Unintended phenotypic effects of single gene insertions in potatoes – assessing developmental dynamics and leaf morphology

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Zusammenfassung

In diesem Artikel werden eine Reihe unbeabsichtigter phänotypischer Effekte einer genetischen Modifikation bei der Kartoffelsorte Bintje vorgestellt. In Pflanzen dieser Sorte wurden zwei verschiedene Gen-Konstrukte eingeführt: Ein Viscotoxin Gen aus der Mistel und ein Aminolävulinat Gen aus der Bäckerhefe. Die gentechnisch veränderten (GV) Pflanzen und entsprechende Kontrollen wurden in zwei unterschiedlichen Wuchsbedingungen untersucht. Die GV Pflanzen zeigten Unterschiede in Entwicklungsdynamik und Alterungsprozess; Abweichungen in der Pflanzengestalt, in der Form der Knollen und der Blätter konnten beobachtet werden. Einige Unterschiede in der Blattform zeigten Interaktionen zwischen Pflanze und Umgebung und weisen somit auf eine veränderte Anpassungsplastizität hin.

Die begleitende Untersuchung von phänotypischen Unterschieden zwischen den drei kommerziellen Sorten Bintje, Appell und Naturella ergab, dass das Set der verwendeten morphologischen Charaktere auch geeignet ist, Sortenunterschiede festzustellen. Die Ergebnisse zeigen, dass phänotypische Unterschiede zwischen Pflanzen derselben Sorte mit oder ohne Fremdgen ebenso ausgeprägt sind, wie diejenige zwischen Pflanzen mit unterschiedlichen genetischen Hintergründen.

Die vorliegende Studie steht im Kontrast zu Analysen, in denen das Profil von Eiweissen (Proteom) oder Stoffwechselprodukten (Metabolom) untersucht wurde und in welchen die Unterschiede zwischen den GV Varianten und den Kontrollpflanzen kleiner waren, als diejenigen zwischen Varietäten oder Sorten.

Summary

Unintended phenotypic effects of insertion of the v*iscotoxin gene* from mistletoe and the *aminolevulinate synthase gene* from yeast into the potato (*solanum tuberosum*), cultivar Bintje have been assessed under two different growth conditions. The genetically modified plants exhibit deviations in developmental dynamics and plant senescence. Changes in plant and tuber shape, leaf metamorphosis and leaf architecture are reported. A few traits of leaf architecture reveal plant-environment interactions, and thus hint at a divergent plasticity of the transgenic variants compared to the non-modified controls.

Assessment of phenotypic differences between the cultivars Bintje, Appell and Naturella indicates that the set of morphological characters used in this study is suited to discriminate phenotypes of commercial cultivars. The results show that unintended phenotypic changes due to gene insertion are as pronounced as those due to the different genetic backgrounds.

The present study contrasts analyses of profiling studies of the proteome and metabolome of potatoes, which show that compositional differences between

genetically modified and non-modified variants are smaller than those between varieties and landraces.

Introduction

Risk assessment analysis of genetically modified (GM) crop plants includes food safety, ecological impacts and unintended effects on phenotype and substance composition (*Cellini et al.* 2004; *Haslberger* 2003; *Kuiper et al.* 2001). With the advent of profiling techniques, changes in the spectrum and abundance of transcripts (*Holtorf et al.* 2004), proteins (*Lehesranta et al.* 2005) and metabolites (*Charlton et al.* 2004; *Griffin* 2003; *Le Gall et al.* 2003; *Tretheway* 2004) have been documented. Although such alterations do not suggest a risk *per se*, they are indicative for an integral reaction of the plants upon random insertion of foreign genes and justify paying closer attention to possible adverse effects.

The same holds true for unintended phenotypic effects of genetic modifications, since the phenotype is the manifestation of the plant's developmental history. However, such changes are not as thoroughly investigated as differences in RNA, protein or metabolite composition. Two reasons account for this deficit. First, dependent on the origin of the plant material, planting time, and subtle changes in growth conditions, phenotypes exhibit a pronounced plasticity. Second, during propagation GM crop plants are selected for normal phenotypes over several generations. As a consequence, reports on unintended phenotypic effects are considered to be the exception rather than the rule. But they have repeatedly been documented: dwarfism in tomatoes with constitutive expression of a fruit synthase gene (*Fray et al.* 1995); enhanced cross pollination in *Arabidopsis* with a herbicide resistance gene (*Bergelson et al.* 1998) and reduced seed production (*Purrington and Bergelson* 1999), or multiple effects in soybeans harbouring a herbicide resistance gene (*Gertz et al.* 1999).

In this paper we investigate unintended phenotypic effects in GM potatoes, *Solanum tuberosum*, cv. Bintje, with two gene constructs for putative resistance to late blight (*Phytophtora infestans*), harbouring the *viscotoxin I gene* (Visco) from mistletoe and the *aminolevulinate synthase gene* (Ala) from yeast, respectively (*Malnoë et al.* unpublished results). The study includes the assessment of the developmental dynamics, the plant morphology, the sequence of dried leaves and the architecture of individual leaves (*Bockemühl* 1980; *Holdrege* 1996). Leaf sequences following the order along the shoot axis provide a valuable tool to assess species and plant specific characteristics of leaf formation. They give insight into the formative capacity of the plant and are a sensitive agency for monitoring alterations of the genetic set-up, as well as changes in environmental conditions. The observations are complemented with a similar analysis of two other commercial cultivars.

Material and Methods

Three commercial cultivars and two transgenic constructs were investigated under two different growth conditions. Appell and Naturella are varieties with partial natural resistance to late blight (*Hebeisen* 2000). Bintje, a widely grown cultivar, is highly sensitive to the oomycete *Phytophtora infestans* and was engineered to circumvent or at least to reduce chemical treatments by two strategies. In a first approach the *Viscotoxin I* (Visco) *gene* from mistletoe with the CaMV 35S promotor was inserted (*Malnoë et al.* unpublished results), whose product elicits fungicidal activity (*Holtorf et al.* 1998). Second, the gene for *aminolevulinate synthase* (Ala) from yeast was fused to GST-1m promotor to induce localized necrosis after spore contact (*Malnoë et al.* unpublished results).

Tubers from plants grown and stored under the same conditions were obtained from each of the constructs (two transgenic lines Ala 3, Ala 20, and three transgenic lines Visco 1, Visco 2, Visco 9) and of the non transformed commercial variety (Bintje nt). Together with two other commercial cultivars (Appell, Naturella) they were planted in pots in the greenhouse and in the field. In the field, the plants were protected against late blight infestation by chemical spraying.

Growth dynamics were documented every ten days in the greenhouse and three times in the field trial. Measurements included plant height, number of shoots, length of selected leaves, the appearance of floral buds and changes in leaf colour. Leaves from the bottom to the first inflorescence were collected, dried, arranged into sequences (*Bockemühl* 1980) and photocopied. Leaf sequences were inspected according to their overall organisation and evaluated on the basis of leaf size, the architecture of leaves and leaflets, leaf silhouettes, the number of intercalary leaflets and the length and width of terminal leaflets.

Qualitative assessment of the leaf sequences was performed, using a protocol of description and comparison, including the above criteria and the integral dynamics of the sequence in terms of progressive changes from one leaf to the next.

For quantitative analysis, leaves of two plants of each Bintje nt, Appell, Naturella, Ala 3, Visco 2 and Visco 9, and of three plants of each Ala 20 and Visco 1 were collected in the greenhouse. In the field, six plants of each Bintje nt and the transgenic lines, and two plants of Appell and Naturella were assessed. Leaves number 5 to 9 were scaled down to 35.5 percent of the original size, scanned and submitted to digital analysis (http://www.scioncorp.com/pages/scion_image_windows.htm). Parameters extracted included leaf area and leaf perimeter, length and maximal width of the terminal leaflet and the number of the intercalary leaflets (Fig. 1). In addition, derived morphological characteristics were defined: the normalized perimeter (leaf perimeter over the square root of the leaf area) allows to compare perimeter length on the basis of a unit area. The silhouette parameter delta (difference between perimeter and circumference of the best fitting ellipse) is indicative for the compactness of leaf shape. The relative length of the terminal leaflet (ratio between leaflet length and leaf length) is a measure for the leaf proportionality. The majority of these traits belong to the UPOV standards (1986) for cultivar distinction. The data were subjected to ANOVA and pairwise comparison by Tukey HSD test (SAS package, SAS Institute, Cary, NC and Statistica, StatSoft Inc.).

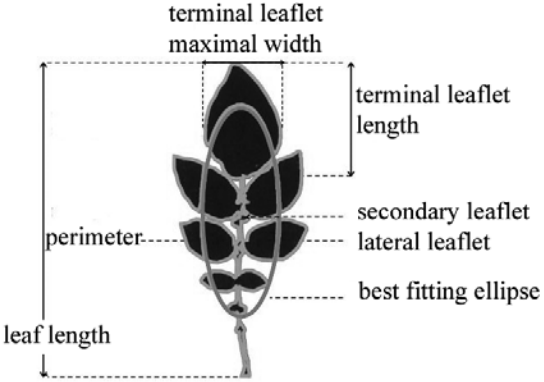

Figure 1: Leaf with indications of the parameters assessed and the best fitting ellipse

Results

Developmental dynamics and growth pattern of plants in the greenhouse

Conspicuous differences between the non-modified Bintje control plants (nt) and the varieties bearing the Ala gene construct (two independent lines Ala 3 and Ala 20) or the Visco gene construct (three independent lines Visco 1, Visco 2, Visco 9) could be observed during the entire growth period. Three weeks after planting, the GM plants showed a pronounced growth retardation (Fig. 2).

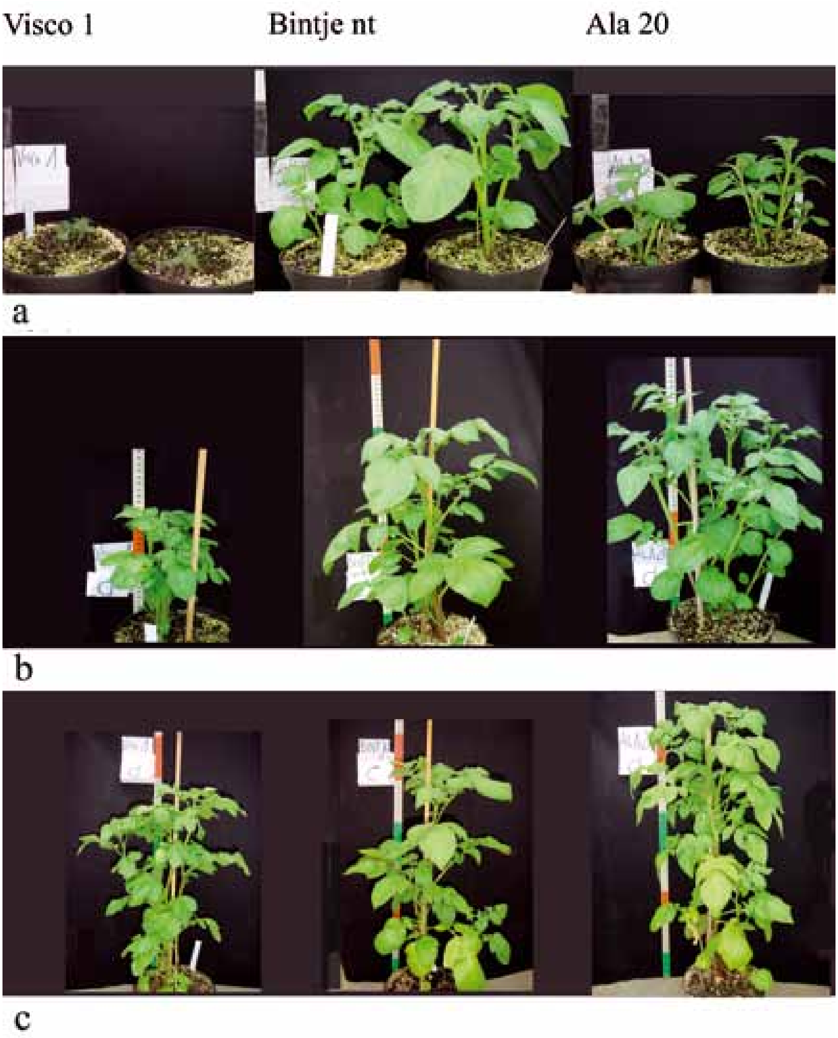

Figure 2: Developmental stages of selected plants grown in the greenhouse. a: three weeks after planting; b: five weeks after planting; c: ten weeks after planting.

The tubers from the Visco plants still had not completed the process of bulking; the plants with the Ala gene showed a slight retardation both with respect to shoot length and leaf size. Five weeks after planting, the Visco plants still lagged behind both Bintje nt and the Ala plants, but the latter had equalled the height of the non-modified control. Ten weeks after planting the Ala plants had overgrown both the controls and the plants bearing the Visco construct. Among the plants with the Visco construct, line-specific growth dynamics was observed. Visco 9 looked similar to Bintje nt (data not shown), whereas Visco 1 and Visco 2 plants grew slower during the whole vegetation period and remained smaller until harvest of the tubers. In contrast to line-specific differences in growth dynamics, the onset of flowering was two weeks earlier in all the transgenic lines compared to Bintje nt. Plant senescence exhibited line-specific progression. It was accelerated for plants of the lines Ala 20 and Visco 9 and delayed in the case of Visco 1, Visco 2, Ala 3 and Bintje nt (data not shown).

Twelve weeks after planting the mean height of the plants was 53.25±2.63 cm for Bintje nt (n=4), 51.50±5.23 cm for the Visco constructs (n=12) and 57.13±3.48 cm for the Ala constructs (n=8), respectively. The differences between Ala and Visco were significant ($P<0.05$). Fifteen weeks after planting, both constructs exhibited significantly reduced tuber weight compared to Bintje nt. It read 218.4±14.9 g per plant (n=4) for Bintje nt, 160.3±29.1 g per plant for the Visco constructs (n=12, $P<0.005$) and 194.7±17.0 g per plant for the Ala constructs (n=8, $P<0.05$). Plants from the Visco 1 and 2 lines showed conspicuously elongated tubers, whereas tuber shape of Ala 3 and Ala 20, Visco 9 and Bintje nt plants could not be distinguished (Fig. 3).

Figure 3: Tubers from four plants per variant collected in the greenhouse. a: Bintje nt, b: Ala 3, c: Ala 20, d: Visco 1, e: Visco 2, f: Visco 9

Visual examination of leaf sequences from plants grown in the greenhouse

In a first step, leaf morphology was qualitatively assessed. Fig. 4 shows selected leaf sequences of GM and non-modified plants grown in the greenhouse. We observed a variety of alterations between Bintje nt and the transgenic lines. A prominent feature of the leaf sequences as a whole is the pattern of leaf size expansion and reduction. Leaves of Bintje nt reach their maximal size gradually at leaf number six or seven and reduce leaf area and petiole length towards the formation of the inflorescence. In contrast, leaves from the plants bearing transgenic constructs lack the same degree of regularity, most obviously depicted in the Visco lines.

Both Ala and Visco constructs exhibit maximal leaf size in positions different from Bintje nt and show disrupted dynamics of size increase and reduction (Visco 1 is an obvious example). Another characteristic is the change in shape from leaf to leaf. A steady addition of leaflets along the sequence up to the climax leaf can be observed for the controls, whereas slow progression (Ala 3) and sudden increase in leaf area and number of leaflets (Ala 3, leaf 5 to leaf 6) are a frequent feature of the Ala-constructs.

The leaf architecture of transgenic plants is affected as well. Leaves from all the Visco lines are more compact with rounded terminal leaflets and show a reduced length of leaflet petioles. The leaf sequences of the Ala lines are similar to those of Bintje nt but exhibit a higher number of intercalary leaflets, which allow for a clear distinction with respect to both, Bintje nt plants and Visco lines. Ala 20 shows more strongly pointed leaves than the controls. Finally, the terminal leaflets of leaves close to the maximal size in the sequence are always larger in the case of Bintje nt than those of the Ala and – even more pronounced – Visco plants. Both, Ala and Visco constructs exhibit a clear reduction in leaf size during the early phases of leaf formation.

Leaves from plants grown in the field were processed to sequences and revealed qualitative characteristics similar to those from the greenhouse (data not shown).

Comparison of Bintje nt with two other commercial cultivars shows an increased number of leaves with a more compact leaf shape in the case of Naturella, whereas the leaves of Appell are larger, the leaflets more pointed and the overall silhouette more open with longer leaflet petioles. Both cultivars display a regularity of leaf metamorphosis similar to Bintje nt and exhibit a discernible cultivar specific character.

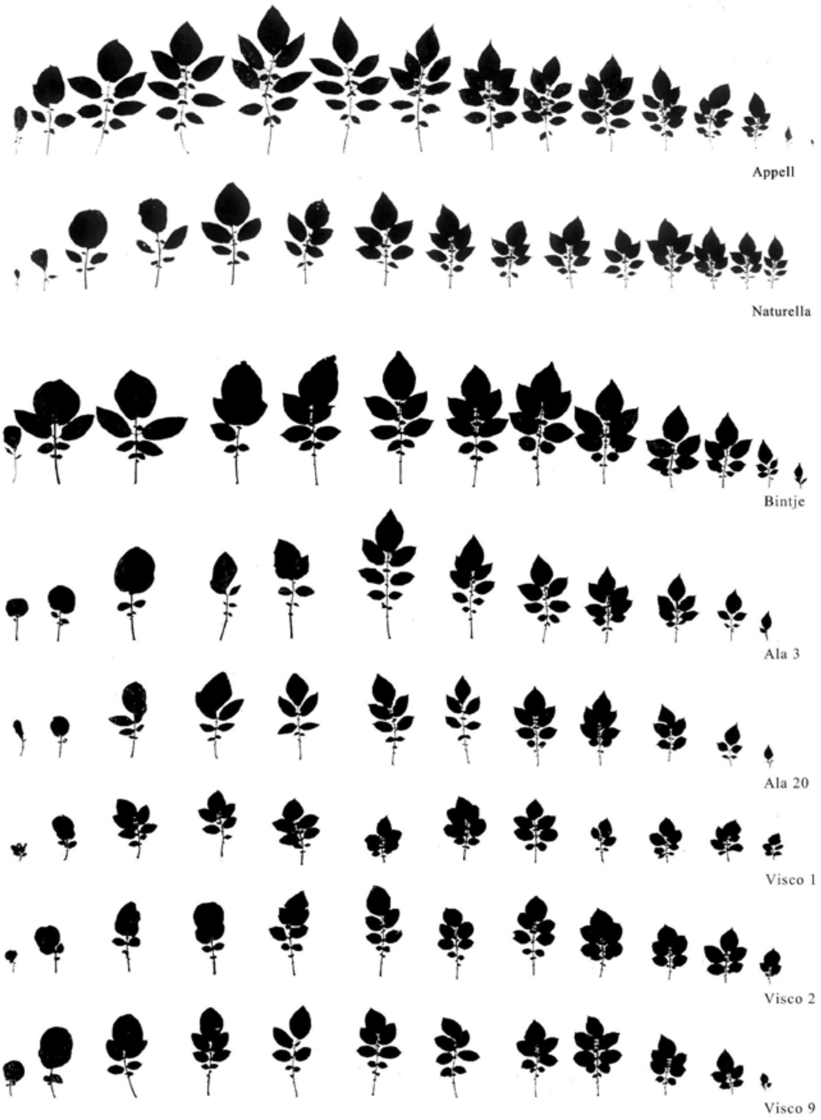

Figure 4: Representative leaf sequences from plants grown in the greenhouse; orientation from left to right corresponds to the alignment along the shoot axis from bottom to the first inflorescence. The first three rows exhibit sequences of the commercial cultivars Appell, Naturella, Bintje; rows Ala 3 and Ala 20 show sequences of two independent lines with the Ala construct; Visco 1, Visco 2 and Visco 9 represent three independent lines harboring the Visco construct.

Quantitative analysis of leaf morphology including plants from the greenhouse and the field

For quantitative analysis, leaves five to nine were pooled from all variants in both locations. Mean values and standard errors were calculated for each data set of area, perimeter, delta, normalized perimeter, leaf length, length of the terminal leaflet, the relative length of the terminal leaflet, the maximal width of the terminal leaflet and the numbers of intercalary leaflets (Table 1), and subjected to ANOVA analysis (Table 2). The significant differences can be classified in four groups: construct-specific effects, line-specific effects, indicative for position sensitive integration of the transgene into the genome, location effects (different growth conditions), and location-construct interactions.

loc	cultivar	#	area [cm]		perimeter [cm]		Delta [cm]		norm perim		leaf length [cm]		term leafl [cm]		rel length term leaflet		max w idth[cm]		# sec. leaflet	
field	Bintje	30	**10,41**	0,75	**32,06**	1,33	**20,23**	1,19	**10,26**	0,37	**18,18**	0,57	**6,96**	0,18	**0,391**	0,013	**4,88**	0,16	**5,63**	0,52
"	Ala 3	30	**11,66**	0,71	**31,89**	1,45	**19,42**	1,25	**9,45**	0,30	**18,37**	0,52	**7,11**	0,17	**0,392**	0,011	**4,89**	0,15	**10,03**	0,77
"	Ala 20	30	**10,44**	0,57	**33,64**	1,69	**21,80**	1,47	**10,46**	0,37	**17,95**	0,57	**6,29**	0,21	**0,355**	0,011	**4,18**	0,14	**12,63**	0,83
"	Visco 1	30	**9,57**	0,51	**29,48**	1,01	**17,94**	0,88	**9,71**	0,34	**17,50**	0,44	**5,31**	0,12	**0,307**	0,008	**3,71**	0,11	**8,57**	0,34
"	Visco 2	30	**10,52**	0,42	**27,07**	1,07	**15,14**	0,98	**8,40**	0,28	**16,73**	0,40	**5,30**	0,13	**0,319**	0,008	**4,11**	0,08	**11,03**	0,53
"	Visco 9	30	**10,12**	0,53	**23,40**	0,95	**11,80**	0,74	**7,44**	0,21	**15,98**	0,46	**5,85**	0,15	**0,370**	0,009	**3,92**	0,13	**9,17**	0,54
"	Appell	30	**10,88**	0,76	**43,42**	1,38	**31,29**	1,19	**13,52**	0,33	**19,93**	0,90	**6,94**	0,30	**0,351**	0,007	**4,08**	0,18	**8,83**	0,54
"	Naturella	30	**9,29**	0,58	**30,40**	1,21	**19,35**	1,02	**10,19**	0,31	**15,51**	0,40	**6,46**	0,16	**0,419**	0,008	**4,81**	0,14	**7,43**	0,65
green	Bintje	10	**11,31**	0,93	**33,59**	2,10	**20,93**	1,85	**10,08**	0,50	**20,89**	0,86	**8,66**	0,69	**0,412**	0,023	**5,77**	0,52	**9,30**	1,07
"	Ala 3	10	**8,79**	0,57	**34,30**	2,30	**23,12**	1,94	**11,52**	0,48	**19,33**	0,97	**7,21**	0,30	**0,376**	0,012	**4,85**	0,30	**10,80**	1,29
"	Ala 20	15	**6,05**	0,29	**27,92**	1,30	**18,65**	1,23	**11,43**	0,54	**16,19**	0,42	**6,32**	0,23	**0,391**	0,012	**4,12**	0,25	**10,47**	0,93
"	Visco 1	15	**6,31**	0,41	**22,60**	1,51	**13,36**	1,32	**9,03**	0,53	**14,01**	0,74	**4,96**	0,19	**0,361**	0,013	**3,65**	0,13	**8,07**	0,80
"	Visco 2	10	**5,11**	0,23	**19,75**	1,35	**11,14**	1,17	**8,70**	0,46	**13,46**	0,53	**5,03**	0,25	**0,374**	0,013	**3,99**	0,24	**9,80**	1,04
"	Visco 9	10	**5,35**	0,33	**22,62**	1,22	**13,99**	1,07	**9,82**	0,43	**14,35**	0,52	**5,10**	0,18	**0,357**	0,010	**3,24**	0,18	**8,10**	0,55
"	Appell	10	**10,42**	1,51	**41,93**	4,07	**29,86**	3,33	**13,25**	0,64	**21,01**	1,76	**7,52**	0,58	**0,361**	0,007	**4,16**	0,31	**10,20**	0,39
"	Naturella	10	**9,20**	0,61	**34,22**	2,33	**22,83**	2,05	**11,26**	0,50	**18,59**	0,88	**7,35**	0,42	**0,399**	0,022	**5,21**	0,26	**10,80**	1,40

Table 1: Average values (in bold) and standard errors of parameters from leaves number five to nine
Loc: growth conditions; #: total number of leaves analysed; delta: silhouette parameter (see text); norm perim: normalized perimeter; length term. leafl: length of terminal leaflet; rel length term leaflet: relative length of terminal leaflet; max width: maximal width of terminal leaflet; # sec. leaflet: number of secondary leaflets.

With exception of the numbers of leaves, which exhibit location dependent differences only (data not shown), all analysed parameters show construct specific effects. In the first group of parameters – leaf perimeter and silhouette parameter delta – variations exhibit construct effects, exclusively. It should be noted that both, Ala 3 and Bintje nt behave in a similar way, i.e. slightly increased values in the greenhouse, whereas all the other transgenic variants reveal an opposite reaction.

		area		perimeter		delta		norm perim.		leaf length		length term. leaflet		rel length term. leafl.		width term. leaflet		number of sec leaflet	
	df	F value	Pr > F																
location	1	15,78	***	2,06		0,22		5,50	*	0,61		2,09		4,28	*	0,33		0,23	
construct	2	5,30	**	16,28	****	17,81	****	16,44	****	12,79	****	51,37	****	7,38	**	23,32	****	9,49	****
line (construct)	3	1,03		0,97		0,97		1,35		1,04		2,76		1,10		3,19	*	2,09	
location*construct	2	3,83	*	1,43		0,63		1,84		4,57	*	7,49	***	0,44		2,98		4,62	*
location*line	3	0,48		1,66		2,22		3,38	*	0,79		0,19		2,10		0,47		0,86	

Table 2: Analysis of variation (ANOVA) of all traits from cultivars Bintje, Ala 3, 20, Visco 1, 2, 9, as listed in Table 1
Location: Growth conditions (greenhouse and field); line: comparisons of Ala 3 & 20, and Visco 1, 2, 9, respectively; location*construct: analysis of interactions between locations and constructs; location*line: comparison of interactions between locations and lines; delta: silhouette parameter; norm perim: normalised perimeter; length term. leafl: length of terminal leaflet; rel length term leaflet: relative length of terminal leaflet; width term. leaflet: width of terminal leaflet; number of sec. leaflet: number of secondary leaflets; df: degree of freedom; Asterisks indicate the level of significance: *): $P<0.05$; **): $P<0.01$; ***): $P<0.005$; ****): $P<0.001$

Closer inspection by pairwise comparison (Table 3) between Bintje nt, and the transgenic cultivars and between Visco versus Ala constructs respectively, reveals significant variations to be the result of pronounced differences of the Visco plants. The average values of the perimeter and of the silhouette parameter delta confirms the results of the t-test and is in full agreement with the visual investigation, which shows Bintje nt and Ala to be more similar to each other than to the plants of the Visco construct (Table 1).

	area	perimeter	Delta	norm perim	leaf length	length term leafl.	rel length term leafl.	width term leafl.	number of sec. leaflet
Ala vs. Visco		*****	*****	*****	*****	*****	*****	*****	***
Bintje vs. Ala						*		***	*****
Bintje vs. Visco	*	*****	*****	*****	*****	*****	*****	*****	***
Bintje vs. App		*****	*****	*****				*****	**
Bintje vs. Nat					***	*	*		
App vs. Nat		*****	*****	*****	*****			*****	

Table 3: Pairwise comparisons (Tukey-Test) of gm constructs, the non-modified control Bintje nt and the two commercial cultivars Appell and Naturella; abbreviations as in Table 2
Asterisks indicate the level of significance *): $P<0.05$; **): $P<0.01$; ***): $P<0.005$; ****): $P<0.001$; *****): $P<0.0005$

A second group of parameters – normalized perimeter and relative length of the terminal leaflet – show both construct and moderate location effects (Table 2). Pairwise comparison (Table 3) indicates these effects again to be the result of the variation of Visco constructs, exclusively. Indeed, the leaves of the Visco plants exhibit reduced proportions due to short terminal leaflets. The reduced normalized perimeter is indicative for the compact shape of the leaves. The differences between the two different growth conditions are relatively small ($p<0.05$).

A third group of parameters – leaf area, leaf length, length of the terminal leaflet and the number of intercalary leaflets – exhibit pronounced construct-specific effects and moderate interactions between genetic background (construct) and growth conditions (Table 2). Pairwise comparisons reveal significant changes in the length of the terminal leaflets and the number of intercalary leaflets between Bintje nt, Ala and Visco, whereas the leaf length allows for discrimination between Bintje nt and Visco, as well as between Ala and Visco plants, respectively (Table 3). Leaf size (area) allows for discriminating Bintje nt and Visco plants, exclusively (Table 3). Fig. 5 depicts the range and dynamics of plant environment interactions for the four parameters. In all cases, the transgenic variants show polar reactions towards changes of two growth conditions, i.e. larger values in the field than in the greenhouse, whereas the opposite is true for Bintje nt.

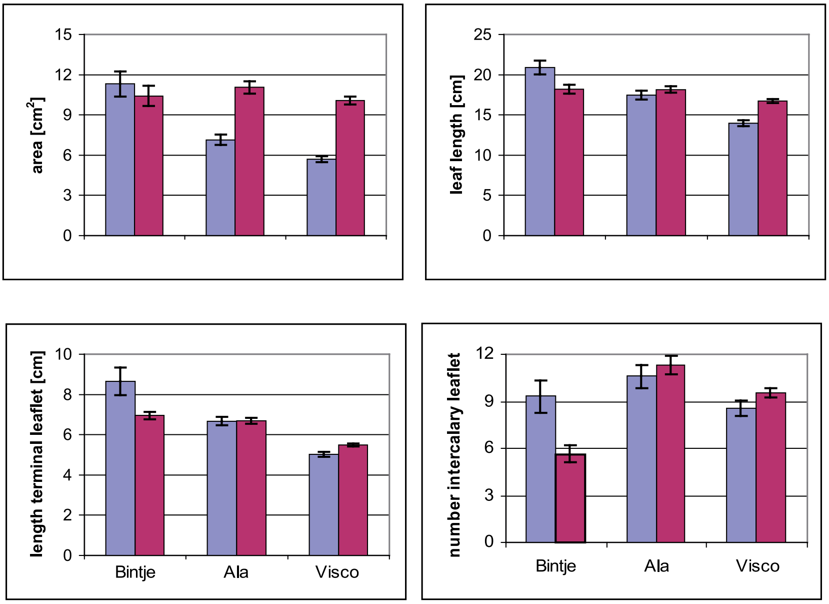

Figure 5: Location-construct-interactions of four parameters. Means and standard errors (bars and whiskers) from tables 1 & 2; blue: plants from the greenhouse; magenta: plants from the field.

Finally, the ANOVA analysis reveals construct-specific and line specific-effects for one trait (maximal width of terminal leaflets) and interactions between line and location for another (normalized perimeter). Inspection of average values and standard errors (Table 1) and pairwise comparisons (data not shown) reveal line-specific effects for the Ala constructs and line-location interaction for Visco 9, respectively.

In conclusion, pairwise comparisons on the construct level indicate pronounced differences between Bintje nt and Visco, Visco and Ala and a reduced number of differences between Bintje nt and Ala.

In order to estimate the significance of the changes observed between Bintje nt and the GM variants, leaves of the two commercial cultivars Appell and Naturella were examined and analysed (Fig. 4 and Table 1). The leaf sequences of the three cultivars can easily be distinguished. If different leaf size is neglected – as can be done since the pairwise comparison does not result in significant changes – the sequences of Bintje nt and Naturella show a stronger resemblance to each other than either Bintje nt to Appell or Appell to Naturella. The pairwise comparison of the morphological parameters (Table 3) exhibits five significant scores between Bintje and Appell, three differences between Bintje and Naturella and five significant changes between Appell and Naturella, respectively. Comparison of these results with the score of changes between Bintje nt and the two transgenic constructs, and between the Visco and Ala plants, shows that the morphological dissimilarity between the three commercial cultivars is of the same order of magnitude as that between Bintje nt and its genetically modified sister plants (*Richter* 2002).

Discussion

We have documented multiple unintended phenotypic effects after gene insertion in potatoes. They include developmental dynamics, differences in growth and senescence patterns, plant shape, leaf metamorphosis, leaf architecture and tuber weight and shape. These findings are in agreement with early work on genetically modified potatoes, where observations of altered plant height, tuber weight and number of potatoes bearing a GUS gene (*Dale and Mc Partlan* 1992) and of altered leaf morphology after insertion of a kanamycin resistance gene (*Conner et al.* 1994), respectively, have been reported.

The analysis of morphological traits allowed for both an unequivocal discrimination of transgenic cultivars from their non-modified controls, and for distinction between the two different constructs Ala and Visco. Eight out of nine traits of leaf architecture, plant height and tuber weight showed significant variations between Visco and Ala constructs. Three out of nine parameters significantly varied between Ala and Bintje nt controls, and all the nine leaf parameters scored significant alterations between Visco and Bintje nt. Since the different variants could be grouped unambiguously irrespective of growth conditions, the morphological traits are robust. The changes in the developmental dynamics and leaf architecture correlate to a considerable extent with the different genetic constructs inserted.

Recent studies on tuber proteomes and metabolomes may challenge our findings. Minor differences in protein composition between tubers of genetically modified plants and controls compared to alterations between varieties and landraces have been reported (*Lehesranta et al. 2005*) and have provided evidence for substantial equivalence (*Catchpole et al.* 2005). In addition, changes in metabolite composition could be attributed to regeneration processes and/or insertion of vector-only constructs (*Shepherd et al.* 2006). The lack of control experiments with vector-only constructs and plant regeneration without genetic transformation sounds a note of caution with respect to the effects described in this study. However, there is good circumstantial evidence for the correlation of phenotypic effects with a particular gene construct inserted, and against somaclonal variation, for two reasons. First, all the traits assessed with exception of the leaf area discriminate unambiguously between Ala and Visco constructs. Second, without exception, the differences are always more pronounced between constructs than among lines within constructs, and thus manifest specific responses of the plants to the transgenes inserted. Random genetic events during the regeneration processes would result in less concerted phenotypes. Similar conclusions have been drawn from a study using metabolic profiling methods on potato tubers (*Roessner et al.* 2001), where cluster analysis and principal component analysis allowed for discrimination of different transgenic constructs. Moreover, profiles of wild-type samples and, in some cases, all the different lines of a single construct could be grouped in a single cluster in each case.

However, for the time being no causal explanation for the phenotypic changes between Bintje nt and the genetically modified variants can be offered. Molecular analysis showed that the site of the gene insertion varies between the transformants, as well as the copy number of inserted sequences, and the comparison of RNA and protein expression patterns of the transgenes revealed conspicuous variations among individual plants, even within the same replication unit (*Malnoë et al.* unpublished results).

The plant environment interactions deserve particular attention. The four traits reveal a divergent plasticity after genetic modification. Predictions of genetically modified plant behaviour under different growth conditions may not easily be extracted from knowledge of non-modified cultivars and hence should be performed with caution.

Our results indicate that the genetic modification of the cultivar Bintje by Ala and Visco gene-constructs results in unintended phenotypic changes that are comparable to or even more pronounced than the differences between commercial cultivars. The GM variants are morphologically not equivalent and could be denoted as new varieties.

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