium into 5 ml of ice-cold MS medium without sugar and filtered immediately through Whatman GF/C membranes. The filters were washed with 10 ml of the same medium, and the radioactivity was determined as indicated above. The intracellular water volume was determined based on the quantification of the relative distribution of two radioactive compounds in a cellular suspension: [¹⁴C]methoxyiminulin to which biomembranes are impermeable and [¹⁴H]H₂O that equilibrates across biomembranes (Oliveira et al. 2002). A value of 7.8±2.5 µl of intracellular water mg⁻¹ DW was obtained and used to calculate the intracellular sugar concentration.

**Calculation of kinetic parameters.** The data of the initial uptake rates of labelled glucose were analysed by computer-assisted non-linear regression analysis software (GraphPad software; San Diego, CA, USA). Transport kinetics best fitting the experimental initial uptake rates were determined, and then estimates for the kinetic parameters were obtained. Error bars indicate the standard error (SE) of triplicate independent experiments.

**Inhibition studies.** To evaluate the effect of inhibitors of signal transduction pathway components, transcription and protein synthesis on the increase of glucose transport activity mediated by low sugar levels and fungal elicitation, glucose transport experiments were performed in pine cells upon incubation with staurosporine (5 µM), cycloheximide (50 µg ml⁻¹), DPI (50 µM), LaCl₃ (1 mM) and α-amanitin (250 µM).

Changes in transport activity mediated by sugar depletion were studied in pine cells cultivated with an initial glucose concentration of 1.2%. Inhibitors were added at day 12, when glucose in the medium had fallen to residual levels, and cells were incubated for 24 h. d-[¹⁴C]Glucose transport was measured on washed cell aliquots. To study the effect of elicitation on sugar transport activity, pine cells were harvested at day 9, when glucose in the medium had fallen to around 0.7% (w/v), and washed in sugar-free MS medium. Cells were resuspended in the same medium in the absence or presence of 2% glucose. *B. cinerea* spores were added to a final concentration of 6×10⁴ or 2×10⁵ spores ml⁻¹. Inhibitors were added to pine cells 15 min prior to elicitation, and d-[¹⁴C]glucose transport was measured on washed cell aliquots, 24 h after challenging.

**Acknowledgments**

The authors would like to thank Serge Defrot (University of Poitiers, France) for helpful suggestions and critical reading of the manuscript. H.A. was supported by FCT (Foundation for Science and Technology), grant ref. SFRH/BD/3194/2000.

**References**


(Received June 28, 2005; Accepted December 15, 2005)