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Apoplastic Expression of Yeast-Derived Invertase in Potato[1]

Effects on Photosynthesis, Leaf Solute Composition, Water Relations, and Tuber Composition

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ABSTRACT

In potato plants (*Solanum tuberosum*), a chimeric yeast-derived invertase gene fused to a 35S cauliflower mosaic virus promoter has been expressed. The protein was targeted to the cell wall by using the signal peptide of proteinase inhibitor II fused to the amino terminus of the yeast invertase. The transformed plants had crinkled leaves, showed a reduced growth rate, and produced fewer tubers. Although in the apoplast of the leaves of the transformed plants the content of glucose and fructose rose by a factor of 20, and that of sucrose declined 20-fold, 98% of the carbohydrate in the phloem sap consisted of sucrose, demonstrating the strong specificity of phloem loading. In the leaf cells of the transformed plants, glucose, fructose, and amino acids, especially proline, were accumulated. Consequently, the osmolality of the cell sap rose from 250 to 350 mosmol/kg. Our results show that the observed 75% decrease of photosynthesis is not caused by a feedback regulation of sucrose synthesis and is accompanied by an increase in the osmotic pressure in the leaf cells. In the transformed plants, not only the amino acid to sucrose ratio in the phloem sap, but also the amino acid and protein contents in the tubers were found to be elevated. In the tubers of the transformed plants, the protein to starch ratio increased.

In most plants, sucrose is the main photosynthesis product exported from the source cells in the leaves to the sink tissues. The expression of a chimeric yeast acid invertase gene in the apoplast of plant cells, as achieved with tobacco (*Nicotiana tabacum*) (19, 21), *Arabidopsis* (21), tomato (*Lycopersicon esculentum*) (5), and potato (*Solanum tuberosum*) (shown here), to decrease the phloem loading of sucrose, provides a means to change source-sink interactions and to study the response of the metabolism of the leaf mesophyll cells (representing the source) on such a change. Results available so far show that the various plants listed above are affected differently by such a transformation.

The expression of yeast invertase in *Arabidopsis* did not affect the photosynthesis metabolism of young leaves. Due to the invertase activity, the leaf contents of glucose and fructose were found to increase with increasing leaf age, accompanied by an increased discoloration and a reduced photosynthesis (21). In the transformed tobacco (21) and tomato plants (5), the growth was inhibited, and the leaves developed a curly structure and bleached regions with increasing age. Qualitative studies with transgenic tomato plants indicated that the starch contained in the leaves of these plants did not disappear during the night, in contrast to wild-type plants, in which most of the leaf starch is consumed during the night.

In the transformed tobacco plants, acid invertase was highly expressed in all leaves, but its activity increased with leaf age and was especially high in the bleached sectors of the leaves (20, 21). Compared to the wild type, the level of starch in the green sectors was found to be increased at the end of the illumination period. The contents of glucose, fructose, and sucrose, as measured after 6 or 12 h of illumination, however, were in the green regions of the leaves of transformed plants not markedly different from those in the wild type. Moreover, the rates of photosynthesis in the green sectors of the leaves of transformed plants, as measured in saturating light and CO_2 by a leaf electrode, did not differ from the rates measured with wild-type leaves. Apparently, the transformation affected the photosynthesis metabolism in the green leaf areas of transgenic tobacco plants only to a limited extent. In contrast, large alterations in metabolism were observed in the bleached regions of these leaves, where a high accumulation of glucose, fructose, sucrose, and starch was found. A large decrease in the rate of photosynthesis was accompanied by a decline in the leaf content of Chl and the activities of enzymes involved in photosynthetic carbon metabolism (20, 21). It appeared that in the transgenic tobacco plants, presumably induced by the accumulation of glucose, the cells in the bleached leaf areas had been gradually dedifferentiated from photoautotrophic to heterotrophic cells. Such a dedifferentiation from photoautotrophic to heterotrophic cells can be experimentally induced by adding glucose to suspensions of autotrophic higher plant cell sus-

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pensions (4), or even by feeding glucose into spinach (*Spinacia oleracea*) leaves (12).

In the present report, the effect of the expression of yeast invertase in the apoplast was studied with potato, which because of its high sink capacity (10), probably responded differently to the transformation. Although an enlarged formation of bleached areas could also be observed in the transformed potato plants, it occurred only with old leaves, which were close to senescence, and these were not been studied for the present report. It will be shown that a decrease of sucrose export from the leaves, as achieved by the introduction of the yeast invertase into the apoplast, affects photosynthesis of green leaves, the sucrose and amino acid composition of the phloem sap, and the protein to starch ratio in the tubers.

MATERIALS AND METHODS

Potato plants (*Solanum tuberosum* var Desirée) were transformed using the *Agrobacterium* system as described in ref. 16, propagated from tissue cultures, and grown in a climatized chamber on 12 h light/12 h dark cycle at a temperature of 23/18°C and a light intensity of 300 μmol photons m^{-2} s^{-1}. The construction of chimeric yeast invertase expression vector is described in ref. 21. The plant leaves were harvested 10 to 14 weeks after propagation. Assimilation and evaporation rates were determined on leaflets attached to the plants with a gas exchange system (18). Invertase activity was measured from leaflets frozen in liquid nitrogen and extracted in a buffer containing 50 mM Hepes KOH, pH 7.4, 5 mM $MgCl_2$, 5 mM DTT, 2 mM benzamidine, 2 mM ε-aminocaproic acid, 1 mM EDTA, 1 mM EGTA, 0.5 mM PMSF, 0.1% Triton X-100, 10% glycerol. An aliquot of this extract (equivalent to 10–30 μg of Chl) was added to 0.6 M sucrose and 0.125 M sodium acetate, pH 5.0. After 10 min, the reaction was completed by adding 75 μL of 0.15 M $Ba(OH)_2$ and subsequently 50 μL of 0.3 M $ZnSO_4$, and the amount of glucose released was determined (15). This extract was also used for the determination of Rubisco activity (9). Metabolites were determined from leaflets harvested at the end of the light and dark period.

Glucose, fructose, sucrose, starch, and the amino acids were measured after chloroform/methanol extraction (15), and phosphorylated metabolites and nucleotides were determined after extraction with perchloric acid (7). The nucleotides were determined by UV detection at 260 nm after HPLC separation. An aliquot was loaded on an anion exchange column Nukleogel SAX 1000/8–46 (Macherey und Nagel, Düren, Germany) and eluted by a gradient from 20 mM Pi, pH 2.65, to 500 mM Pi, pH 4.0, within 30 min. Phloem sap was collected by the aphid technique (15). Phloem exudate was collected from detached leaves placed with their petiole into 20 mM EDTA KOH, pH 9.0. During the collection of the phloem exudate, the leaves were illuminated at the same light intensity as used during growth. Apoplast sap was sampled from potato leaves as described by Mimura et al. (13). Tubers were harvested from plants with intact source leaves, their fresh weight was determined, and from each tuber, a disk was cut across the stolon. From this disk, three small disks with a diameter of 5 mm were stamped and frozen in liquid nitrogen. One set of disks was lyophilized to determine the water content, the second was extracted by the chloroform/methanol method to determine the metabolite content, and the third was extracted by the same buffer used for the determination of invertase activity to measure the protein content. Protein was then precipitated by 10% TCA, taken up in 0.1 M NaOH, and determined by the Bradford method (2). The osmolality of the leaves was determined in the leaf sap with a water vapor osmometer (Wescor 5100 C).

RESULTS AND DISCUSSION

Expression of Yeast Acid Invertase in the Apoplast of the Leaves of the Transgenic Plants

A chimeric yeast invertase gene, fused to the 35S cauliflower mosaic virus promoter and given the poly-A site of the octopine synthetase gene, was transferred into potato using the *Agrobacterium* system. As shown previously for tobacco and *Arabidopsis* plants, the invertase is targeted into the apoplast due to the presence of the proteinase inhibitor II signal peptide at its amino terminus (21). When analyzing the metabolic consequences of a chimeric gene transferred into plants, it is important to ascertain that the alterations observed in the metabolism of the transgenic plants were due to the expression of the chimeric gene and are not the result of independent mutation, e.g. somaclonal variation during the transformation. For this reason, most of our measurements were carried out with three different transformants. Multiple plants were propagated from the three transformants along with wild-type plants as controls. Because two of the transformants yielded very similar results in all aspects, for the sake of brevity, we report here only the results obtained with the two transformants, A 41–8 (T_8) and A 41–12 (T_{12}), and omit those of the transformant A 41–11.

The activity of the acid invertase in the transformants T_8 (11.9 ± 4.2 μmol mg $^{-1}$Chl min^{-1}) and T_{12} (14.5 ± 9.1 μmol mg Chl^{-1} min^{-1}) was at least 2 orders of magnitude higher than that detected in wild-type plants (<0.1 μmol mg^{-1} Chl min^{-1}). To check whether the yeast invertase present in the leaves of transformed plants is functionally active in the apoplast, the carbohydrate content in the apoplastic space of wild type and also of leaves of transformed plants was

Table I. *Carbohydrate Content in the Leaf Apoplast Space and in Whole Leaves of Potato Plants Illuminated for 6 h*

Mean values of eight analyses ± SD.

	Total Leaf	Leaf Apoplast	Percent of Total
	nmol (mg Chl)$^{-1}$		
Wild type			
Sucrose	4100 ± 1100	36 ± 19	0.9
Fructose	2060 ± 520	20 ± 6	1.0
Glucose	1410 ± 600	17 ± 8	1.2
Transformant-8			
Sucrose	4050 ± 1100	2 ± 3	<0.05
Fructose	3820 ± 870	342 ± 180	9
Glucose	2050 ± 260	314 ± 160	15

determined. The extracellular space of the leaves was rapidly infiltrated with a solution containing an osmoticum, and the carbohydrate content of the infiltrate was analyzed and compared with the carbohydrate contents of whole leaves. As shown in Table I in the leaves of transformed plants, the apoplastic content of sucrose was reduced to about one-twentieth, whereas that of glucose and fructose increased by almost 20-fold when compared to the corresponding values in the wild-type leaves. This result clearly demonstrates that the yeast invertase expressed in the potato leaves was indeed functionally active in the apoplast.

Effect of the Transformation on the Phenotype and the Growth of Plants and Tubers

The potato plants expressing yeast invertase in the range of activities analyzed in our experiments show a modified phenotype characterized by a reduced number and size of the leaflets and an earlier senescence, resulting in a loss of leaves in the lower region of the plants (Fig. 1A). Unlike the apoplastic invertase transformants of tobacco (21) and tomato (5), necroses and pale areas were not observed in fully expanded young source leaves. The leaves of the transformed plants showed a tendency to curl (Fig. 1B). The transformed plants exhibited a slower growth than the wild-type plants, but they flowered (Fig. 1C) and also produced tubers, although in reduced quantities (shown later in Table X).

Expression of the Invertase in the Apoplast Does Not Lead to Changes in the Chl Content

Metabolic activities and metabolite contents of leaves are usually related to the Chl content of the leaves. As the leaves of the transgenic potato plants differed markedly in their appearance from those of the wild type, the question arose whether Chl was a suitable basis to compare data of different plants. To answer this question, we compared the Chl content and the weight per leaf area of wild-type and transgenic plants (Table II). The Chl content and the fresh weight per leaf area were only slightly increased in the transformed plants, leading to an unaltered Chl to fresh weight ratio. In the transformed plants, a slightly higher dry weight to fresh weight ratio was observed. The implication of this increase in dry matter will be discussed later. As the Chl content per fresh weight of leaf was almost constant in the different plants, it appears appropriate to relate metabolic parameters of wild type and transgenic plants to the Chl content.

Transgenic Potato Plants Show a Lower Rate of Photosynthesis and a Decreased Transpiration Rate

Photosynthesis of leaflets attached to the plants was determined with IR gas analyzers at ambient concentration of CO_2 and a light intensity similar to that in the growth chamber (Table III). Under this condition, rates of photosynthesis were almost constant over the entire illumination period in all three plant types. In comparison with the wild-type plants, however, rates of photosynthesis of the transgenic plants were considerably lower than those of the wild type (T_8 by

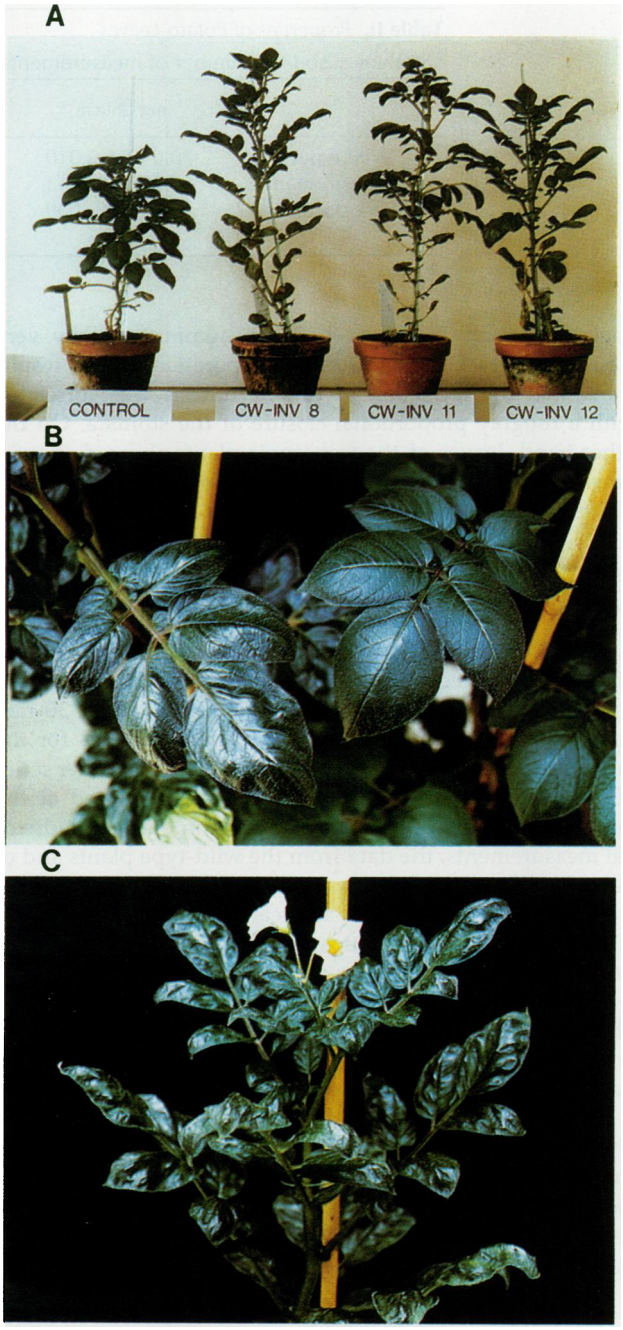

Figure 1. A, Phenotype of wild-type and three independent transgenic potato plants expressing yeast invertase in their apoplast. The plants are grown for 12 weeks from the tissue culture before they were used in these studies. B, Leaf curling of a leaf of a transformed plant (left) in comparison to wild-type leaf (right). C, Flowering of a transformed plant.

Table II. *Properties of Potato Leaves*

Values ± SD (n = number of measurements).

	mg Chl cm^{-2}	mg fresh $weight^{-2}$	mg Chl g fresh $weight^{-1}$	g dry weight g fresh $weight^{-1}$
Wild type	0.048 ± 0.010	25.1 ± 1.5	1.91	0.098 ± 0.009
Transformant-8	0.050 ± 0.013	27.9 ± 3.8	1.79	0.105 ± 0.010
Transformant-12	0.055 ± 0.009	28.4 ± 1.0	1.93	0.111 ± 0.010
	$n = 6$	$n = 10$		$n = 10$

about 50% and T_{12} by even 75%), accompanied by very similar decreases of the rates of evaporation. These results show that the reduction of photosynthesis was correlated with a roughly proportional closure of the stomata. On the other hand, leaves of the transgenic plants showed about 5 times higher rates of dark respiration than those of wild-type plants.

Leaf Contents of Phosphorylated Intermediates of Carbohydrate Metabolism

Table IV shows the results of analyses of the leaf content of phosphorylated intermediates of carbohydrate metabolism. For the measurements, leaf samples harvested 30 min before the end of illumination were used (same as for the measurements shown in Table V) in addition to another series of measurements carried out with leaves harvested at the middle of the day. Because of the variation between individual measurements, the data from the wild-type plants and of the two different transformants, T_8 and T_{12}, of the two series were pooled and presented as mean values. The leaf ATP/ADP and UDPGlc/UTP ratios were measured in the samples from the experimental series of Table V only.

Because hydrolysis of sucrose to hexoses followed by a phosphorylation of these hexoses and resynthesis of sucrose represents a futile cycle, the cellular ATP/ADP system in the transgenic plants might have been strained. Apparently this is not the case, as the cellular ATP/ADP ratios in the wild-type and transgenic plants were found to be similar. The supposed additional ATP demand of the cytosol can obviously be supplied by the cellular ATP-regenerating reactions. An inhibition of the CO_2 fixation by a lack of ATP can, therefore, be excluded.

Compared to the wild-type plants, in the transformed plants, the levels of sucrose (see Tables I and V) and hexose phosphates and the UDPGlc/UTP ratios (Table IV) were not found to be markedly increased. These results suggest that the observed decrease of photosynthesis upon the lowering of sucrose export is not a simple feedback control of Calvin cycle or sucrose synthesis enzymes caused by an accumulation of sucrose or the intermediates of sucrose synthesis. It remains to be elucidated to what extent the enzymes involved in photosynthesis have been changed in their activity. At least the Chl content was not significantly lowered in the transgenic plants, and the activity of Rubisco was found to be unchanged. In leaves of wild-type plants and of the transformants T_8 and T_{12}, Rubisco activities of 128 ± 21, 122 ± 15, and 146 ± 32 μmol (mg Chl·h)$^{-1}$, respectively, have been determined (±SD from four to six measurements). In the transformed plants, the contents of RuBP were found to be elevated (Table IV), indicating that the decrease of photosynthesis observed in the transgenic plants was not caused by a decreased rate of regeneration of RuBP.

Leaf Content of Starch, Hexoses, and Amino Acids

The leaf content of starch was analyzed in the same sample used in Table V and also in leaves harvested 30 min before the end of the following night (Fig. 2A). In contrast to the wild-type plants, where most of the leaf starch was degraded during the night, we found that in transgenic plants, the starch content declined only slightly in the following night. Obviously, the presence of yeast invertase activity in the apoplast, causing the decrease of sucrose export via the sieve tubes, resulted in a decline of starch consumption during the night.

Table III. *Photosynthesis, Evaporation, and Respiration by Potato Leaves Determined at Ambient CO_2 and O_2 Concentration by IR Gas Analyzer at the Times Indicated*

Beginning of illumination, 7 h; light intensity, 225 μmol photons m^{-2} s^{-1}.

	Photosynthesis		Evaporation		Respiration
	11.00	16.00	11.00	16.00	During darkness
	μmol CO_2 (mg Chl·h)$^{-1}$		mmol H_2O (mg Chl·h)$^{-1}$		μmol CO_2 (mg Chl·h)$^{-1}$
Wild type	61.9	53.8	4.81	4.74	1.14
Transformant-8	31.5	28.0	2.95	2.90	5.11
Transformant-12	13.7	19.6	1.07	1.54	5.10

Table IV. *Metabolite Contents in Leaves of Potato During Illumination*

The data have been obtained from leaf samples as analyzed in Table V. For brevity, data obtained with transformants T_8 and T_{12} have been combined into one mean value (±SD).

	Wild Type	Transformant
	nmol (mg Chl)$^{-1}$	
RuBP	202 ± 48	362 ± 187
Glc-6-P	144 ± 65	169 ± 60
Fru-6-P	72 ± 43	74 ± 30
Phospho*enol*pyruvate	17 ± 7	20 ± 13
Pyruvate	17 ± 3	19 ± 9
ATP/ADP	2.5 ± 0.15	3.3 ± 0.15
UDPGlc/UTP	9.6 ± 2.4	7.5 ± 2.3

Table V. *Metabolite Contents of Potato Leaves Quenched 30 min before the End of the Light Period*

The data are mean values obtained with two sets of plants. In each set, two to four control plants and two to four plants of the transformants were individually analyzed.

	Wild Type	Transformants	
		T_8	T_{12}
	nmol/mg Chl (transformant/wild type)		
Sucrose	7,630	5,770 (0.8)	8,230 (1.1)
Glucose	630	2,880 (4.6)	5,520 (8.8)
Fructose	1,490	9,300 (6.2)	12,770 (8.6)
Σ sugars	9,750	17,950	26,520
Malate	13,100	15,600 (1.2)	15,500 (1.2)
Glutamate	2,006	2,503 (1.2)	2,504 (1.2)
Glutamine	317	394 (1.2)	922 (2.9)
Aspartate	617	715 (1.2)	739 (1.2)
Asparagine	60	70 (1.2)	94 (1.6)
Serine	504	408 (0.8)	598 (1.2)
Glycine	191	536 (2.8)	555 (2.9)
Alanine	146	203 (1.4)	213 (1.5)
Valine	51	86 (1.7)	151 (3.0)
Leucine	12	32 (2.7)	57 (4.8)
Isoleucine	25	54 (2.2)	98 (3.9)
Tyrosine	13	62 (4.8)	98 (7.5)
Histidine	12	32 (2.7)	81 (6.8)
Lysine	19	34 (1.8)	50 (2.6)
Proline	28	2,140 (76)	4,270 (153)
Σ Amino acids	4,001	7,269	10,430
Sucrose/Σ Amino acids	1.91	0.79	0.79

Whereas the sucrose content was not much different in wild-type and transgenic plants, the amounts of glucose and fructose in the leaves of transformed plants were up to 9 times higher than in the wild-type leaves (Tables I and V). The highest accumulation was observed in the transformant T_{12}, which also showed the lowest rate of photosynthesis (Table III). Since only 15 and 9%, respectively, of the total amount of glucose and fructose contained in the leaves of the transformed plants were found to be present in the apoplast, and since there is virtually no glucose and fructose present in the phloem sap (see above), the difference between the glucose and fructose contents in whole leaves and the apoplastic compartment of these leaves has to be allocated to the leaf cells. It can be concluded from these results that 85% or more of the glucose and fructose found in the leaves of transformed plants originating from sucrose hydrolysis in the apoplast were ultimately present in the leaf cells. The contents of soluble carbohydrates declined during the following night in both the wild-type and transgenic plants (Fig. 2B). These results indicate that during darkness in transgenic plants, oxidative metabolism proceeded primarily at the expense of soluble carbohydrates.

In the transformed plants, a marked increase in the levels of amino acids was found (Table V). It may be noted that in another series of analyses performed with four sets of plants harvested at the middle of the illumination period, the contents of glutamate, glutamine, aspartate, and serine were found to be 2.3, 2.2, 2.6, and 2.3 times higher, respectively, in the transgenic plants than in the wild-type plants. The accumulation of amino acids in the leaves of the transformant plants is probably the result of the decreased sucrose translocation, as the export of amino acids from the source leaves via the sieve tubes occurs in the mass flow of sucrose (24).

In all our measurements, we found consistently that for valine, leucine, isoleucine, tyrosine, and histidine, the relative increase in the transgenic plants was markedly higher than that of the more abundant amino acids like glutamate, aspartate, and serine. We have recently shown that in barley leaves during the night period, when amino acid export from the leaves is reduced, the leaf contents of certain amino acids such as leucine and tyrosine were elevated. This increase could be caused by a persistence of the biosynthesis rates at a reduced export rate (24). The same explanation may hold for the unproportionately elevated levels of valine, leucine, isoleucine, tyrosine, and histidine found in the transformed plants (Table V). Most striking, however, is the dramatic increase in proline content found in the transgenic plants. Whereas this amino acid is contained in wild-type plants in minute amounts only (in about 50% of our analyses it could not be detected at all), it comprises 30 and 40% of the total amino acids in the transformant T_8 and T_{12}, respectively.

In both the transgenic and wild type plants, the amino acid contents did not markedly differ between the light and dark periods (Fig. 2C). During darkness, the consumption of amino acids by protein synthesis or by export via the sieve tubes appeared to have been either totally suppressed or the remaining amino acid-consuming processes were fully compensated by nitrate assimilation at the expense of carbohydrate oxidation and by protein degradation.

Transgenic Plants Appear To Be Under Stress

A very striking phenomenon observed in leaves of transgenic, invertase-expressing plants is the large accumulation of proline, an amino acid known to be a signal of water stress in various plants (8). Two further indications support the

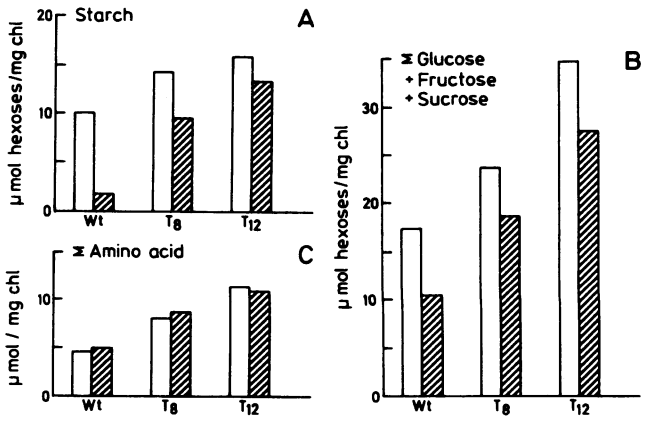

Figure 2. Metabolite contents in leaves of wild-type and invertase transformed potato plants. The leaves were quenched in their metabolism in liquid nitrogen 30 min before the end of the day (□) and 30 min before the end of the night (■). The measurements are mean values of the same series as in Table V.

Table VI. *Organic Solutes in Potato Leaves*
Calculated from data of Table II and Table V.

	mL H_2O (mg Chl)$^{-1}$	Σ Soluble Carbohydrates + Malate + Amino Acids	
		μmol (mg Chl)$^{-1}$	mmol (L cellular H_2O)$^{-1}$
Wild type	0.472	26.9	57.0
Transformant-8	0.500	40.8	81.6
Transformant-12	0.461	52.5	113.9

assumption of water stress in the transformed plants: (a) Summing the contents of the soluble carbohydrates, malate, and amino acids present in the leaves of the various potato plants studied here and relating these contents to the water contained in these leaves (as determined from the difference between leaf fresh and dry weight), the total content of solutes was 57 mmol/L in the wild-type plant and 114 in the transformant T_{12}, respectively (Table VI). (b) Osmolality measurements in the cell sap obtained from the various potato leaves (Table VII) showed that in the sap of the transgenic plant T_{12}, the osmolality was about 100 mosmolal higher than in the wild-type plants. These results demonstrate that in the leaves of the transgenic potato plants, the osmotic pressure was increased. The curly appearance of the leaves of transformed plants may be caused by osmotic inhomogeneity. Apparently, a reduced sucrose export from the mesophyll cells of the transformed plants, leading to an accumulation of hexoses and amino acids, resulted in an increase of the osmotic pressure in the leaf cells. Determination of water potential and turgor are required for a further characterization of this phenomenon.

The question arises as to the subcellular location of these compounds. Because subcellular metabolite analysis by nonaqueous fractionation has not yet been done with potato leaves, this question can be answered only from results obtained with leaves of other plant species. Subcellular fractionation of tobacco leaves revealed that the glucose and fructose contained in these leaves in relatively high amounts are located primarily in the vacuoles (D. Heineke, unpublished observations). Nonaqueous fractionation of leaves from spinach (15), barley (24), and maize (23) showed that the amino acids are more or less excluded from the vacuoles. The apparent vacuolar concentrations of all the major amino acids in these leaves were 1 to 2 orders of magnitude lower than in the cytosol or the chloroplast stroma.

The leaves of the plant species listed above contained under the conditions of the measurements very little proline, and therefore data for the subcellular distribution of proline are not yet available. In spinach and barley leaves, amino acids in total were found to be the main organic solutes in the cytosolic and stromal compartment. A generalization of this finding suggests that in the transgenic plants expressing yeast invertase in the apoplast, the increase in the osmotic pressure in the cytosol and in the stroma will be primarily due to an increase of the concentrations of amino acids.

There are numerous reports indicating that water stress can induce an inhibition of photosynthesisis (17). Probably in the leaves of transformed potato plants studied here, the observed increase in osmolality of the cellular fluids is involved in the inhibition of photosynthesis. As shown in Table III, the lowered photosynthesis rate in the transgenic plants is accompanied by a decrease in the evaporation rate, indicating that the stomata have been closed to large extent. The increased level of RuBP observed under these conditions could be caused by a limitation of CO_2 supply. One explanation could be that the increase in the osmolality of the cellular fluids leads to a closure of the stomata, which in turn results in an inhibition of photosynthesis. This would explain in a simple way how an inhibition of the phloem export of sucrose ultimately causes an inhibition of photosynthesis to prevent an excessive accumulation of photosynthesis products that would destroy the osmotic balance of the source cells. Further experiments including determinations of water potential and turgor are required to verify this hypothesis.

Phloem Sap Composition

For the analysis of carbohydrate composition, the phloem sap was collected using the aphid technique, where the stylets of aphids attached to leaves are severed by a laser beam (1, 6). The results of Table VIII show that in the transgenic plants, the carbohydrate pattern in the phloem sap was not altered. Although it is well established that in many plants phloem transport is specific for sucrose (3), phloem loading of hexoses at high levels of hexoses has been postulated (11). Our results clearly show that in transgenic plants, in which the apoplast the ratio of sucrose to hexoses is decreased by 2 orders of magnitude (Table II), sucrose is still the only carbohydrate translocated by the phloem system. This demonstrates the very high specificity of the phloem loading of sucrose.

The phloem sap samples that we obtained from the transgenic potato leaves were too small for analyzing carbohydrates or amino acids. To obtain information about the amino acid to carbohydrate ratio in the phloem sap, the exudate from excised potato leaves, which were placed with their petiole into 20 mM EDTA solution, was analyzed.

Table VII. *Osmolality of Potato Leaf Sap*
The results from each plant were documented separately.

	mosmol (kg)$^{-1}$		
Wild type	254	250	224
Transformant-8	306	299	325
Transformant-12	361	381	330

Table VIII. *Distribution of Carbohydrates in the Phloem Sap Obtained from Illuminated Potato Leaves by the Aphid Technique*

	Wild Type	Transformant	
		T_8	T_{12}
	% of total		
Sucrose	95	97	96
Fructose	2.4	1.5	1.0
Glucose	2.7	1.2	2.6

Table IX. *Comparison of the Contents of Sucrose and Amino Acids in Illuminated Potato Leaves and in the Exudate Obtained from Excised Illuminated Leaves*

Mean values from six experiments with transformant-8 and transformant-12.

	Sucrose/Σ Amino Acids
Wild type	
Leaf content[a]	1.91
Leaf exudate	28
Transformant	
Leaf content[a]	0.79
Leaf exudate[b]	9.60

[a] Data from Table V. [b] ½ Glc + ½ Fru added to sucrose.

According to a recent study by Weibull et al. (22), leaf exudates obtained as described above can be employed for an approximate measurement of the amino acid composition of the phloem sap. Taking into account that the glucose and fructose contained in the leaf exudate represent artificially hydrolyzed sucrose (D. Heineke, unpublished observations), we determined the sucrose to amino acid ratio from the contents of sucrose and hexoses and from the sum of the amino acids analyzed in the leaf exudates (Table IX). In the exudates, this ratio is found to be 1 order of magnitude higher than in the leaves.

A similar value was found between the corresponding ratios in the phloem sap obtained by aphid technique from spinach leaves and whole spinach leaf contents (15). Taking the composition of the leaf exudate as some measure for the composition of the phloem sap, the data indicate that in the phloem sap of transgenic plants, the amino acid content in relation to the sucrose content is higher than in the phloem sap of wild-type plants. It may be noted that a similar increase in the amino acid to sucrose ratio has been observed in the phloem sap collected from wild-type plants during darkness as compared to that collected during illumination (24). From this observation, the increase in the amino acid to sucrose ratio in the phloem sap of the transformed plants can be explained as a consequence of the decreased phloem loading of sucrose.

Influence of the Altered Composition of the Phloem Sap on the Metabolite Composition of the Tubers

From a set of wild-type and transformed plants grown simultaneously in the same growth chamber for the same length of time, the tubers were analyzed from duplicates of each plant type. A comparison of the individual data shows marked differences between the tubers of the wild-type and transformed plants (Table X). The transformed plants produced fewer tubers. In the transformed plants, a slight increase in the water content was observed, although a larger number of analyses may be required to verify this. Clearly, in the tubers of transformed plants, the content of hexoses was found to be elevated. Because in the transformed plants the sieve tube contents of hexoses in relation to sucrose are unaltered, the observed increase in hexoses in the tubers is probably the result of the invertase activity expressed in the apoplast of the tubers, as 35S cauliflower mosaic virus promoter used for the transformation is not tissue specific. An increase of hexose levels in the tubers is not consistent with a symplastic way of phloem unloading of sucrose, as proposed by Oparka (14).

In the tubers of transformed plants, the amino acid content also was markedly increased. The amino acid pattern of this increase, however, is very different from that found in leaves. Glutamine and asparagine, which are the most abundant amino acids in the wild-type tubers, show a higher increase in the tubers of transformed plants than the other amino acids, amounting to more than 50% of the total amino acids. Proline, representing up to 40% of the total amino acids in the leaves of the transformed plants (Table V), was also detected in the tubers of the transformed plants, but it never amounted to more than 5% of the total amino acids (data not shown).

Compared to the wild-type plants, the content of starch in the tubers of the transformed plants was found to be decreased, and that of protein increased (Table X). The increase in protein content was mainly due to an increase of the main

Table X. *Potato Tubers Harvested from Wild-Type and Transformed Plants Grown Simultaneously in the Same Growth Chamber and for the Same Time Period (12 weeks after Propagation from Cell Culture)*

The results were obtained with two plants each of the wild-type and the transformed plants. The results of each plant were documented individually.

	Wild Type		Transformant-8		Transformant-12		
Number of tubers per plant	5	12	7	5	3	5	
Total weight of tubers per plant	186	216	121	121	83	83	g
Dry weight/fresh weight	0.23	0.23	0.20	0.21	0.21	0.19	
Glucose	1.23	1.94	2.19	4.07	1.37	8.24	μmol (g fresh weight)$^{-1}$
Fructose	0.11	0.15	1.58	1.26	0.58	1.71	μmol (g fresh weight)$^{-1}$
Sucrose	3.71	4.44	2.76	4.58	3.71	2.57	μmol (g fresh weight)$^{-1}$
Glutamine	10.5	7.1	21.5	18.7	23.6	13.5	μmol (g fresh weight)$^{-1}$
Asparagine	21.2	14.9	24.4	29.1	35.3	18.8	μmol (g fresh weight)$^{-1}$
Σ Amino acids	55.1	41.1	69.3	72.8	88.1	55.2	μmol (g fresh weight)$^{-1}$
Protein	2.61	3.94	4.49	5.46	5.80	5.23	mg (g fresh weight)$^{-1}$
Starch	83.5	81.7	66.1	78.5	67.3	49.1	mg (g fresh weight)$^{-1}$
Protein/Starch	0.031	0.048	0.068	0.070	0.086	0.107	

storage proteins, patatin and the 22-kD protein (data not shown). As a result of these changes, the ratio of protein to starch was markedly increased in the transformed plants. A comparison of the two transformants shows that this increase in the ratios is correlated to the different changes in the rates of photosynthesis, metabolite levels, osmolality of the leaf sap and tuber production, as shown in Tables III through VII and X.

CONCLUSION

The effect of the yeast invertase expressed in the apoplast demonstrates that in potato leaves, phloem loading of sucrose involves the apoplastic compartment. Our results show that a decrease of sucrose export from the plant source cells to the sink tissues, as caused by the introduction of the yeast invertase gene, affects the partitioning of the products of photosynthesis between carbohydrates and amino acids, which ultimately leads to an increase in the protein to starch ratio in the potato tubers.

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