

# Salicylic acid-independent induction of pathogenesis-related protein transcripts by sugars is dependent on leaf developmental stage

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**Abstract** Soluble sugars have been found to regulate a number of genes involved in functions associated with sink metabolism, defense reactions and photosynthesis. As viruses and pathogens induce the expression of pathogenesis-related (PR) protein genes and have also been reported to lead to localized sugar accumulation in leaves, it was investigated whether a salicylic acid-independent but sugar-dependent pathway for PR-protein gene induction may exist in plant cells. Leaf discs of tobacco plants were floated on different sugar solutions, transcript accumulation and salicylic acid (SA) levels were subsequently determined. PR-Q and PAR-1 transcripts were found to be inducible by glucose, fructose and sucrose. No significant change in SA content could be detected, following incubation. On the other hand, SAR8.2 transcripts were repressed by elevated levels of soluble sugars and sorbitol, respectively, suggesting sensitivity to turgor pressure. Since leaves undergo sink to source transition during growth, sugar responsiveness was investigated in leaves of different developmental stages. Interestingly, induction of PR-Q and PAR-1 by soluble sugars was essentially restricted to fully expanded leaves and was independent of plant age. Induction by salicylate was not confined to the source capacity of a leaf but was dependent on the age of the respective leaf. Repression of transcripts encoding photosynthetic genes (ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbcS*) and chlorophyll *a/b* binding protein (*cab*)) by soluble sugars were largely independent from the leaf developmental state. These findings hint to the possibility of salicylic acid-independent defense reactions of plants against pathogens by induction of a set of PR proteins in source leaves. Furthermore, the data suggest different mechanisms for the induction of PR-protein genes and the repression of photosynthetic genes by soluble sugars.

**Key words:** Pathogenesis-related protein; Photosynthetic gene expression; Salicylic acid; Sugar sensing; Tobacco

## 1. Introduction

Sucrose is the major photoassimilate transported from source to sink tissues in most higher plants. Besides its role to fuel growth and development of plants, sucrose is believed to act directly or indirectly as mediator between sink and source tissues regulating photosynthesis according to sink demand [1–6]. These functions suggest that assimilates may act as signal molecules to modulate the expression of genes involved in those processes. Indeed, a large number of genes such as genes encoding storage proteins, enzymes involved

in starch metabolism and photosynthesis were shown to be regulated by sugars (summarized in [7]). Jang and Sheen [8] observed that the repression of photosynthetic genes in maize protoplasts by soluble sugars required phosphorylation of hexoses but no further metabolism. They therefore suggested that hexokinase may act as sensor and transmitter of photosynthetic gene repression by sugars in higher plants.

Interestingly, infection of leaf tissues by viruses and pathogens may lead to elevated carbohydrate levels [9–11] possibly resulting from an export block of photoassimilates caused by the intruder. In addition to modulating gene expression in primary metabolism, soluble sugars have also been found to activate some defense-related genes [12–14]. Moreover, cell wall invertases have been observed to be induced during bacterial and fungal infections [15,16]. Taken together these data suggest that elevated hexose levels resulting from the postulated increase in sucrose breakdown after pathogen attack could lead to increased expression of defense-related functions and might possibly explain the phenomenon of high-sugar resistance in plants [17]. This hypothesis has been supported by the finding that systemic acquired resistance is induced in transgenic sugar-accumulating plants expressing yeast invertase in the cell wall [18]. Furthermore, by means of subtractive cDNA cloning four cDNAs encoding pathogenesis-related proteins (PR-1b, PR-Q, SAR8.2, and PAR-1) were isolated from transgenic sugar-accumulating tobacco plants [14]. PR-1b and PR-Q belong to classes 1 and 3 of PR proteins, respectively. PR-Q encodes a chitinase while the functions of the other PR-proteins remain to be elucidated.

Signal transduction pathways for both the repression of photosynthetic genes and activation of defense-related genes by sugars are largely unknown. Jang and Sheen [8] postulated a common mechanism for sugar sensing in both instances. Supportive to this hypothesis was the finding that the same threshold level of hexoses was required for the induction of transcripts encoding a pathogenesis-related protein (PR-Q) and repression of transcripts specific for chlorophyll *a/b* binding protein (*cab*), respectively [18]. However, the data obtained from sugar-accumulating plants expressing yeast invertase in the cytosol, vacuole and cell wall imply that sensing is no intracellular event but instead occurs in the secretory system which argues against hexokinase-mediated signal transduction [18].

In this study the expression of those PR-transcripts isolated from sugar-accumulating tobacco plants was analyzed in response to soluble sugars in wild-type plants to distinguish between their accumulation resulting from elevated levels of sugars or salicylic acid. Photosynthetic gene expression using either *cab* or *rbcS* was also investigated to get further evidence for common sugar sensing of these different transduction pathways. Furthermore, we asked whether the developmental

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stage and the age of leaves may determine responsiveness to soluble sugars and salicylate.

## 2. Materials and methods

### 2.1. Northern blot experiments

RNA preparation, separation of RNA in denaturing agarose gels, blotting and hybridization of nylon membranes were performed as described [14].

### 2.2. Plant material

*Nicotiana tabacum* L. cv. Samsun NN was obtained from Vereinigte Saatzuchten AG (Ebendorf, Germany). Plants in the greenhouse were maintained in soil under a light-dark regime of 16 h/8 h (mean temperatures: 25°C/20°C; mean irradiance: 300  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) at 60% humidity.

### 2.3. Floating experiments

Tobacco plants used for floating experiments had 4–19 leaves depending on the experiment (see figure legends). Leaf discs were derived from sink to mature source leaves as described in the figure legends. All experiments were started 6 h after illumination and incubations were done for 22–24 h in the dark under otherwise greenhouse conditions.

### 2.4. Measurement of soluble sugars

After floating on sugars, leaf discs were washed with water to remove adhering sugars, dried with paper towel and quickly frozen in liquid nitrogen. Soluble sugars and starch were quantified as described by Sonnwald [19].

### 2.5. Determination of salicylic acid

After floating on sugars and salicylate, leaf discs were washed with water, dried with paper towel, quickly frozen in liquid nitrogen and lyophilized. Free and bound salicylic acid was extracted and quantitated using ortho-anisic acid as an internal standard as described previously [20].

## 3. Results

### 3.1. PAR-1 and PR-Q transcripts accumulate in response to soluble sugars

RNA levels specific for SAR8.2, PR-1b, PR-Q and PAR-1 accumulate in transgenic tobacco plants with elevated sugar levels [14,18]. To study whether these genes would respond to soluble sugars, floating experiments on different sugar solutions were performed with wild-type tobacco plants. Excised leaf discs from mature tobacco leaves were floated on water, and different concentrations (100–500 mM) of glucose, fructose and sucrose for 22–24 h. RNA was isolated, separated on denaturing agarose gels, and hybridized with PAR-1, PR-1b, PR-Q and SAR8.2 cDNAs, respectively.

The response of the different PR-protein genes to sugar incubations was not uniform. PR-1b expression was not affected by soluble sugars at all (data not shown). Surprisingly, SAR8.2 transcripts accumulated after floating on water and sugar solutions of low osmotic strength (Fig. 1). This result suggested that osmotic changes rather than the sugars themselves were the cause for SAR8.2 mRNAs accumulation. Therefore, leaf discs of tobacco plants were floated on different concentration of the penetrating osmolyte sorbitol and for comparison on the non-penetrating osmolyte polyethylene glycol 8000. Similar to the data obtained with soluble sugars, SAR8.2 transcripts accumulated at low concentrations of sorbitol (80 mM) while at a concentration of 250 mM, SAR8.2 mRNA levels were indistinguishable from the non-induced *in planta* state. Incubating leaf discs on 7% polyethylene glycol

8000 (PEG8000) also did not lead to a significant increase in SAR8.2 mRNA transcripts (data not shown).

Analysis of PR-Q and PAR-1 transcripts in wild-type leaves floated on water and soluble sugars (100–500 mM) revealed that these transcripts strongly accumulated in response to sucrose, glucose and fructose (Fig. 1 and [14]). Incubating leaf discs on sorbitol (80 and 250 mM) or 7% PEG8000 did not influence PAR-1 and PR-Q levels significantly (data not shown). We therefore suppose that the response of the PAR-1 and PR-Q genes to sugars is mediated by the sugar molecules themselves and not by osmotic effects exerted by them.

### 3.2. SA levels in leaf discs after floating on sugars are not sufficient to drive expression of PR-Q and PAR-1 transcripts

Both PAR-1 and PR-Q genes are inducible in tobacco leaves by salicylate [14,21]. We asked whether leaves would produce salicylic acid after floating on soluble sugars which could be responsible for PAR-1 and PR-Q induction. For this reason, free and bound SA levels were determined in leaf discs floated on water and different concentrations of glucose and sucrose. No increase in free or bound SA was observed after floating leaf discs on glucose solution of different concentrations in comparison to water (Table 1). On the other hand, there was a slight increase in bound SA after floating leaves on different sucrose solutions (Table 1). For comparison, SA levels have been determined in leaf discs after floating on 100  $\mu\text{M}$  and 1 mM salicylate. Commonly, 1 mM salicylate is used in floating experiments for induction of PR-protein expression, 100  $\mu\text{M}$  is on the brink of being effective (data not shown). Levels of SA in leaf discs were elevated by 1–2 and 2–3 orders of magnitude after floating on 100  $\mu\text{M}$  and 1 mM salicylate, respectively, in comparison to those floated on sucrose (Table 1). This suggest that the slightly induced SA levels in leaves after floating on sucrose are not sufficient to induce PAR-1 and PR-Q genes.

### 3.3. Accumulation of PAR-1 and PR-Q transcripts in response to soluble sugars depends on leaf maturity

During maturation, leaves develop from a heterotrophic to an autotrophic organ which process is called the sink to source transition of leaves (reviewed in [22]). This development is accompanied by dramatic physiological changes reflected at the molecular and metabolic level, particularly affecting carbohydrate metabolism. It can therefore be

Table 1  
Free and bound SA in leaf discs after floating on different sugar solutions

	Free SA	Bound SA
Water	1.10	52.12
100 mM Glc	0.36	28.72
300 mM Glc	0.00	42.46
500 mM Glc	0.32	31.57
100 mM Suc	10.32	70.38
300 mM Suc	2.87	80.47
500 mM Suc	1.52	106.99
0.1 mM SA	102.56	3744.33
1 mM SA	1710.95	14883.97

Measurements were performed with samples containing leaf discs from six different plants floated for 22 h in the dark. Values are expressed as ng/cm<sup>2</sup>.

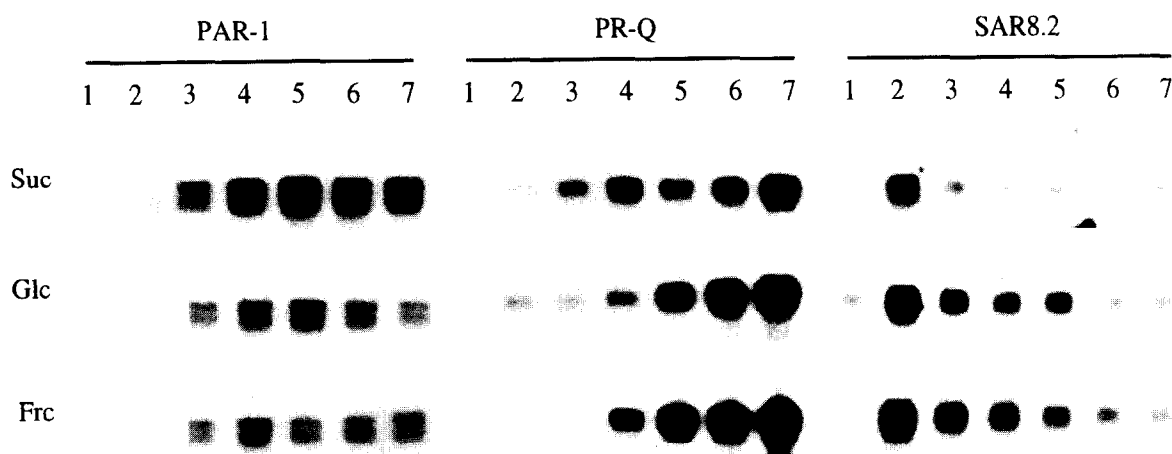


Fig. 1. Expression analysis of PAR-1, PR-Q and SAR8.2 transcripts in leaf discs of tobacco plants floated on water (lane 2) and different concentrations of sucrose (Suc), glucose (Glc) and fructose (Frc) for 22 h in the dark. Sugar concentrations varied between 100, 200, 300, 400 and 500 mM (lanes 3–7, respectively). RNA from leaves prior to floating is shown in lane 1. Each RNA was a pool from samples of six mature source leaves taken from different plants at the 15-leaf stage. 15  $\mu$ g of total RNA were loaded per lane.

presumed that different leaves of a plant differ in their ability to sense sugars leading to the activation of defense and repression of photosynthetic genes, respectively. To check this assumption differently aged leaves of 3-month-old plants (19-leaf stage) were floated on water, glucose and sucrose, respectively. The length of those leaves used in the experiment was determined and the size calculated as percentage of those leaves which were largest (taken to be 100%) to get a measure for leaf maturity.

Sugar inducibility of PAR-1 and PR-Q transcripts was highly dependent on leaf maturity (Fig. 2). Only minor amounts of RNA transcripts encoding PAR-1 and PR-Q were detectable in leaves which had reached 15, 25, 40, 55, or 75% of final length after floating on glucose and sucrose, respectively (Fig. 2, lanes 1–5). In contrast, floating leaf discs from fully developed leaves (100% final length) on glucose or sucrose gave rise to high levels of PAR-1 and PR-Q transcripts (Fig. 2, lane 6). This experiment was repeated with plants at the 15-leaf stage using leaves of 20, 40, 60 and 100% final length which essentially gave the same results (data not shown).

Due to their varying metabolic functions, different leaves of the same plant can be expected to take up and metabolize soluble sugars to different degrees which could be the basis for the difference in responding to soluble sugars by activating PAR-1 and PR-Q genes. For this reason, soluble sugars (glucose, fructose and sucrose) were measured in leaves prior to and after floating on water, 300 mM glucose or 300 mM sucrose using responsive (100% final size, about 20 cm in length) and non-responsive (40% final size, about 8 cm in length) leaves of 3-month-old plants at the 19-leaf stage. As shown in Fig. 3, steady-state levels of sugars in the 40% and 100% leaves were comparable when floated on glucose or sucrose. Plants floated on glucose mainly accumulated glucose and relatively low amounts of fructose and sucrose while floating on sucrose resulted in high steady-state levels of all soluble sugars. Steady-state levels of glucose and fructose were similar indicating that sucrose was efficiently taken up and hydrolyzed by invertase in leaves of both stages. This result indicates that the exogenous sugars can be taken up and metabolized by both responsive and non-responsive leaves.

Floating differently aged leaves of tobacco plants at the 15-

leaf stage on 1 mM salicylate revealed increasing induction of PAR-1 and PR-Q genes from upper to lower leaves (data not shown).

#### 3.4. Repression of *rbcS* and *cab* transcripts in response to soluble sugars is independent from leaf maturity

To further elucidate the hypothesis that the induction of PR-protein genes and repression of photosynthetic genes might be initiated by similar sugar sensing mechanism, photosynthetic gene transcripts (*rbcS* and *cab*) were analyzed in the same leaves after floating on sugars. In contrast to the activation of PR-protein genes, repression of *rbcS* and *cab* mRNAs by glucose and sucrose was similarly effective in all leaves under study (Fig. 2, lanes 1–6, data are shown for *rbcS*).

#### 3.5. Inducibility of PAR-1 and PR-Q transcripts by soluble sugars is linked to the source capacity of leaves

The largest leaves of plants differing in age have adopted similar physiological roles despite their difference in absolute age because they already have to sustain sink growth. In order to discriminate between a possible influence on inducibility exerted by age and/or metabolic state, the largest leaves of differently aged plants at the 4-, 5-, 8- and 15-leaf stage were floated on 300 mM sucrose and water, respectively, and analyzed as described above. As shown in Fig. 3, PAR-1 and PR-Q transcripts strongly accumulated in response to sucrose in all leaves analyzed with levels being slightly lower in leaves taken from very young plants at the 4- and 5-leaf stage. Again, all leaves were able to respond to sucrose by repressing *rbcS* transcripts (data not shown). For comparison, leaf discs from the same leaves were floated on 1 mM salicylate. Interestingly, induction was considerably more effective in leaves of the older plants (Fig. 4). Thus it appears that responsiveness of leaves to salicylate is more dependent on plant age while responsiveness to sugars depends more on the metabolic state of the respective leaf.

## 4. Discussion

The present report described (1) the different expression of several PR-protein genes in response to soluble sugars (PR-1b, PR-Q, SAR8.2 and PAR-1) and (2) the varying abilities of

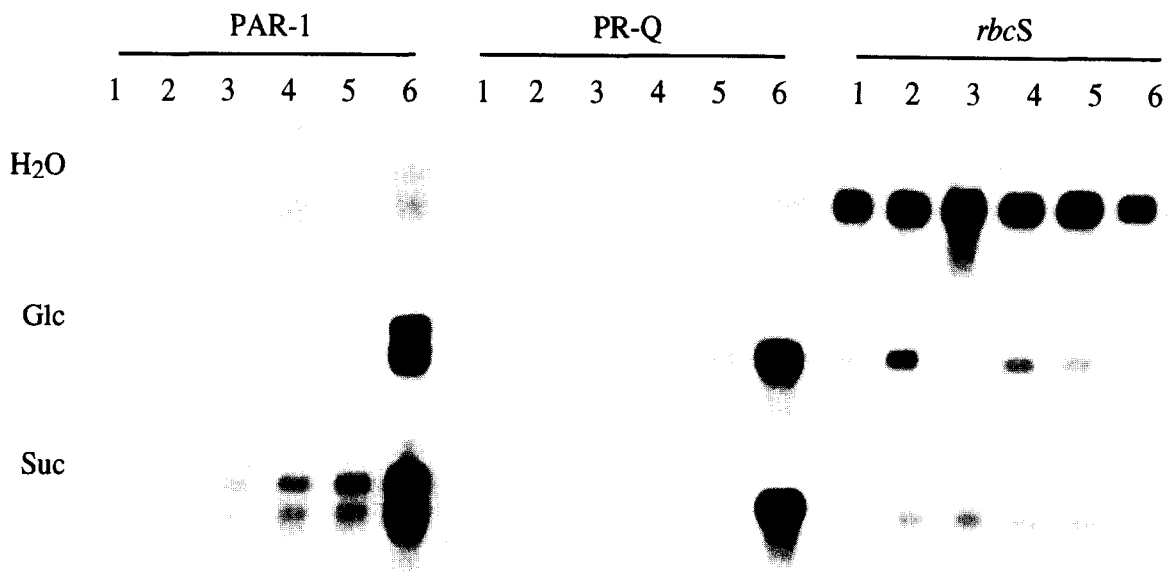


Fig. 2. Expression analysis of PAR-1, PR-Q and *rbcS* transcripts in leaves of different maturity after floating on water, 300 mM glucose (Glc) and 300 mM sucrose (Suc), respectively, for 22 h in the dark. Leaves floated had obtained approximately 15, 25, 40, 55, 75 and 100% final leaf length (lanes 1–6, respectively). Each RNA was a pool from samples of at least six leaves taken from different plants at the 19-leaf stage. 15  $\mu$ g of total RNA were loaded per lane.

differently matured leaves to answer to soluble sugars by activating PR-protein (PAR-1, PR-Q) and repressing photosynthetic genes, respectively, as well as to respond to salicylate.

#### 4.1. Expression of PR-1b, PR-Q, SAR8.2 and PAR-1 in response to soluble sugars

PR-protein genes are known to be induced under a number of stress conditions like pathogen attack, UV light, ozone, chemical treatment, salicylic acid, ethylene and other hormones (see [21,23]). We were interested to investigate sugar induction of several PR-protein transcripts (PR-1b, PR-Q, SAR8.2 and PAR-1) because they had previously been isolated from, and found to accumulate in sugar-accumulating transgenic tobacco plants, expressing cytosolic *Escherichia coli* pyrophosphatase or apoplasmic and vacuolar yeast invertase [18,14].

Expression of PAR-1 and PR-Q transcripts turned out to be similar and to differ fundamentally from SAR8.2 and PR-1b expression in wild-type tobacco leaves floated on different sugar solutions.

PR-1b transcripts did not respond to soluble sugars at all. So far, PR-1b transcripts have been found to accumulate locally and systemically in response to TMV and salicylate [24], ozone [25], UV-B light [23,26],  $\alpha$ -aminobutyrate and xylanase [27]. It remains unknown so far why levels of this transcript are also increased in sugar-accumulating tobacco plants expressing *E. coli* pyrophosphatase (*ppa-1*). Levels of free and bound SA were comparable between wild-type and *ppa-1* plants (unpublished results). SAR8.2 transcripts accumulated in response to water and solutes of low osmotic strength indicating that the respective gene(s) are turgor-responsive. Floating on water or low concentrations of solutes would allow water to be taken up by the plant vacuole and increase turgor pressure. This argument is supported by the fact that *ppa-1* plants suffer from turgor pressure that is higher by 9-fold as compared to wild-type plants [28]. Thus, in addition of being inducible by agents leading to systemic acquired resist-

ance [29,30], the expression of SAR8.2 appears to be sensitive to turgor pressure. This fact renders SAR8.2 unique among the PR-protein genes. *Osmotin*, belonging to class 5 of the PR-protein genes, has also been found to accumulate under con-

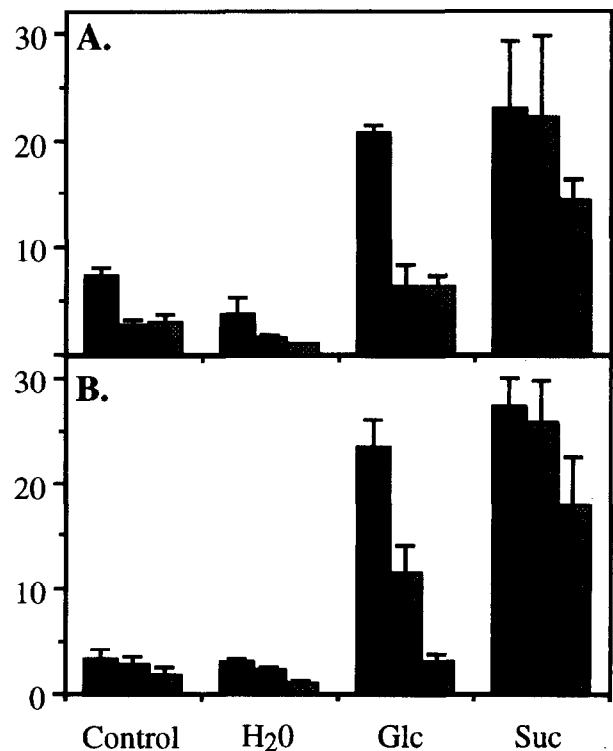


Fig. 3. Levels of soluble sugars prior to (control) and after floating on water (H<sub>2</sub>O), 300 mM glucose (Glc) and 300 mM sucrose (Suc) respectively, for 22 h in the dark. Leaves taken for analysis had reached about 40% (A) and 100% (B) final leaf length. Values are means of four samples taken from leaves of different plants and are expressed as mmol/m<sup>2</sup>. Glucose (solid bars), fructose (striped bars) and sucrose (dotted bars).

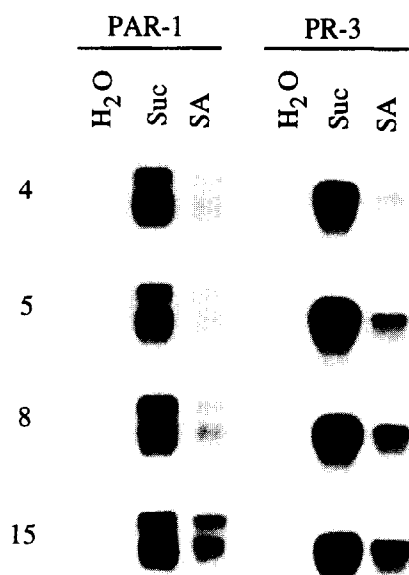


Fig. 4. Expression analysis of PAR-1 and PR-Q (PR-3) transcripts in leaf discs of tobacco plants floated on water, 300 mM sucrose (Suc) and 1 mM salicylate (SA). Each RNA was isolated from six leaf discs taken from different plants at the 4-, 5-, 8- and 15-leaf stage. Only the most fully developed leaves of the respective plants were used. 15  $\mu$ g of total RNA were loaded per lane.

ditions of osmotic stress. Yet, it accumulates in suspension tobacco cells adapted to high NaCl concentrations which reduces water potential and turgor pressure [31].

PAR-1 and PR-Q were found to be inducible by glucose, fructose and sucrose in a SA-independent manner. No induction was obtained by sorbitol and PEG8000 indicating that the sugar molecules themselves or their metabolism but no osmotic effects were responsible for induction. These results support our hypothesis that a SA-independent sugar-dependent pathway for some pathogenesis-related functions has evolved in plants which might be important in early phases of local pathogen attack prior to SA action.

#### 4.2. Leaf maturity governs responsiveness to sugars and salicylate

Inducibility of PAR-1 and PR-Q genes by soluble sugars was strongest in bottom leaves of tobacco plants. Yet, the absolute age of a leaf was not determining responsiveness to sugars. This suggests that inducibility is likely to be linked to the physiological and metabolic qualities of source leaves. The difference in sugar sensing between upper non-responsive leaves and older responsive leaves does not reside in different sugar uptake capacities. We suppose that source leaves sense a strong metabolic disturbance when faced with elevated levels of exogenous soluble sugars because their metabolism is adapted to synthesize and export sucrose. On the other hand, sink leaves are tailored to import transport sugar which is directed into catabolic pathways to sustain growth of the leaves. Therefore, exogenously supplied sugars do not impose a conflict on their basic physiology.

The activation of PAR-1 and PR-Q genes in mature leaves contrasts the results obtained with the repression of *rbcS* and *cab* genes which could be observed in leaves of all stages. This finding argues against common sensing mechanism leading to the activation of defense-related and repression of photo-

synthetic genes, or it may be present in mature leaves only [8,18]. Hexokinase-mediated signal transduction of photosynthetic gene expression as suggested by Jang and Sheen [8] cannot be excluded by the results reported in this study.

Accumulation of PAR-1 and PR-Q transcripts by salicylate increases from top to bottom leaves of plants. Experiments with bottom leaves of differently aged plants showed that induction was associated with the absolute age of a leaf rather than with its physiological state as a source leaf. As salicylate has been found to be required for systemic acquired resistance [32] it can be hypothesized that the higher susceptibility of older leaves to salicylate may be related to the phenomenon of adult plant resistance [33].

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