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Repression of Acetolactate Synthase Activity through Antisense Inhibition

Molecular and Biochemical Analysis of Transgenic Potato (*Solanum tuberosum* L. cv Désirée) Plants

Rainer Höfgen[1], Bernd Laber, Iris Schüttke[2], Ann-Kristin Klonus, Wolfgang Streber, and Hans-Dieter Pohlenz*

Hoechst Schering AgrEvo GmbH, D-13476 Berlin, Germany

Acetolactate synthase (ALS), the first enzyme in the biosynthetic pathway of leucine, valine, and isoleucine, is the biochemical target of different herbicides. To investigate the effects of repression of ALS activity through antisense gene expression we cloned an ALS gene from potato (*Solanum tuberosum* L. cv Désirée), constructed a chimeric antisense gene under control of the cauliflower mosaic virus 35S promoter, and created transgenic potato plants through *Agrobacterium tumefaciens*-mediated gene transfer. Two regenerants revealed severe growth retardation and strong phenotypical effects resembling those caused by ALS-inhibiting herbicides. Antisense gene expression decreased the steady-state level of ALS mRNA in these plants and induced a corresponding decrease in ALS activity of up to 85%. This reduction was sufficient to generate plants almost inviable without amino acid supplementation. In both ALS antisense and herbicide-treated plants, we could exclude accumulation of 2-oxobutyrate and/or 2-aminobutyrate as the reason for the observed deleterious effects, but we detected elevated levels of free amino acids and imbalances in their relative proportions. Thus, antisense inhibition of ALS generated an in vivo model of herbicide action. Furthermore, expression of antisense RNA to the enzyme of interest provides a general method for validation of potential herbicide targets.

The amino acid biosynthetic pathways are well studied in bacteria and fungi but only recently have they received attention in higher plants, because various enzymes of the biosynthetic pathways for essential amino acids have proved to be the molecular targets for several commercially successful herbicides (La Rossa and Falco, 1984; Kishore and Shaw, 1988; Ray, 1989). Another driving force for the study of plant amino acid biosynthesis and its regulation were attempts to manipulate biosynthetic capacities in order to influence the nutritional value of crop plants, either by mutation or by genetic engineering (Coruzzi, 1991; McGrath and Coruzzi, 1991; Karchi et al., 1993). Due to the complex regulation of amino acid biosynthesis in plants, the analysis of auxotrophic mutants (Negrutiu et al., 1985; Chapple et al., 1992) and of deregulated mutant plant enzymes (Dotson et al., 1990; Frankard et al., 1992) was helpful in elucidating the regulatory hierarchies. In vivo expression of bacterial enzymes with regulatory properties different from those of the corresponding plant enzymes allowed the analysis of effects of changes of the content of single amino acids on plant growth (Shaul and Galili, 1992; Karchi et al., 1993).

Within the biosynthetic pathway of the branched chain amino acids Val, Leu, and Ile, the enzyme ALS (EC 4.1.3.18) has attracted a great deal of attention as the target for several structurally distinct classes of commercially successful herbicides, namely sulfonylureas, imidazolinones, and triazolopyrimidines (Mazur and Falco, 1989). Plants treated with these herbicides respond with growth retardation caused by inhibition of DNA synthesis and cell division, followed by chlorosis and necrosis of meristematic tissues (Ray, 1984; Scheel and Casida, 1985a; Ray, 1986). Mature tissues die slowly and death of the whole plant can take several weeks. Biochemical and genetic studies have demonstrated that inhibition of ALS is the primary reason for the observed effects (Ray, 1984; LaRossa et al., 1987a). Overexpression of plant ALS genes in heterologous backgrounds allowed for the enzymatic properties to be characterized, especially with respect to the interaction with herbicides (Smith et al., 1989; Singh et al., 1991). Proof that ALS is the only site of action of these herbicides was provided by cloning genes for herbicide-resistant ALS enzymes (Haughn and Somerville, 1986; Smith et al., 1988; Sathasivan et al., 1990; Hattori et al., 1992) and their transfer to susceptible plants to generate herbicide-resistant transgenic plants (Haughn et al., 1988).

It has been speculated that toxicity elicited by ALS-inhibiting herbicides is due to the accumulation of the acetolactate precursor 2-oxobutyrate (La Rossa et al., 1987b). Alternatively, amino acid starvation was suggested to be responsible for the slow action of these herbicides, leading to the appearance of morphological effects only after the pool of free amino acids has been depleted (Scheel and Casida, 1985b; Schloss, 1989). A recent study (Shaner and Singh, 1993) excluded accumulation of 2-oxobutyrate

[1] Present address: Max-Planck-Insitut für Molekulare Pflanzenphysiologie, D-14415 Potsdam, Germany.

[2] Present address: Schering AG, Institute of Cellular and Molecular Biology, D-13342 Berlin, Germany.

* Corresponding author; fax 49–30–401–4333.

Abbreviation: ALS, acetolactate synthase or acetohydroxyacid synthase.

or of its transamination product 2-aminobutyrate as reason for the observed effects on plant growth. The authors suggest a starvation for branched chain amino acids as the primary cause for the phytotoxicity of the ALS inhibitor imazaquine.

Because the effects of herbicide application on plants are of a transient nature, we attempted to generate an in vivo plant model that allows the analysis of the physiological changes accompanying the permanent inhibition of ALS. Therefore, we have cloned an ALS gene from potato and transferred a constitutively expressed antisense construct of this gene to potato plants. We then isolated transgenic plants with reduced ALS activity, which mimicked the effects of herbicide application to potato plants. Analysis of these plants with respect to enzyme activity and metabolite content are presented. The implications of this approach for the in vivo validation of potential herbicide targets are discussed.

MATERIALS AND METHODS

Plant Material

The potato cultivar Désirée (*Solanum tuberosum* L.), obtained through Vereinigte Saatzuchten eG (Ebsdorf, Germany), was propagated under greenhouse conditions. Surface-sterilized tuber slices of potato were used as starting material for axenic tissue cultures and transformations.

Plant Transformation

Tissue culture and transformation of leaf discs was essentially performed as described by Rocha-Sosa et al. (1989). The binary vector pH29 carrying the ALS antisense gene was transformed into *Agrobacterium tumefaciens* strain pGV2260 according to Höfgen and Willmitzer (1988) and used for potato leaf disc transformation. Agrobacterial growth was inhibited by claforan and transgenic callus was selected on kanamycin. After shoot induction about 80 plantlets were rooted, still on kanamycin. Further propagation and replication of plantlets was performed on claforan-containing medium (Murashige and Skoog medium with 2% [w/v] Suc). During all tissue culture procedures, as well as for later propagation, the medium was enriched with 0.5 g/L casein hydrolysate (casamino acids, Merck, Darmstadt, Germany) to complement for the expected ALS antisense-mediated deficiency in amino acid biosynthesis (Negrutiu et al., 1985).

Nucleic Acid Analysis

RNA and DNA preparation, blotting procedures, hybridization with random-primed-labeled probes (Boehringer Mannheim kit), and standard recombinant DNA techniques were performed as described previously (Sambrook et al., 1989; Jackson et al., 1993). Usually, 30 μg of total RNA or 50 μg of DNA digested with a suitable restriction enzyme were applied to the gels. Sequencing was done using a Pharmacia T7 Sequencing Kit and sequence analysis was performed on a VAX III using the University of Wisconsin Genetics Computer Group programs.

Construction of an ALS Antisense Gene

The potato ALS gene was cloned by a nested PCR approach using the PCR 1000 vector system because the first set of primers yielded only faint and ambiguous bands. The primers were homologous to the sequence of the tobacco *SurB* gene (Lee et al., 1988). The first pair comprised positions 415 to 444 and 2376 to 2409 (representing nucleotides encoding amino acid residues 1–10 and 655–664, respectively), and the second, more inward pair of primers comprised positions 445 to 477 and 2341 to 2375 (representing nucleotides encoding amino acid residues 11–21 and 643–654, respectively). The 5′ oligo was provided with an *Eco*RI site and the 3′ oligo was provided with an additional *Bam*HI site for cloning purposes. An *Asp*718/*Not*I fragment of the ALS gene was cut from this vector, treated with T4 DNA polymerase, and ligated into the *Sma*I site of pUC19 to yield pUC-ALS. A *Kpn*I/*Sal*I fragment with suitable orientation was cut from pUC-ALS and cloned into the expression cassette of a plant binary vector, BinAR (Höfgen and Willmitzer, 1990), resulting in a potato ALS gene in antisense orientation with respect to the cauliflower mosaic virus 35S promoter of the expression cassette. Plant termination sequences were provided by an octopine synthase 3′ end. The plasmid was designated pH29.

Assay of ALS Activity

ALS activity was determined essentially as described by Miflin (1971). Protein was extracted from about 100 mg of plant material followed by $(NH_4)_2SO_4$ precipitation (25–70%, w/v). About 0.1 to 1 mg of the precipitated protein were used for enzyme assays with 40 mM Na-pyruvate, 0.32 mM thiaminpyrophosphate, 0.5 mM $MnSO_4$, and 20 mM Na-phosphate buffer, pH 7.5. After incubation for 1 h at 30°C, the reaction was terminated by the addition of $ZnSO_4$ (5 mM). After centrifugation the supernatant was acidified with HCl. After addition of 1.7% (w/v) α-naphthol and 0.17% (w/v) creatin and 1 h of incubation at room temperature, the absorbance was recorded at 530 nm.

Amino Acid Analysis

Amino acids were extracted from about 100 to 500 mg of leaf tissue by homogenizing the frozen plant material in methanol:chloroform:water (12:5:3; 5 μL/mg). After the addition of water and brief centrifugation, the supernatant was vacuum dried and the pellet was redissolved in water. Quantitative amino acid analyses were performed by HPLC after precolumn derivatization with *o*-phthaldialdehyde (Hurst, 1984).

Analysis of 2-Oxoacids

Quantitative determination of individual 2-oxoacids was performed as described previously (Laber et al., 1994). Plant material was frozen in liquid nitrogen and homogenized in 25 mM Mops-NaOH buffer, pH 7.1 (300 μL/g fresh weight). After centrifugation, 50 μL of the supernatant was directly used for derivatization. For vacuum infiltration experiments leaves or leaf discs of potato plants were

incubated overnight at ambient temperature with either 50 mM potassium phosphate buffer, pH 7.0, or phosphate buffer supplemented with 5 mM Thr.

RESULTS

Cloning of a Partial ALS Gene from Genomic DNA of Potato

The published sequences of different ALS genes do not contain introns (Lee et al., 1988). Therefore, we cloned an ALS gene directly from genomic DNA of potato by a nested PCR approach utilizing two sets of oligonucleotide primers homologous to the tobacco ALS *SurB* gene (Lee et al., 1988). The resulting fragment of approximately 2 kb correlates to the PCR product of 1952 bp based on the expected homology to tobacco ALS. This should correspond to approximately 94% of the coding sequence of 1991 bp, which we thought would be sufficient for antisense inhibition. The identity of the cloned fragment was confirmed by comparing partial 5′ and 3′ sequences with previously cloned ALS genes. The sequence of the 3′ end exhibited 94% homology to the *SurB* nucleotide sequence and 92% identity to the derived amino acid sequence. The homology of the 5′ sequence to *SurB* was 82% on the nucleotide level and the identity of the derived amino acid sequence was 70%. The 5′ sequence is still within the putative transit peptide, which might explain the lower homology. The homology to the yeast (Falco et al., 1985) and *Escherichia coli* enzyme (Lawther et al., 1981) amounts to 35 and 30% identical but 60 and 41% similar amino acids, respectively.

ALS Antisense Inhibition Leads to Phenotypical Changes Comparable to Herbicidal Effects

An ALS antisense gene (pH29) was constructed under control of the heterologous, constitutive 35S cauliflower mosaic virus promoter and transferred to *A. tumefaciens* strain pGV2260. This *Agrobacterium* strain was used for a standard leaf disc transformation procedure of potato plants. Rooted plantlets were replicated and one copy of each plant was transferred to Murashige and Skoog medium without casamino acid complementation to screen for plants unable to grow without an exogenous supply of amino acids. Two potato plants (P.H29–2 and P.H29–41) exhibited clear growth retardation under these conditions, but recovered when returned to complementing medium.

About 40 individual replicas of transgenic potato plants were put in soil and grown under greenhouse conditions for phenotypic evaluation. Three plants (P.H29–2, P.H29–23, and P.H29–41) showed phenotypically overt symptoms with different degrees of severity. P.H29–23 displayed only slight growth retardation immediately after transfer from tissue culture to soil but recovered quickly to control plant phenotype. P.H29–2 showed growth retardation and leaf chlorosis but partially recovered during later growth, staying only slightly smaller than controls. P.H29–41, however, exhibited constantly severe phenotypical symptoms: growth retardation and stunting, leaf chlorosis and necrotic lesions on older leaves, bushy growth, and alteration of the leaf morphology to crumpled, small, simple leaves without formation of the compound leaves typical of potato. Several siblings of P.H29–41 usually died in the first week after transfer from tissue culture to soil.

Antisense inhibition of ALS should impair the flux through the branched chain amino acid biosynthetic pathway. This in turn should give rise to a phenotype comparable to that of plants treated with herbicidal inhibitors of ALS. For comparison, potato control plants were treated with the imidazolinone herbicide Scepter (1 or 0.1 kg/ha). This treatment resulted in growth retardation, formation of phenotypically altered leaves at the shoot apex (small, narrow, and pale green leaves showing a crumpled morphology), and a delay in leaf expansion. At the higher concentration (1 kg/ha) necrotic spots appeared on the surfaces of older leaves, and shoot tips and young leaves turned brownish, became desiccated, and finally died. Our transgenic ALS antisense plants mimicked these symptoms, especially P.H29–2 and P.H29–41. Moreover, the phenotype of the transgenic plants was stable in a second vegetatively grown generation sprouted from harvested tuber material.

ALS Antisense Plants Are Severely Impaired in Biomass Production

The primary phenotypical effect of ALS-inhibiting herbicides is severe growth retardation (Scheel and Casida, 1985a, 1985b; Ray, 1986). Similarly, transgenic ALS antisense plants revealed as the most prominent phenotypical effect severe growth retardation resulting in a drastically reduced production of biomass. The severely growth-retarded transformant P.H29–41 grew to only a few centimeters in height (Fig. 1). The fresh weight of green matter of 1-month-old transgenic plants reached only about 5% of the fresh weight of control plants (Table I). The plants produced tiny white tubers (Fig. 1), and the average tuber

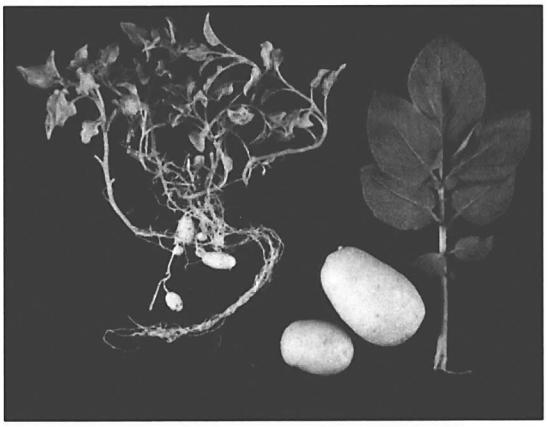

Figure 1. The effect of expression of ALS antisense RNA on greenhouse-grown potatoes. Comparison of a 2-month-old ALS antisense plant P.H29–41 (left) with a control plant (right) reveals severe growth retardation, bushy growth, alteration in foliage morphology, and reduced tuber growth. Leaves showed slight chlorosis and necrotic lesions.

number was reduced by a factor of about 2. The fresh weight per tuber was reduced to 4% of the fresh weight of controls and total tuber yield per plant was reduced to about 2% (Table I).

Molecular Analysis of ALS Antisense Plants

Genomic DNA of potato plants was isolated and a Southern blot analysis was performed by hybridization with the cloned potato ALS gene. ALS seems to be a single- or low-copy number gene in the potato genome, since only one hybridizing band for *Bam*HI-digested DNA could be detected, correlating to the absence of an internal restriction site in the ALS gene. Due to the presence of one internal *Hin*dIII site, two hybridizing bands were detected in *Hin*dIII-digested genomic DNA. Antisense plants P.H29–2, P.H29–23, and P.H29–41 showed additional bands in genomic southern blot analyses, thus proving that they contain integrated copies of the ALS antisense gene (data not shown).

Total RNAs isolated from different organs of potato plants were analyzed by hybridizing northern blots with the cloned potato ALS gene. A band of approximately 2 kb in size (Fig. 2A) was detected. Almost similar expression levels of ALS were found in leaves, stems, stolons, and tubers. Expression seemed to be slightly higher in young leaves and buds (sink tissues) than in fully expanded leaves (source tissues). Root tissues exhibited a reduced expression of ALS. Flowers or fruits have not been analyzed.

Northern blot analysis of leaf (P.H29–2 and P.H29–41) and tuber material (p.H29–41) of transgenic ALS antisense plants revealed drastically, although not completely, reduced steady-state levels of ALS mRNA (Fig. 2B). Total RNA isolated from leaf material of potato plants treated with the herbicide Scepter showed no alteration in ALS expression (Fig. 2B). Thus, chemical inhibition of enzyme activity did not affect expression levels of ALS, whereas antisense inhibition reduced the content of ALS mRNA.

To investigate possible *trans*-regulatory effects of ALS antisense inhibition, northern blot analyses of total leaf RNAs of P.H29–2, P.H29–41, and control plants were performed. Hybridization of probes of two genes coding for biosynthetic enzymes of the aspartate family, i.e. Thr deaminase (from potato; Hildmann et al., 1992) and dihydrodipicolinate synthase (from *Nicotiana sylvestris*, V. Frankard, unpublished data), and of a gene coding for another enzyme of the branched chain amino acid biosynthetic pathway, i.e. isopropylmalate dehydrogenase (from potato; Jackson et al., 1993) did not reveal alterations in the expression levels of these three genes (data not shown). Apparently, there are no major changes with respect to the expression of other amino acid biosynthetic genes to compensate for reduced expression of ALS mRNA or reduced enzyme amounts.

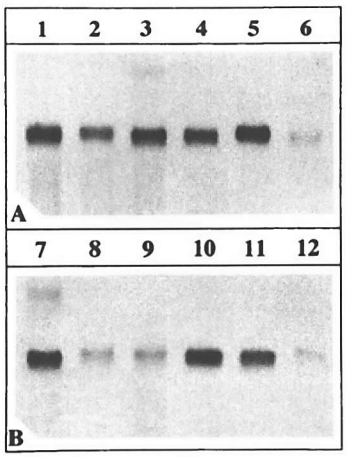

Figure 2. Northern blot analysis of expression of ALS mRNA in potato plants. Total RNA extracted from different tissues of control plants, ALS antisense plants, and Scepter-treated control plants was hybridized with a radiolabeled potato ALS gene. A, ALS mRNA expression in sink leaves (lane 1), source leaves (lane 2), stems (lane 3), stolons (lane 4), tubers (lane 5), and roots (lane 6) of control plants. B, ALS mRNA expression in leaves of a control plant (lane 7), the ALS antisense plants P.H29–2 (lane 8) and P.H29–41 (lane 9), and a control plant treated with Scepter (lane 10), as well as in tubers of a control plant (lane 11) and of the ALS antisense plant P.H29–41 (lane 12).

ALS Antisense Plants Show Reduced Levels of Enzymatic Activity

Antisense inhibition of ALS led to a reduced availability of the corresponding mRNA for translation, which in turn should result in a reduced concentration of this enzyme. To test this assumption, we performed assays of ALS activity in different plant tissues. In control plants the highest enzyme activity was measured in very young tissues near the vegetative bud (1.03 ± 0.05 ΔE_{530}/mg fresh weight), whereas fully developed and expanded leaves, and old but

Table I. *Biomass allocation of potato contol plants and ALS antisense plants P.H29–41*
Greenhouse-grown plants were harvested 4 weeks after transfer to soil.

	Control Plants	ALS Antisense Plants	Percent
Green Matter			
Number of samples	3	6	
Fresh weight (g)	41.5 ± 2.8	2.2 ± 0.7	5.3
Tubers			
Number of samples	4	5	
Tubers per plant	11 ± 2	5 ± 3	45.5
Fresh weight per plant (g)	105 ± 11	2.0 ± 1.3	1.9
Fresh weight per tuber (g)	10.4 ± 1.9	0.4 ± 0.14	3.8

still green leaves at the stem base, showed about 5-fold-reduced ALS activities (0.20 ± 0.03 and 0.18 ± 0.02 ΔE_{530}/mg fresh weight, respectively). Since the variability of ALS activity in tissues of different developmental stages was high, all additional ALS assays were carried out using carefully selected tissues of the same developmental stage, i.e. young developing tissue. In antisense plants the ALS activity was significantly reduced: in plant P.H29–2 to 33% of wild-type activity and in the phenotypically more severely affected plant P.H29–41 to only 15% of wild-type activity (Table II).

Inhibition of ALS Activity Is Accompanied by Changes in the Free Amino Acid Pool

Inhibition of ALS, either by antisense inhibition or herbicide treatment, should influence the pool of free amino acids in plant tissues. Therefore, we determined the free amino acid content of young and old leaves of antisense plants, control plants, and control plants treated with the herbicide Scepter (1 kg/ha) 2 weeks prior to sampling (Table III). Immature young (sink) leaves of control plants contained about 2.4 times more free amino acids than older, mature (source) leaves. In Scepter-treated plants we measured an increase of the total amount of free amino acids, which was more pronounced in sink tissues (10.0-fold) than in source tissues (2.3-fold). Thus, sink tissues contained 10.4 times more free amino acids than source tissues. In both ALS antisense plants P.H29–2 and P.H29–41, the total increase in free amino acid content was not as high as for Scepter-treated plants, and it was almost uniform for sink (factors 3.4 or 2.3) and source tissues (factors 4.0 or 3.1). This resulted in a ratio between sink and source tissues comparable to that of control plants (factors 2.1 and 1.8).

The levels of individual amino acids were increased in both herbicide-treated and antisense plants. However, the increases differed for various amino acids. Despite ALS inhibition, which is expected to reduce the amounts of Val, Leu, and Ile, we detected elevated amounts, with the single exception of a severe reduction of Val in sink tissues of Scepter-treated plants.

2-Oxobutyrate and/or 2-Aminobutyrate Does Not Accumulate in ALS Antisense or Herbicide-Treated Plants

Hypothetically, inhibition or down-regulation of ALS activity should lead to accumulation of 2-oxobutyrate or its transamination product 2-aminobutyrate. Since high levels of 2-oxobutyrate are toxic to *Salmonella typhimurium* (LaRossa et al., 1987b) and 2-aminobutyrate has been shown to disrupt cell division in *Allium* (Langzagorta et al., 1988), it was concluded that accumulation of 2-oxobutyrate and/or 2-aminobutyrate is responsible for toxicity of ALS inhibitors (for refs., see Shaner and Singh, 1993). Therefore, we determined the 2-oxoacid content of greenhouse-grown plants and detected high levels of pyruvate but only trace amounts of 2-oxobutyrate in leaf extracts of antisense and control plants (data not shown). However, we could induce an approximately 10-fold increase of 2-oxobutyrate content in leaf tissues of P.H29–41, but not in controls, by feeding excess amounts of Thr by vacuum infiltration to isolated leaf discs (Fig. 3). Thr feeding to P.H29–2 induced accumulation of intermediate levels of 2-oxobutyrate (data not shown). Simultaneous infiltration with imazaquine or chlorsulfurone (1 μM each) plus Thr provoked an even more pronounced accumulation of 2-oxobutyrate, whereas neither buffer nor herbicides alone had any enhancing effects. 2-Aminobutyrate could not be detected in controls, in the antisense plant P.H29–2, or in Scepter-treated plants. However, low levels were present in tissues of P.H29–41 (Table III).

Table II. *Comparison of the ALS activities of control and ALS antisense plants*

Samples were taken from young leaves of greenhouse-grown plants 4 weeks after transfer to soil.

Plant	Number of Samples	Enzyme Activity	Percent
		ΔE_{530}/mg fresh wt	
Control plant	20	1.60 ± 0.24	100.0
P.H29–2	10	0.53 ± 0.11	33.1
P.H29–41	20	0.23 ± 0.06	14.4

DISCUSSION

By introducing a potato ALS antisense gene into potato plants, we isolated transgenic plants specifically reduced in ALS activity. This approach made possible detailed investigations on the physiology of transgenic potato plants specifically disturbed in the branched chain amino acid biosynthetic pathway and on the influence of this inhibition on whole-plant physiology.

Primary Effects of ALS Antisense Inhibition on Gene Expression and Enzyme Activity

We demonstrated a reduction in the steady-state levels of ALS mRNA in transgenic plants as the primary effect of ALS antisense gene expression. The ALS signal in northern blots of transgenic plants was reduced, although only partially. Assaying for ALS activity revealed for control plants some variations between comparable tissues of individual plants as well as for different developmental stages within one plant. These results correspond to those found for tobacco ALS (Keeler et al., 1993). Despite these variations, we are able to show a significant reduction of ALS activity in ALS antisense plants. Furthermore, the decrease in enzyme activity was positively correlated to the reduction of the steady-state levels of mRNA and to the severity of the observed phenotype. A reduction to 15% of wild-type activity (P.H29–41) constituted a sufficient threshold level to impair growth of potato plants almost irreversibly. Threshold levels of ALS activity necessary to generate phenotypically overt symptoms might still be slightly lower, because part of the residual activity, measured as formation of acetoin, might be caused by other enzymatic activities present in the crude extracts of plants (Bryan, 1980).

It has been shown that amino acid biosynthetic pathways are highly regulated on the enzyme level (Bryan, 1980).

Table III. *Free amino acids in leaves of control plants, Scepter-treated control plants, and ALS antisense plants*

Amino acids were extracted from young and old leaves of control plants, Scepter (1 kg/ha)-treated control plants, and ALS antisense plants P.H29–41 and P.H29–2 4 weeks after transfer to soil. Scepter treatment was done 2 weeks prior to sampling. Values are the means of three replicates. nd, Not detectable; Aba, aminobutyrate; Hser, Homoserine.

Amino Acid	Control		Control + Scepter		ALS Antisense Plant			
					P.H29–41		P.H29–2	
	Young	Old	Young	Old	Young	Old	Young	Old
				nmol g^{-1} fresh wt				
Asp	610	453	790	453	649	414	850	637
Glu	2,665	812	3,541	127	641	261	2,299	1,874
Asn	427	51	29,170	722	3,744	439	1,171	220
Ser	547	297	1,019	550	1,526	714	1,098	898
Gln	1,164	465	19,975	2,235	7,585	3,787	6,005	1,790
His	nd	nd	1,167	70	709	203	78	nd
Hser	24	nd	50	nd	nd	nd	56	18
Gly	67	37	416	72	399	234	150	124
Thr	246	126	787	166	882	303	447	326
Arg	49	26	13,335	53	104	72	42	24
Ala	523	263	1,487	475	2,918	2,114	1,049	901
γ-Aba	576	268	2,045	984	4,228	2,583	2,827	2,085
Tyr	61	28	446	87	790	234	157	92
α-Aba	nd	nd	nd	nd	94	121	nd	nd
Met + Trp	258	88	1,000	108	392	204	342	294
Val	171	94	nd	236	238	238	305	115
Phe	63	57	386	154	364	161	108	125
Ile	67	41	384	55	269	280	146	69
Len	42	18	153	65	324	171	127	81
Lys	43	30	218	54	346	153	124	75
Total	7,603	3,154	76,369	7,366	26,202	12,686	17,381	9,748

However, it remains possible that additional transcriptional regulation of the appropriate genes might occur. Therefore, ALS antisense plants were scored for transcriptional regulation of other enzymes within amino acid biosynthetic pathways. Steady-state mRNA levels of Thr deaminase, isopropylmalate dehydrogenase, and dihydrodipicolinate synthase were found not to be altered.

Phytotoxicity Can Be Correlated to Changes in Amino Acid Content But Not to Accumulation of 2-Oxobutyrate

Phytotoxicity of ALS-inhibiting herbicides has been assigned to accumulation of nonphysiological amounts of 2-oxobutyrate and/or 2-aminobutyrate (La Rossa et al., 1987b; Schloss, 1989). Under physiological conditions we were unable to detect accumulation of these two metabolites in ALS antisense or in herbicide-treated plants. Thus, we exclude accumulation of 2-oxobutyrate and/or 2-aminobutyrate as the reason for the observed detrimental effects. Upon feeding of Thr to isolated leaves, 2-oxobutyrate was accumulated in antisense and herbicide-treated plants, but not in control plants. Apparently, the reduced capacity to metabolize 2-oxobutyrate generates a bottleneck in the pathway, which becomes evident only under these nonphysiological conditions.

Another hypothesis linked the activity of ALS-affecting herbicides to a general starvation for amino acids. Chlorsulfuron effects on growth of excised pea roots (Ray, 1984) or on soybean suspension cultures (Scheel and Casida, 1985a) could be reversed by application of casein hydrolysate or, when individual amino acids were tested, by Val and Leu. Protective effects could be slightly increased by additionally supplying Ile. We could also, although only partially, alleviate the symptoms caused by antisense inhibition of ALS by supplementation with casamino acids, but not by supplementation with Val, Leu, and Ile (data not shown). For soybean suspension cultures a decrease of the Val, Leu, and possibly Ile content, but no effect on other amino acids, was detected after sulfonylurea herbicide treatment (Scheel and Casida, 1985a). In contrast to this observation, Royuela et al. (1991) measured an accumulation of free amino acids and an increase in relative proportion of some of them in chlorsulfuron-treated wheat and maize. We also determined an increased content of total free amino acids as well as a perturbed composition in ALS antisense and in Scepter-treated potato plants. Detrimental effects on plant growth caused by imbalances in amino acid contents have been reported in the literature (reviewed by Bryan, 1980). For example, feeding of Thr and Lys inhibits growth of most plants, presumably by limiting Met biosynthesis. Growth inhibition was also observed to result from application of each of the branched chain amino acids, but in particular by combinations of Leu plus Val or Leu plus Ile. Tobacco plants expressing a feedback-insensitive dihydrodipicolinate synthase accumulated high amounts of Lys, resulting in severe growth retardation and morphologically altered plants (Frankard et al., 1992; Shaul and Galili, 1992).

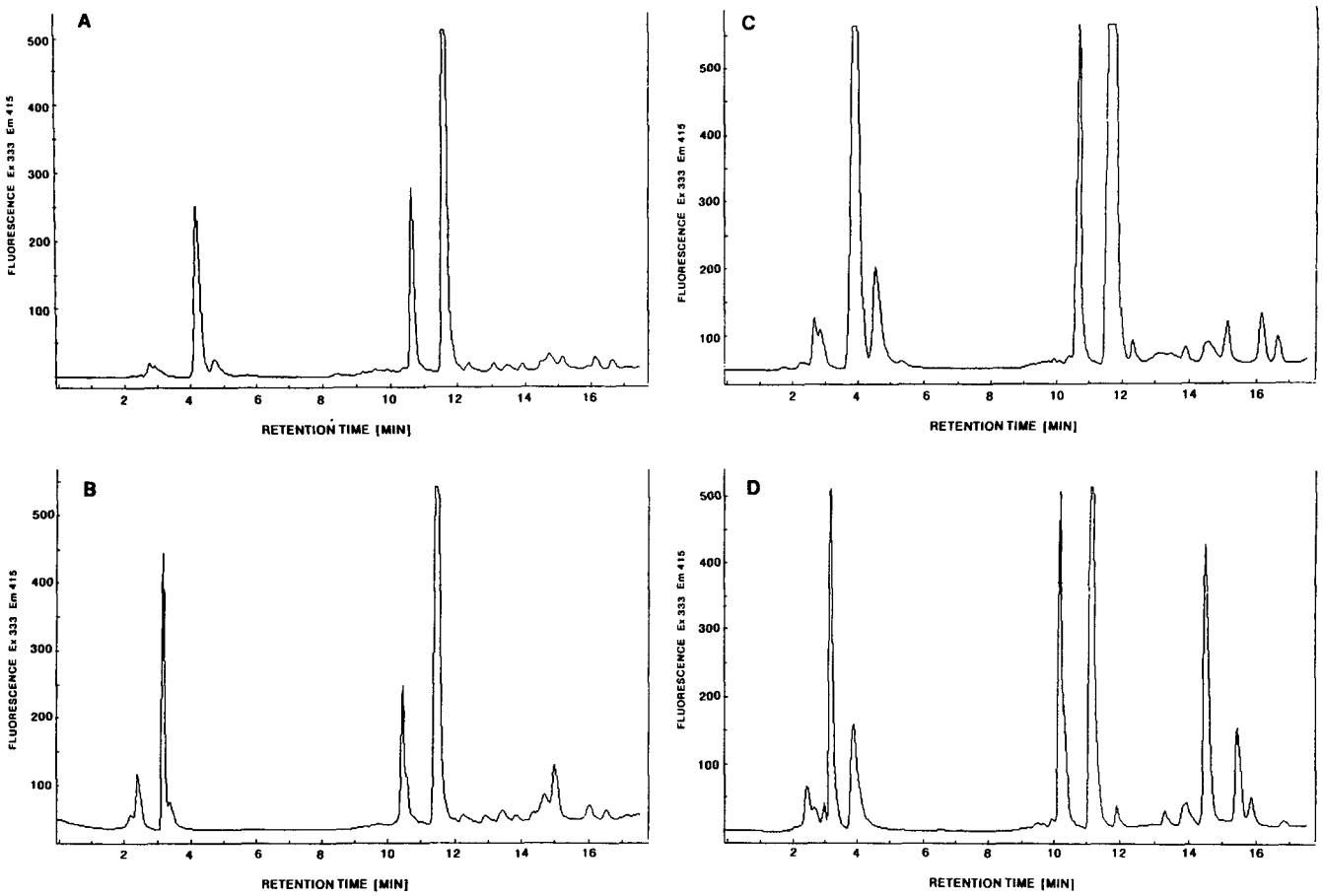

Figure 3. Analysis of 2-oxoacids extracted from leaves of control plants (A and B) and of the ALS antisense plant P.H29–41 (C and D). Leaves were vacuum infiltrated either with buffer (A and C) or with 5 mM Thr (B and D). Pyruvate elutes from the HPLC column at about 11.5 min, and 2-oxobutyrate elutes at about 14.5 min. Only the ALS antisense plant accumulated high amounts of 2-oxobutyrate and only upon Thr feeding (D).

In our opinion the increase in the total amount of free amino acids accompanied by imbalances in their relative proportions reflects a general deregulation of amino acid biosynthesis. This might entail retardation of plant growth, cell division, and further pleiotropic effects. On the other hand, the accumulation of Thr and Lys, especially in sink organs, might in itself be sufficient to explain the phytotoxic effects caused by ALS inhibition.

Antisense Gene Expression as a Tool for Evaluating Herbicide Targets

The fundamental difference between herbicide application and antisense inhibition is that herbicides can block ALS activity completely (Scheel and Casida, 1985a), whereas constitutive antisense inhibition results in a permanent but incomplete down-regulation of enzyme content. However, the similarity of phenotypical effects and of changes in metabolite profiles convinced us that comparable physiological processes occur in antisense and in herbicide-treated plants. Thus, these plants represent phenocopies of the effects caused by ALS-inhibiting herbicides. Based on these results, antisense inhibition of specific plant enzymes might provide a general approach for the validation of putative herbicide targets.

For this approach the complete elimination of residual enzyme activity would be desirable. However, due to the lethal effects caused by constitutive expression of antisense RNA for essential metabolic enzymes, this usually cannot be achieved. Even supplementation with exogenously supplied metabolites of the corresponding pathway does not completely overcome this problem and usually allows only regeneration of plants with partially reduced enzyme content. Therefore, it would be especially advantageous to develop an inducible antisense RNA expression system that allows the control of the antisense effect at any stage of plant development. However, experiments we performed using heat shock, tetracyclin, and jasmonic- or ABA-inducible promoters have been unsuccessful so far (data not shown).

For the application of antisense technology as an in vivo model system for validating potential herbicidal targets, no general quantitative predictions can be given for the decrease in RNA and protein amounts necessary to produce effects on plant growth. The efficacy of the system depends on whether the targeted gene codes for an essential indispensable protein, for example, Rubisco (Rodermel et al.,

1988), or for a "dispensable" protein, e.g. the potato tuber storage protein patatin. In the latter case a more than 90% reduction of protein content exerted no effect on the plant (Höfgen and Willmitzer, 1992). Antisense inhibition of the small subunit of Rubisco, however, impaired photosynthesis already at 50% reduction in protein content. A further decrease led to growth retardation and alteration in leaf morphology as well as accompanying secondary pleiotropic effects (Quick et al., 1991). Determination of these threshold levels helps to discern "good" and "bad" herbicide targets because "not all enzymes, even in the same biosynthetic pathway, are equal" (Schloss, 1989). By antisense inhibition we could not demonstrate lethal effects due to the reduction of Thr deaminase levels (data not shown). Therefore, we cannot suggest Thr deaminase as a suitable target for herbicide design. But we have proven the already known "good" target nature of ALS and also validated glutamate-1-semialdehyde aminotransferase as a potential herbicide target (Höfgen et al., 1994). Our antisense approach could be a powerful tool to sustain biochemical herbicide design projects.

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