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Salinity Response of Transgenic Potato Genotypes Expressing the Oxalate Oxidase Gene

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Abstract: In vitro and in vivo responses of transgenic and non-transgenic potato (*Solanum tuberosum* L.) genotypes to salinity were investigated. A gene responsible for the production of oxalate oxidase enzyme, whose expression has been associated with plant stress tolerance, was used. Transgenic potato plants expressing the oxalate oxidase enzyme were produced using *Agrobacterium*-mediated transformation. The transgenic nature of the plants was verified by their antibiotic (kanamycin) resistance and then in vitro enzyme assays. Four independent transgenic genotypes obtained from 2 cultivars, Maris Bard and Desiree, were tested at 5 levels of NaCl (0%, 0.25%, 0.50%, 0.75% and 1.00% w/v) in vitro. The same genotypes were also tested at 3 levels of NaCl (0%, 0.50% and 0.75% w/v) under glasshouse conditions (in vivo). The results revealed that the growth of all genotypes was significantly inhibited by increasing salt treatments under both in vitro and in vivo conditions. Transgenic genotypes showed a relatively higher salinity tolerance than the non-transgenic genotypes in vitro. On the other hand, in the glasshouse, the results were less consistent but some transgenic genotypes showed superior tuber yield characteristics to the non-transgenics under salt stress.

Key Words: Enzyme, Oxalate oxidase, salinity, *Solanum tuberosum*

Oksalat Oksidaz Geni Taşıyan Transgenik Patates Genotiplerinin Tuzluluğa Yanıtları

Özet: Bu çalışmada, transgenik ve transgenik olmayan patates (*Solanum tuberosum* L.) genotiplerinin in vitro ve in vivo koşullarda tuzluluğa karşı yanıtları araştırılmıştır. Bitkide sentezlenmesi stres ile ilgili olan oksalat oksidaz enzimi üretiminden sorumlu olan oksalat oksidaz geni kullanılmıştır. Oksalat oksidaz enzimi üreten transgenik patates genotipleri *Agrobacterium*-gen transferi metodu ile elde edilmiştir. Bitkilerin transgenik olup olmadıkları antibiyotiğe (kanamycin) dayanıklılıkları ve daha sonra enzim testleri ile belirlenmiştir. İki patates çeşidi (Maris Bard ve Desiree) ve bunlardan elde edilen dört transgenik genotip, in vitro koşullarda beş farklı NaCl düzeyinde (%0, %0.25, %0.50, %0.75 ve %1.00) test edilmişlerdir. Aynı genotipler üç farklı NaCl düzeyinde (%0, %0.50 ve %0.75) in vivo (sera) koşullarda da test edilmiştir. Bulgular göstermiştir ki; hem in vitro hem de in vivo koşullarda artan tuz uygulamaları ile tüm genotiplerin gelişimleri önemli düzeyde azalmıştır ve in vitro koşullarda transgenik genotipler, transgenik olmayan genotiplere göre tuzluluğa daha iyi dayanım göstermiştir. Buna karşılık serada, elde edilen sonuçlar biraz farklı olmuştur. Fakat, yine tuz stresi altında, bazı transgenik genotipler yumru ile ilgili karakterler bakımından daha iyi performans göstermişlerdir.

Anahtar Sözcükler: Enzim, oksalat oksidaz, tuzluluk, *Solanum tuberosum*

Introduction

Over the course of history, salinity has been a recurrent environmental problem in agriculture (Jacobson and Adams, 1968; McWilliam, 1986). It is now an increasing global problem as all continents have salt-affected soils. Today the estimated total area of the salt-affected soils in the world is around 954 x 10^6 ha (Szabolcs, 1994).

In the past, conventional breeding methods developed various crop species with improved environmental stress tolerance (Cullins, 1991). However, breeding progress and crop improvement are time consuming and labour intensive. Novel breeding methods such as somaclonal variation, interspecific hybridisation, somatic hybridisation and gene transformation provide potential for improvement in important characters required in many cultivated varieties. Today, these unconventional breeding methods combined with conventional methods are used as powerful tools in plant breeding programmes by an enormous number of researchers.

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Recently, much research has focused on stress-induced gene expression, which is thought to be an important indicator for the discovery of new genes responding to various plant stresses (Wang et al., 2003). The sequence for the gene for oxalate oxidase, an enzyme which catabolises oxalic acid, has been found to possess close homology to the wheat protein, germin (Lane et al., 1993). In another investigation, 2 polypeptides, Gs1 and Gs2, induced during salt stress of a salt-tolerant barley cultivar, were identified as germin-like proteins by amino acid sequencing (Hurkman et al., 1991), suggesting that members of the germin family may also have a protective function during osmotic stress. In addition, wheat germins are thought to have a role in cell wall expansion in cereals (Jaikaran et al., 1990) as Gs1 and Gs2 were found in a cell wall fraction from barley roots (Hurkman, 1992). Furthermore, studies of changes of protein synthesis during salt stress of the facultative halophyte *Mesembryanthemum crystallinum* (Michalowski and Bohnert, 1992) led to the cloning of a cDNA induced by salt stress that has a close sequence similarity to germin. Recently, antibody-based studies have confirmed that some of these different germin homologues have oxalate oxidase activity (Dumas et al., 1995). Relatively high specific gene expression found in the vascular bundles might have an effect on osmotic or salt stress (Hurkman and Tanaka, 1992). Hurkman and Tanaka (1992) also speculated that hydrogen peroxide (H_2O_2) formation from the conversion of oxalate to CO_2 and H_2O_2 by oxalate oxidase enzyme during salt stress may be a signal of salt stress or generate other signals that induce genes encoding proteins involved in salt tolerance in barley. Finally, it is thought that the oxalate oxidase transgene in potato, which has not been found to normally express the oxalate oxidase enzyme, may play a role in salt and osmotic stress. Therefore, the objectives of this study were to i) introduce the oxalate oxidase gene, ii) screen oxalate oxidase enzyme activity in transformed plants and iii) investigate the potential of transgenic potato genotypes for salinity tolerance under in vitro and in vivo conditions.

Materials and Methods

Plant Material

Two tetraploid potato cultivars, Maris Bard (MB) and Desiree (DS), were obtained as plantlets from the collection in the Department of Agricultural Botany, the University of Reading, UK. Stock shoot cultures were maintained by culturing single node stem sections on MS basal medium (Murashige and Skoog, 1962). The growth room was illuminated with cool white fluorescent tubes at 23 ± 2 °C with a 16 h photoperiod and a photosynthetic photon flux density (PPFD) of 95 µmol m^{-2} s^{-1}.

Transformation Procedure

Agrobacterium tumefaciens strain LBA4404 was used for genetic transformation. Oxalate oxidase transformation vector pSR2 contained a full-length oxalate oxidase clone (710 bp) constructed from a barley root cDNA library with synthetic oligonucleotides. This binary vector (pBin19) also comprises a neomycin resistance gene (*nptII*) and constitutive CaMV35S promoter. This vector was introduced into *A. tumefaciens* by Zeneca Seeds (Thompson et al., 1995). The *Agrobacterium* strain LBA4404, containing the binary vector, was maintained on Luria Broth (LB) (Miller, 1972) medium supplemented with 50 mg l^{-1} of kanamycin, 500 mg l^{-1} of streptomycin and 100 mg l^{-1} of rifampicin. Stem segments from 4-week-old cultures were inoculated with an *Agrobacterium* suspension (OD_{595} = 0.1) for 15 min. Individual shoots from *Agrobacterium*-infected stem segments were transferred onto MS with 100 mg l^{-1} of kanamycin and 400 mg l^{-1} of cefotaxime for rooting and selection of transformed shoots. Afterwards, all plantlets which gave a positive response to the rooting culture were screened using the oxalate oxidase staining test as a second selection procedure.

Oxalate Oxidase Activity Determination

For the determination of oxalate oxidase activity, single tissues from plantlets grown in media with kanamycin and the control were used. Leaf discs (approximately 0.03 g) from independent shoots of *Agrobacterium*-infected stem segments grown on the kanamycin selective media and non-infected controls were ground in Eppendorf tubes containing 300 µl of distilled water, a small amount of sand and polyvinyl pyrrolidone (PVP) using a tissue grinder. The samples were then maintained on ice. For the stain solution, a succinate buffer was prepared by dissolving 1.18 g of succinate acid in 90 ml of H_2O and this was adjusted to pH 3.5 with KOH. Then 0.09 g of oxalic acid was dissolved in the succinate buffer. Horseradish peroxidase (EC.1.11.1.7, Type VI - A) was prepared in advance by

adding 5 ml of distilled water to 250 mg of the enzyme and this was stored at -20 °C. A total of 160 µl l^{-1} of horseradish peroxidase was added to 1 mM oxalic acid dissolved in the succinate buffer. After that, 4-chloronaphthol (200 mg l^{-1}) dissolved in methanol was added dropwise while stirring and heating (30-40 °C) the solution. Then 50 ml of leaf extract for each sample was spotted onto a nitrocellulose membrane, and allowed to air dry for 15 min. They were subsequently bathed in 100 ml of freshly prepared activity stain in a plastic box. After about 30 min, the development of purple coloration in samples from transgenic plants was observed on the reverse of the membrane. In the control (non-transgenic plant) and escapes, no purple coloration was observed. As a result, 4 transgenic potato lines were produced from cultivars Maris Bard and Desiree.

Salt Tolerance Screening

In the second part of the research, the 2 potato cultivars, MB and DS, and their 4 transgenic genotypes (MB_{T1}, MB_{T2}, MB_{T3} and DS_{T1}) were tested on MS basal medium supplemented with 5 levels of NaCl (0%, 0.25%, 0.50%, 0.75% and 1.00% w/v). Each treatment consisted of 15 replicate test tubes, and each tube was inoculated with 1 axillary bud node. The rest of the procedure was the same as for plant material maintenance in vitro. After 4 weeks, the parameters measured were shoot length (SL), number of leaves per plant (LNO), number of usable nodes (NNO) (stem section, 10 ± 2 mm long with a central node suitable for sub-culturing in vitro), number of aerial roots (ART) (the roots on the shoot above the medium), number of true roots (TRT) (the roots initiated from stem explant in the medium), fresh weight of shoot (SFW), dry weight of shoot (SDW) and root score (RSC) based on a visual assessment, recorded on a 1 to 9 scale where 1 was no root and 9 a very well-established root system, considering number of roots, branches, root thickness and general appearance.

The same cultivars (MB and DS) and transgenic genotypes (M_{T1}, M_{T2}, M_{T3} and DS_{T1}) were also tested for salinity in a controlled glasshouse. After transferring plantlets from in vitro to in vivo, surviving healthy plants were transferred into large pots (15.24 cm) filled in with John Innes No. 1 compost (John Innes Centre, UK) in the glasshouse and kept for 4 days without any application of salt for the selection of healthy plants to start the experiment. Each treatment consisted of 5 pots. For 4 days, the plants were watered only with deionised water. After this procedure, they were watered with solutions containing deionised water plus salt concentrations (0%, 0.50% and 0.75% w/v NaCl) determined according to the amount of water held by soil against gravity (Bilski et al., 1988). Each NaCl solution was divided by 4 and added to each pot every other day to avoid sudden shock to the plants. After 8 days, all applications of salt reached their final concentrations. All treatments were subsequently supplied with deionised water to keep the moisture of the soil at about the field capacity. All measurements were taken after 100 days after the beginning of NaCl application. The characters measured were number of leaves (LNO), number of stems (SNO), plant height (PH), shoot fresh and dry weight (SFW and SDW), root fresh and dry weight (RFW and RDW), tuber fresh and dry weight (TFW and TDW) and number of tubers (TNO).

Statistical Analysis

The experimental design for all experiments was a randomised block factorial design. After preliminary analysis of the data, some characters showed skewed and non-normal distribution, in which case square root or logarithmic transformation was used as appropriate to restore normality or to improve the homogeneity of variance (Snedecor and Cochran, 1980). Analyses were carried out for these characters on transformed data. The statistical analyses were carried out using the SAS computer package (SAS, 1985).

Results and Discussion

The first part of the study included production of transgenic plants using *Agrobacterium*-mediated transformation methods. After inoculation of stem segments with *Agrobacterium*, they were transferred onto MS medium supplemented with antibiotics. However, some shoots obtained from these stem segments did not show resistance to kanamycin in the rooting culture (Figure 1). Shoots often show less sensitivity to kanamycin than roots due to a lack of direct exposure to the chemical. For this reason, the non-transformed shoots in Figure 1 referred to as 'escape' may sometimes survive the initial selection process (Horsch et al., 1985). Afterwards, all rooted plantlets had oxalate oxidase enzyme activity. Representative data are given in Figure 2. As a result, 4 transgenic potato

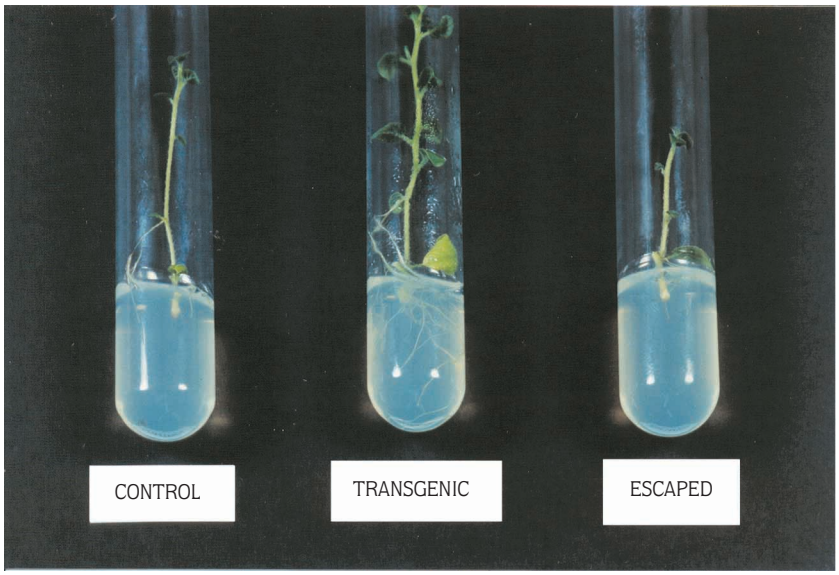

Figure 1. The effect of kanamycin (100 mg l^{-1}) on escapes (right), transgenic (centre) and non-transgenic control material (left).

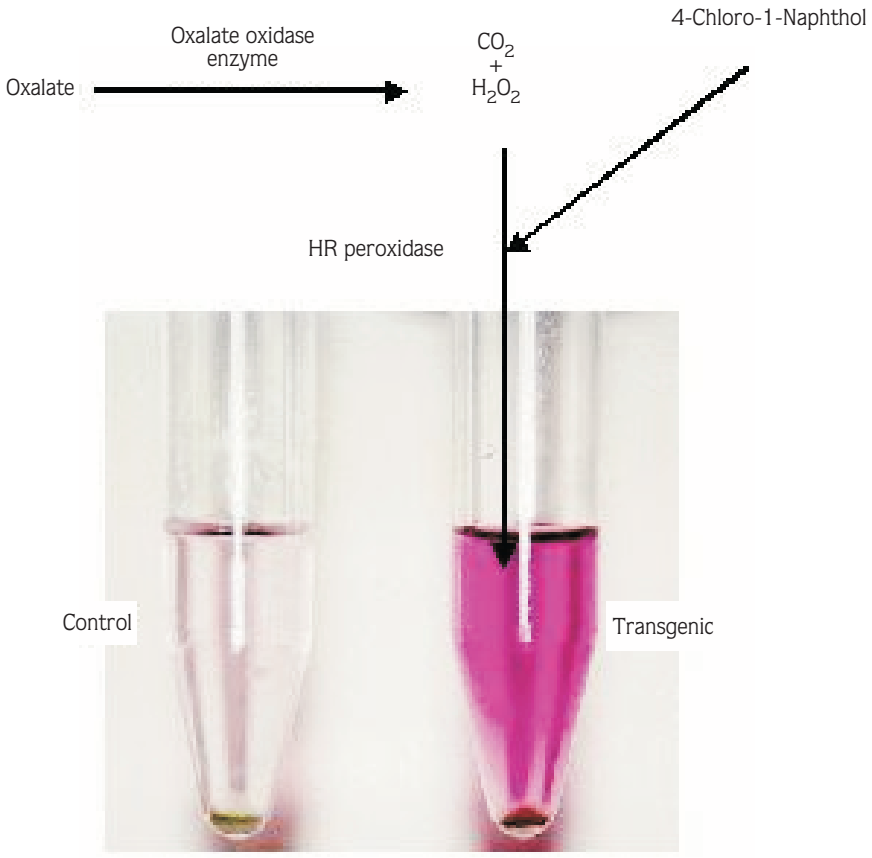

Figure 2. Oxalate oxidase enzyme activity in transgenic and control genotype.

lines were produced from cultivars Maris Bard and Desiree.

In the second part of the study, the responses of the transgenic and non-transgenic genotypes to salinity under in vitro and in vivo conditions were evaluated.

In vitro (Glasshouse) Tests

The analyses of results for the characters measured clearly indicated that salinity had a highly significant effect on all the potato genotypes grown on MS medium supplemented with a range of NaCl concentrations. According to the orthogonal analysis of genotypes, the differences between MB and its transgenic lines for LNO, SFW and SDW were also significant. On the other hand, the differences between DS and DS_{T1} were only significant for RNO and RSC (Table 1).

In order to detect the differences between the genotypes in terms of salinity tolerance, the relationships between the salinity and genotypes for each character were assessed using regression lines. The slopes of the regression lines for the genotypes indicated their performances against salinity for an individual character. The performances of all genotypes with respect to salinity in all characters are summarised in Figure 3a (MB and its transgenics) and Figure 3b (DS and DS_{T1}) after 2-step data transformation was carried out over slope values. In the first step, slope values for each of the 6 genotypes in each character were converted to reciprocal slope values (RSV) for ease of interpretation. In the second step, relative reciprocal slope values ($RRSV_T$) (%) for the transgenic genotypes were calculated using the following formula:

$$RRSV_T\ (\%) = (RSV_T * 100) / RSV_C$$

$RRSV_T$: Relative reciprocal slope value of a transgenic genotype.

RSV_T: Reciprocal slope value of the transgenic genotype.

RSV_C: Reciprocal slope value of a non-transgenic genotype (cultivar MB or DS).

RSV_C of MB and DS were considered to be 100% on Y the axis in Figures 3a and 3b, respectively.

The relative reciprocal slope values allow for the comparison of genotypes and different degrees of effect of salinity on different characters. The relative reciprocal slope values were used here, because the data for some characters showed non-normal distribution and so a square root or logarithmic transformation was used as appropriate to restore normality. Therefore, absolute values would not reflect the actual performance of the genotypes in terms of comparison of the characters. The same process was carried out for the other genotypes, DS and DS_{T1}, in Figure 3b. As a result, the values of

Table 1. Analysis of variance for some characters measured in vitro (SL: Shoot length, LNO: Number of leaves, RSC: Root score and SFW: Shoot fresh weight).

		Mean square			
Sources	d.f.	SL[1]	LNO[1]	RSC	SFW[1]
Genotype	5	18.75***	2.87***	11.76**	142.36***
MB vs MB_{T1}, MB_{T2} & MB_{T3}	1	3.01	13.75***	0.11	161.42***
MB_{T1} vs MB_{T2} & MB_{T3}	1	0.05	0.58	6.01	56.32*
MB_{T2} vs MB_{T3}	1	9.28***	0.03	10.67	1.94
DS vs DS_{T1}	1	0.01	0.99	34.56***	1.36
Salinity	4	313.34***	25.69***	581.71***	1687.40***
Genotype*Salinity	20	9.44***	0.97***	9.92***	52.68***
Replication	14	1.69	0.53	5.21	21.02
Error	406	1.33	0.31	2.95	12.65

[1] Data + 1 were transformed to square root.
*, **, ***, Significant at 0.05, 0.01, 0.001 level, respectively.

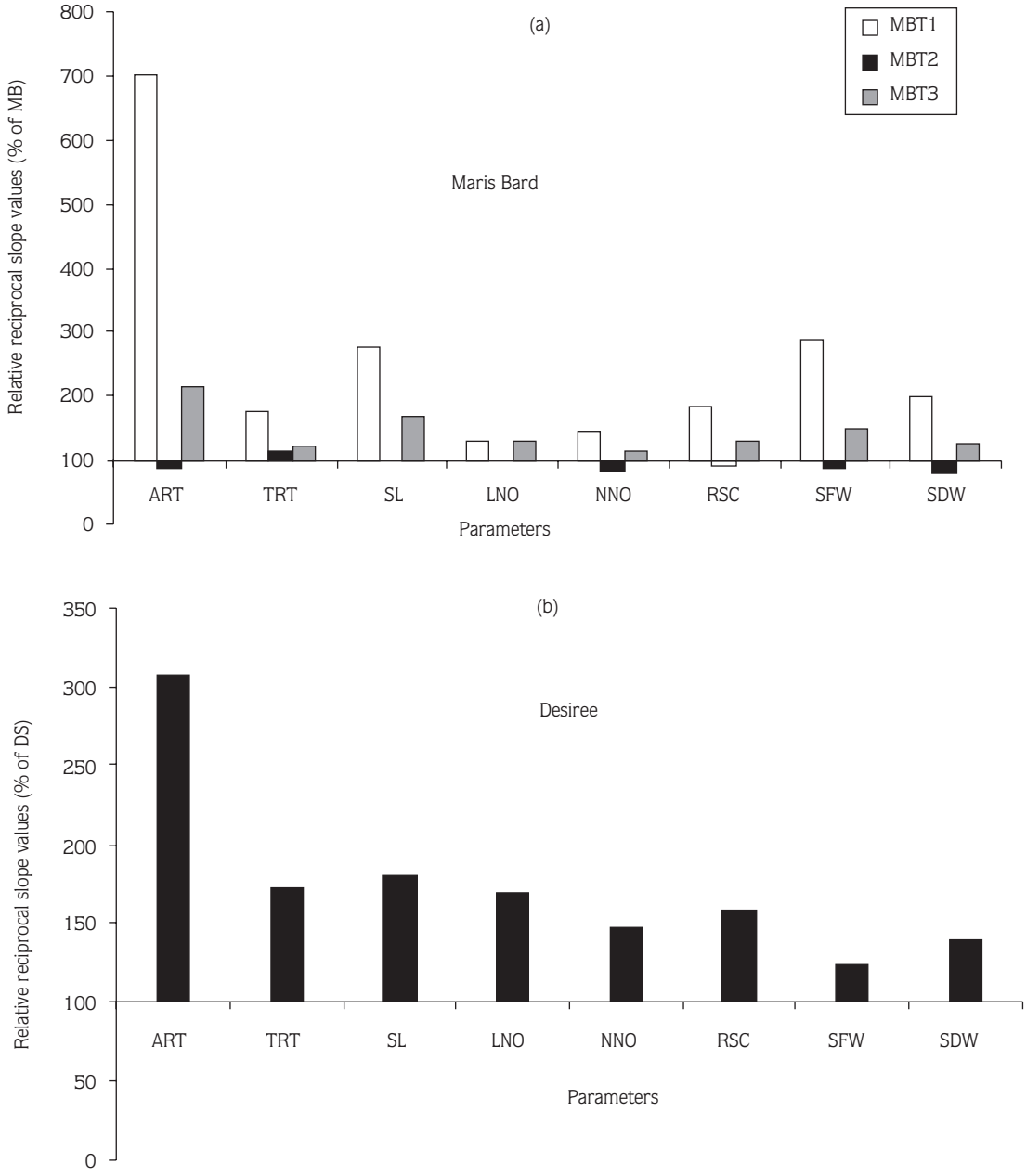

Figure 3. The relationship between all the characters measured and salinity for MBT1, MBT2 and MBT3 compared with MB (a) and for DST1 compared with DS (b) in vitro. (MB and DS are 100% as a control) (SL: Shoot length, LNO: Number of leaves, NNO: Number of usable nodes, ART: Number of aerial roots, TRT: Number of true roots, RSC: Root score, SFW: Shoot fresh weight, SDW: Shoot dry weight).

genotypes which are higher than 100% indicate that those showed a better performance against salinity than the non-transgenic genotype in each character. In contrast, any transgenic genotype with a lower relative reciprocal slope value indicates a poorer performance than the non-transgenic genotype.

Figure 3a reveals that the transgenic genotypes, MB_{T1} and MB_{T3}, had high tolerance to salinity compared to MB according to relative reciprocal slope value for all characters. However, the values for MB_{T2} in ART, NNO, RSC, SFW and SDW were lower than 100 or those of MB, and the same or slightly higher in TRT, SL and LNO. Thus,

MB_{T1} was not superior to MB in all characters. Furthermore, the differences between the transgenic genotypes in response to salinity for each character indicate variation among the transgenic genotypes. Figure 3b also illustrates that the other transgenic genotype, DS_{T1}, showed a better performance than the control. In addition, the results indicate that there are differences between the characters of the genotypes in response to salinity. Higher increases in MB_{T1} and MB_{T3} compared to the control were observed in ART, SL, SFW and SDW. This contrasts with DS_{T1}, for which SFW and SDW accounted for the lowest. These genotypic differences in response to salinity may result from variations in transgene expression level or some other factors affecting gene expression such as plant age, gene integration and number of gene copies (Bavage et al., 2002; James et al., 2004).

In vivo (Glasshouse) Tests

All statistical parameters including salinity, genotype, their interactions and orthogonal comparisons between transgenics and their controls were highly significant for the characters measured (Table 2). Similar to the in vitro results, the genotypes were dramatically affected by salinity levels and there was variation among the genotypes in response to salinity. Unlike the in vitro experiments, the non-transgenic genotype (MB) was superior to its transgenic genotypes in all characters measured, except for tuber and root characters, in response to salinity stress (Figure 4a). The other transgenic genotype (DS_{T1}) exhibited a lower salt tolerance than DS in all the characters (Figure 4b).

As a result, the in vitro responses of the genotypes to salinity were different from their in vivo responses for some characters. Although the in vitro results were encouraging for increased salt tolerance, the same genotypes did not show the same performance for most of the characters in vivo. Similar performances of genotypes in both in vitro and in vivo responses were obtained from only root and tuber related characters. Morpurgo (1991) and Naik and Widholm (1993) also found a significant correlation between in vitro and in vivo for root growth only. These differences between the cultivars in response to salinity in vitro and in vivo might be attributed to different mechanisms being involved under different growing systems.

The results of the present study are not sufficiently clear-cut to be able to attribute enhanced relative salt tolerance in the phenotypically normal transgenic genotypes to the presence of the oxalate oxidase transgene. However, variation between all genotypes indicates that the oxalate oxidase enzyme was involved in this complex process. This limited enhanced salt tolerance indicates that incorporating genes such as oxalate oxidase using genetic transformation could be a potential tool in

Table 2. Analysis of variance for some characters measured in vivo (PH: Plant height, SFW: Shoot fresh weight, RFW: Root fresh weight and TFW: Tuber fresh weight).

		Mean square			
Sources	d.f.	PH[1]	SFW[1]	RFW[1]	TFW[1]
Genotype	5	63.89***	114.34***	30.95***	37.81***
MB vs MB_{T1}, MB_{T2} & MB_{T3}	1	64.63***	38.04**	16.56***	81.19***
MB_{T1} vs MB_{T2} & MB_{T3}	1	0.01	4.33	0.03	21.53*
MB_{T2} vs MB_{T3}	1	8.41***	0.21	0.38	17.42*
DS vs DS_{T1}	1	17.30**	63.92***	5.95**	8.79
Salinity	2	236.68***	143.56***	16.26***	646.80***
Genotype*Salinity	20	12.44***	13.38***	1.96***	10.99***
Replication	14	1.1	4.73	1.11	3.92
Error	406	1.89	3.7	0.55	3.01

[1] Data + 1 were transformed to square root.
*, **, ***, Significant at 0.05, 0.01, 0.001 level, respectively.

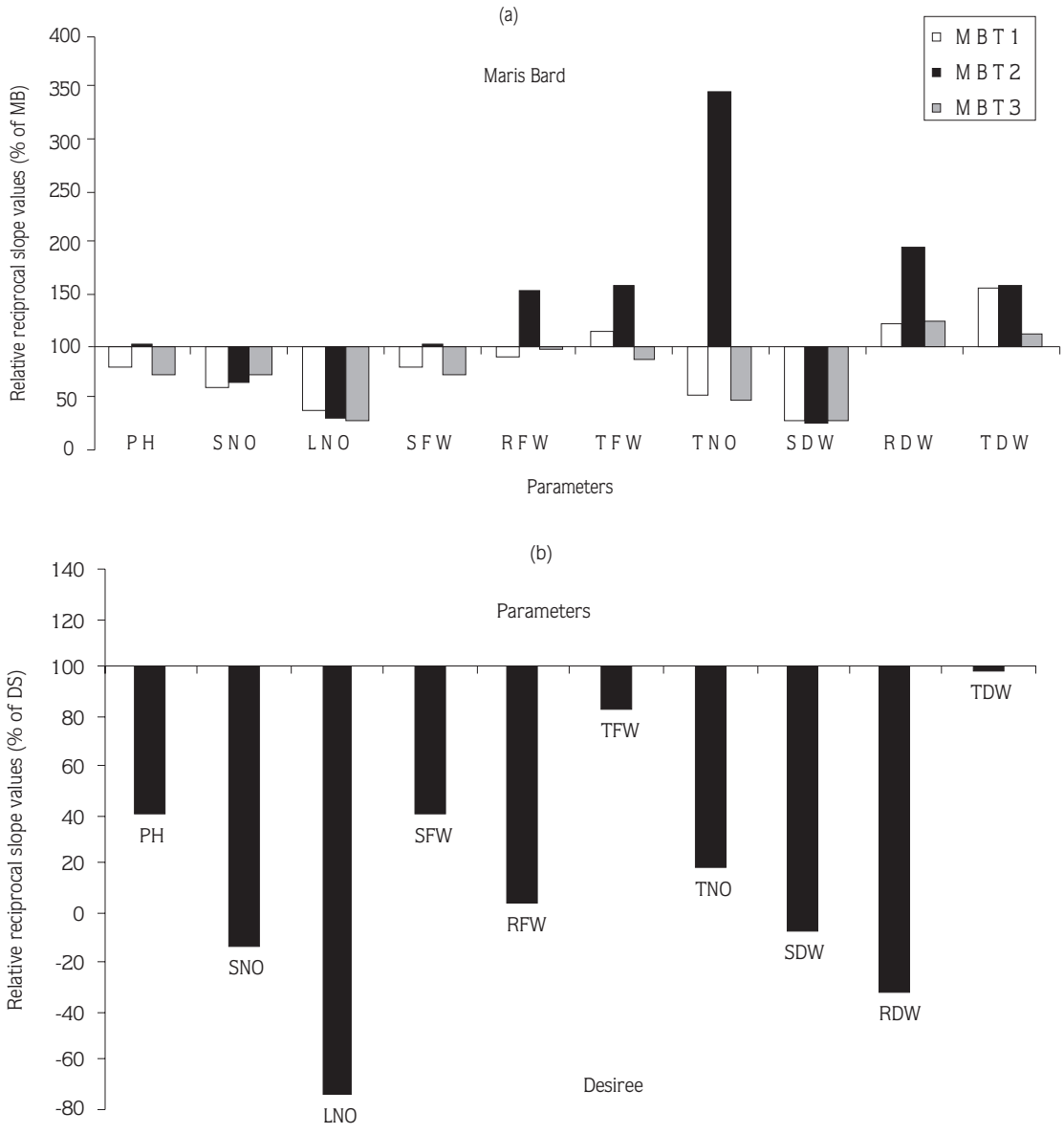

Figure 4. The relationship between all the characters measured and salinity for MB compared with its transgenic genotypes (a) and for DST1 compared with DS (b) at the tuber yield stage in 1996. (MB and DS are 100% as a control) (LNO: Number of leaves, SNO: Number of stems, PH: Plant height, SFW: Shoot fresh weight, SDW: Shoot dry weight, RFW: Root fresh weight, RDW: Root dry weight, TFW: Tuber fresh weight, TDW: Tuber dry weight, TNO: Number of tubers.

plant breeding for the development of salt and drought tolerant plants. Furthermore, one should consider that breeding for this complex character could require multiple gene transformations to obtain commercially desirable salt tolerant crops. Therefore, it would be informative to conduct more investigations at the molecular, cellular and whole plant levels and produce transgenic plants carrying more stress-related genes.

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