Plant Cell Rep
DOI 10.1007/s00299-010-0960-3

ORIGINAL PAPER

Potassium chloride and rare earth elements improve plant growth and increase the frequency of the *Agrobacterium tumefaciens*-mediated plant transformation

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Received: 7 September 2010 / Revised: 8 October 2010 / Accepted: 23 November 2010

Abstract Plant transformation efficiency depends on the ability of the transgene to successfully interact with plant host factors. Our previous work and the work of others showed that manipulation of the activity of host factors allows for increased frequency of transformation. Recently we reported that exposure of tobacco plants to increased concentrations of ammonium nitrate increases the frequency of both homologous recombination and plant transgenesis. Here we tested the influence of KCl and salts of rare earth elements, Ce and La on the efficiency of *Agrobacterium*-mediated plant transformation. We found that exposure to KCl, $CeCl_3$ and $LaCl_3$ leads to an increase in recombination frequency in *Arabidopsis* and tobacco. Plants grown in the presence of $CeCl_3$ and $LaCl_3$ had higher biomass, longer roots and greater root number. Analysis of transformation efficiency showed that exposure of tobacco plants to 50 mM KCl resulted in ~6.0-fold increase in the number of regenerated calli and transgenic plants as compared to control plants. Exposure to various concentrations of $CeCl_3$ showed a maximum increase of ~3.0-fold in both the number of calli and transgenic plants. Segregation analysis showed that exposure to KCl and cerium (III) chloride leads to more frequent integrations of the transgene at a single locus. Analysis of transgene intactness showed better preservation of right T-DNA border during transgene integration. Our data suggest that KCl and $CeCl_3$ can be effectively used to improve quantity and quality of transgene integrations.

Keywords Plant transformation efficiency · T-DNA integration · *Agrobacterium tumefaciens* · *Nicotiana tabacum* · Homologous recombination · Rare earth elements · KCl

Communicated by H. Ebinuma.

Electronic supplementary material The online version of this article (doi:10.1007/s00299-010-0960-3) contains supplementary material, which is available to authorized users.

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Introduction

The process of plant transformation relies on the ability of the transgene to successfully reach the nucleus and integrate in plant's genome. Two major transformation techniques are known, *Agrobacterium*-mediated and gold particle-mediated. Whereas the former one is preferred for transformation of dicots, the latter one is mainly used for monocots. Despite the fact that both methods have been developed as efficient transformation techniques, there are number of plants that are either completely recalcitrant or are very difficult to transform.

Many innovative techniques have been developed that improved the efficiency of transformation. Among the simplest approaches was the modification of temperature of cultivation of *Agrobacterium tumefaciens* and plants. Lowering temperatures during co-cultivation with *Agrobacterium* (Li et al. 2003) and chilling during a regeneration step (Immonen 1996) substantially improved transformation efficiency. Another relatively simple technique is supplementation of growth medium with various

Published online: 05 December 2010

chemicals favoring better transformation outcomes. Supplementation of co-cultivation medium with silver thiosulfate and calcium nitrate resulted in a several-fold increase of transient expression of a reporter gene upon bombardment of plant tissue (Perl et al. 1992).

High rate of transformation requires the establishment of optimal conditions for efficient regeneration of transgenic material from transformed tissues. In many cases, regeneration of transformed cells represents a major challenge in transformation of plants that are susceptible to T-DNA integration but recalcitrant to somatic embryogenesis (SE) (Takumi and Shimada 1997). Various methods can promote SE: increased cytosolic levels of calcium (Racusen and Schiavone 1990; Chugh and Khurana 2002; Malabadi and Staden 2006); the application of silver ions that prevents cellular necrosis caused by ethylene (Dias and Martins 1999; Sahrawat et al. 2003); promotion of SE in wheat by zinc deficiency (He et al. 1991; Kothari et al. 2004) and by exposure to various nitrogen sources (Immonen 1996); promotion or prevention of shoot regeneration by cupric sulphate and high EDTA (Sahrawat et al. 2003; Kothari et al. 2004). Similarly, supplementation of regeneration media with spermidine was shown to improve the recovery of wheat transformants by more than threefold (Khanna and Daggard 2003).

Ammonium and nitrate levels were reported to promote SE in various plant species (He et al. 1989; Choi et al. 1998; Jiménez 2001; Kothari et al. 2004). High concentrations of ammonium nitrate in regeneration media greatly improved SE and increased the yield of somatic embryos in ginseng (Choi et al. 1998). Our recent work showed that an increase in the concentration of ammonium nitrate in the cultivation media resulted in increased regeneration rate of explants and stable transformation rate of tobacco, wheat and triticale plants (Boyko et al. 2009; Greer et al. 2009). These reports also showed that the increase in ammonium nitrate concentration in regeneration medium resulted in substantial, concentration-dependent increase in homologous recombination frequency (HRF) (Boyko et al. 2009). Interestingly, recent reports established a link between the activity of human Rad51 and ammonium-sulphate at the molecular level (Liu et al. 2004; Shim et al. 2006; Sigurdsson et al. 2001). It was demonstrated that ammonium-based salts induce conformational changes in hRad51 leading to an increase in its activity and therefore promoting recombination. These data are important, since transgene typically integrates into the genomic DNA either via non-homologous end joining (NHEJ) or via homologous recombination (HR), two main strand break repair mechanisms (Gorbunova and Levy 1999). Since NHEJ is an error-prone and a predominant repair mechanism in plants, the transgene integration typically leads to deletions in the transgene and genomic DNA (Shrivastav et al. 2008). We previously showed that exposure to ammonium nitrate increases the HR frequency and transformation frequency (Boyko et al. 2009). The transformation events we analyzed included better quality and more intact insertions of the transgene, achieved most probably due to the more frequent involvement of HR, an error-free repair mechanism (Boyko et al. 2009).

The application of a wide spectrum of rare earth elements during a tissue culture stage also holds much promise, as a positive effect of these elements on nitrogen metabolism has been documented (Weiping et al. 2003) and references therein). Since our previous work showed the effectiveness of high level of nitrogen for the improvement of plant transformation, we hypothesized that the salts of rare earth elements such as $CeCl_3$ or $LaCl_3$ would also improve the transformation rate. Rare earth elements were previously shown to enhance the nitrogen metabolism in various plant species, and to have broad positive effects on a plant growth (Cao et al. 2007; Weiping et al. 2003; Yin et al. 2009).

Overall, it has become evident that each species, cultivar, and even tissue has its own unique set of requirements for various salt combinations and concentrations (Maës et al. 1996; He et al. 1989). The foregoing supports the existing need for extensive studies directed on elucidating specific concentrations of macro and micro salts as well as physical conditions optimal for promoting T-DNA integration and SE in various economically important plant species.

In the current study we evaluated the possibility of the application of potassium chloride and two rare earth elements, namely cerium and lanthanum, for influencing the HRF and for increasing the efficiency of plant transformation. KCl was chosen for two reasons: first, previously we showed that chloride ions had a positive influence on homologous recombination rates (RRs) in *Arabidopsis* (Boyko et al. 2010b; Boyko et al. 2006a, b); second, we recently found that the removal of potassium ion from the MS medium results in a decrease in HRF (Boyko et al. 2009). Because the enrichment of growth medium with ammonium nitrate could induce somatic recombination events and increase the frequency of *Agrobacterium*-mediated plant transformation (Boyko et al. 2009), we hypothesized that supplementing plant growth media with rare earth elements would improve nitrogen metabolism and thus could also have a positive effect on plant transformation.

Materials and methods

Plants used in experiment and growth conditions

The impact of potassium chloride on RR was evaluated using transgenic *A. thaliana* line #11 plants (Swoboda et al. 1994).

This line carries the *uidA* (GUS) reporter gene in the genome, which allows the detection of somatic HR events. Upon repair of a DNA strand break in the region of homology via HR, the activity of transgene is restored and can be visualized using the histochemical staining as described (Figure S1; Filkowski et al. 2004a). In brief, 3-week-old Arabidopsis line #11 plants were vacuum infiltrated 2 × 10 min in a sterile staining buffer containing 100 mg 5-bromo-4-chloro-3-indolyl glucuronide (X-glu) substrate (Jersey Labs Inc., USA) in 300 mL 100 mM phosphate buffer (pH 7.0), 0.05% NaN_3, 0.05% Tween 80, 1 mL dimethylformamide. Afterwards plants were incubated at 37°C for 48 h and subsequently bleached with ethanol.

Arabidopsis line #11 plants were germinated and grown on standard solid MS medium (Murashige and Skoog 1962) supplemented with various quantities of potassium chloride (Table 1). Importantly, to get potassium chloride as a single source of potassium in the medium, potassium nitrate was omitted, and potassium dihydrogenphosphate was replaced by ammonium dihydrogenphosphate. To compensate for a total loss of nitrate, the concentration of ammonium nitrate was increased proportionally (Table 1). After 3 weeks, plants were harvested for histochemical staining and somatic RRs were calculated (see below).

The influence of rare earth elements on plant growth and frequency of somatic HR events was studied using transgenic *Nicotiana tabacum* line #LU2 plants (Ilnytskyy et al. 2004). This line carries in the genome a single copy of the *luciferase* (LUC)-based HR substrate. Similar to GUS-based recombination substrate, the LUC-based substrate allows the measurement of HR events at the LUC transgene (Figure S1). Recombination events were analyzed in 4-week-old plants using CCD camera (Gloors Instruments AG, Switzerland) 1 h after the cleavage substrate luciferin was sprayed on plants. *N. tabacum* line #LU2 plants were germinated and grown on standard solid MS medium supplemented with 0.1, 0.3, 1.0, 2.0 and 3.0 μM of either cerium (III) or lanthanum (III) chlorides, or both salts simultaneously. Standard solid MS medium was used as a control. Following 4 weeks since germination, HR events were detected using a CCD camera and the frequency of somatic recombination events was calculated (see below). At the same time, the root length was measured, and the number of roots formed on each plant was scored to evaluate the rooting; typically, ten plants were used for a single experiment and five measurements were performed for three independent experiments. Also, the average dry mass of single plant was calculated.

For stable transformation experiments *N. tabacum* cultivar Big Havana wild-type plants were used. The plants were germinated and grown in control or modified liquid MS medium supplemented with various quantities of potassium or cerium (III) chlorides (Table 2). To get the ammonium nitrate as a single source of nitrogen in the medium, potassium nitrate that was originally present in standard MS medium was substituted for potassium sulphate. Modified MS medium containing 20.6 mM of ammonium nitrate, an amount that is naturally present in standard MS medium was used as a control (Table 2). Once grown (at a fully developed leaf stage), the plants were used for transformation with *Agrobacterium*.

In all cases, plants were grown in high light conditions (32.8 $\mu Em^{-2}\ s^{-1}$) at 22°C in a 16 h light regime and at 18°C in an 8 h dark regime, under a constant humidity of 65%.

Calculation of the homologous recombination frequency and recombination rate

Homologous recombination frequency (HRF) was calculated by counting the number recombination events (areas

Table 1 Growth medium compositions used for the analysis of potassium chloride effects on homologous recombination frequency in *Arabidopsis*

Control: MS macro (mM)		Experimental media compositions, all final concentrations listed in mM		
		KCl 1×	KCl 2.5×	KCl 5×
NH_4NO_3	20.6	41.2	41.2	41.2
KNO_3	18.8	–	–	–
$CaCl_2$	3	3	3	3
$MgSO_4$	1.5	1.5	1.5	1.5
KH_2PO_4	1.25	–	–	–
KCl	–	18.8	47	94
$NH_4H_2PO_4$	–	1.25	1.25	1.25

To have potassium chloride as the only source of potassium in growth medium, potassium nitrate was omitted, whereas potassium dihydrogenphosphate was substituted with ammonium dihydrogenphosphate. To compensate for a total loss of nitrate, the concentration of ammonium nitrate was increased proportionally. × indicates the concentration of K^+, where 1× is the amount similar to the one present in the standard MS, whereas 2.5× and 5× are 2.5- and 5-fold larger amounts

Table 2 Growth medium compositions used for the analysis of potassium and cerium (III) chlorides effects on transformation efficiency in *N. tabacum*

Standard MS macro (mM)		Experimental media compositions (mM)							
		NH_4NO_3 1×	NH_4NO_3 2×	NH_4NO_3 3×	$CeCl_3$ 0.1	$CeCl_3$ 0.3	$CeCl_3$ 0.5	$CeCl_3$ 1.0	KCl 50
NH_4NO_3	20.6	20.6	41.2	61.8	20.6	20.6	20.6	20.6	20.6
KNO_3	18.8	–	–	–	–	–	–	–	–
$CaCl_2$	3	3	3	3	3	3	3	3	3
$MgSO_4$	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
KH_2PO_4	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25
K_2SO_4	–	9.4	9.4	9.4	9.4	9.4	9.4	9.4	9.4
KCl	–	–	–	–	–	–	–	–	50
$CeCl_3$	–	–	–	–	0.0001	0.0003	0.0005	0.001	–

To have ammonium nitrate as the only source of nitrogen in the medium, potassium nitrate was substituted for potassium sulphate. Modified MS medium containing 20.6 mM of ammonium nitrate, an amount that is naturally present in standard MS medium was used as a control

of luciferase activity) in the population of ~50 4-week-old tobacco plantlets and relating this number to the total number of plants used for the analysis.

Recombination rate (RR) was calculated by counting the number of recombination events (blue spots/sectors) in the population of ~100 3-week-old Arabidopsis plantlets, relating this number to the total number of plants used for the analysis and then dividing this number by the average number of haploid genomes present in 3-week-old plants grown on specific media.

The number of haploid genomes per plant was calculated by relating the yield of total DNA (in micrograms per plant) to the mean DNA content (0.16 pg) of an *A. thaliana* haploid cell and the number of plants used for DNA preparation. To avoid a bias during DNA preparation, DNA was extracted using two different methods (see Boyko et al. 2009).

Agrobacterium strains and constructs used in experiment

Agrobacterium GV3101 strain containing pPM6000 helper plasmid was used for transformation (Boyko et al. 2009). The T-DNA cassette contained the active *luciferase* (LUC) gene driven by the *N-gene* promoter. The *hph* gene that confers resistance to hygromycin was cloned under control of 35S CaMV promoter, and served as a selection marker.

Liquid cultures of *Agrobacterium* were inoculated from frozen glycerol stock and grown overnight at 28°C in the presence of appropriate antibiotics (kanamycin, 50 mg/ml; rifampicin, 25 mg/ml; and gentamicin, 25 mg/ml). Cells were harvested and prepared for transformation as previously described (Kovalchuk et al. 2000).

Stable transformation of *N. tabacum* cultivar Big Havana wild-type plants was performed as previously described (Boyko et al. 2009). In brief, leaves of 4-week-old tobacco plants grown in sterile conditions were removed and then submersed into a Petri dish laid out with Whatman paper and containing resuspended *Agrobacterium*. Once upside-down and completely submersed, the leaf surface was incised using a sharp surgical blade. Incisions were made in parallel to side veins. The distance between two parallel incisions was 5–7 mm; thus, on average 4–6 incisions were made per single leaf. Four leaves of approximately similar size per each experimental group were used and the number of incisions was kept at 20 per each experimental group. Each experiment was repeated three times. When cutting was completed, leaves were allowed to be submersed for 10 min, then they were blotted dry and placed upside-down in solid standard MS medium. After 3 days of incubation in the dark at 22°C, leaves were removed from plates, well rinsed with sterile distilled water, blotted dry, and transferred to solid standard MS medium containing IAA (0.8 mg/L), kinetin (2 mg/L) for calli induction and regeneration, and the combination of ticarcillin (100 mg/L) with potassium clavulanate (3 mg/L) to control *Agrobacterium* growth. Calli and roots induction were performed under selection conditions (hygromycin, 25 mg/L). The developed plantlets were transferred to soil and checked for *luciferase* gene expression. The effect of selected chemicals on plant transformation was evaluated using two parameters: callus regeneration efficiency and stable transformation frequency (Boyko et al. 2009). The former shows the total number of shoots produced on callus-inducing/regenerating medium per 20 incisions made in 4 leaves during transformation. The latter shows the total number of *luciferase* expressing plants obtained per 20 incisions made in 4 leaves during transformation.

T-DNA segregation analysis

Seeds of self-pollinated T0 tobacco plants were germinated and grown for 3 weeks on MS medium in presence of

hygromycin (25 mg/L). 300–400 seeds per each putative transgenic line were used. The following number of transgenic lines was analyzed: for control, 8 lines; for "KCl" group, 16 lines; for "Ce 0.1 μM", 5; for "Ce 0.3 μM", 7; for "Ce 0.5 μM", 12; and for "Ce 1.0 μM", 8 lines. Plants showing an antibiotic resistance phenotype were scored, and a segregation ratio was calculated. Single locus typically gave a ratio of resistant to sensitive plants of 3:1, whereas two, three and four (or more) loci would give the ratio of 15:1, 63:1 and 255:1, respectively. Statistical significance of calculated segregation ratios was confirmed using Chi square statistic with $\alpha = 0.05$.

Total DNA preparation and PCR amplification of right and left borders

Total DNA was prepared using the *Gene Elute Plant Genomic DNA Miniprep Kit* (Sigma) according to the manufacturer's protocol. The following number of transgenic lines was used for the analysis of PCR intactness: for control, 5 lines; for "KCl" group, 20 lines; for "Ce 0.1 μM", 6; for "Ce 0.3 μM", 3; for "Ce 0.5 μM", 16; and for "Ce 1.0 μM", 3 lines. The intactness of the integrated T-DNA borders was determined using previously published set of PCR primers (Table S1) for left and right T-DNA border analysis (Boyko et al. 2009). Each set consisted of one outward (from integrated T-DNA) and three consecutive inward (into integrated T-DNA) primers. Deletions of T-DNA border sequence limit the number of inward primer binding sites in a deletion-size-dependent manner. The deletion size can be approximated by analyzing the PCR products in agarose gel: an intact border sequence should yield 3 PCR products, and a truncation of a border sequence should reduce the number of PCR products to 2, 1 or 0 depending on a deletion size (Boyko et al. 2009). PCR conditions were the same as previously published (Boyko et al. 2009).

Southern blot analysis

To determine the number of T-DNA copies inserted in plant genome, 10 μg of total genomic DNA prepared from plants regenerated from control, Ce 0.3 and Ce 0.5 media was digested overnight with 10 U of *Nde*I (New England Biolabs, Beverly, MA) in a final volume of 200 μl. Precipitated DNA was dissolved in 10 μl of water. The DNA was separated by electrophoresis on a 1% agarose gel. The gel was treated for 30 min in 0.4 M NaOH, and then for 20 min in 0.25 M HCl. The DNA was blotted onto a positively charged nylon membrane (Roche, Mannheim, Germany) by vacuum transfer; afterward, the DNA was UV-crosslinked. Hybridization and washes of the membrane were performed according to DIG Application Manual for Filter Hybridization (Roche, Mannheim, Germany) with following modifications. The membrane was hybridized with a DIG-labeled probe overnight at 42°C. The probe (575 nt) was synthesized with PCR DIG Probe Synthesis Kit (Roche, Mannheim, Germany) according to the manufacture's protocol using *luciferase*-specific primers (forward 5′ ATTTATCGGAGTTGCAGTTGCGCC 3′, reverse 5′ CCAGCAGCGCACTTTGAATCTTGT 3′) and pCAMBIA NLUC plasmid as a template. The High Stringency membrane wash was done at 65°C.

pCAMBIA NLUC (1 μg) was digested overnight with 2 FDU of FastDigest *Nde*I (Fermentas). Enzyme was removed by phenol–chloroform (1:1) and then chloroform. DNA was precipitated with sodium acetate and ethanol and precipitated DNA was dissolved in water. The digested plasmid DNA was used as a positive control for the blot. The intact T-DNA should give a fragment of at least 6.3 kb (from *Nde*I site to the right border) (see Fig. 6a).

Statistical treatment of data

In all cases, the mean and standard error or standard deviation from at least three independent experiments was calculated. Statistical significance of the experiment was confirmed by either the two-tailed paired Student's *t* test (comparing data from two treatments), or Kolmogorov–Smirnov test (comparing two distributions). Statistical analysis was performed using the *JMP 5.0* software (SAS Institute Inc) and *R* (version 2.10.1) statistical package (freeware).

Results

High concentrations of potassium chloride increase recombination rates in *Arabidopsis*

To analyze whether the exposure to potassium chloride can induce homologous recombination, transgenic *Arabidopsis* line #11 plants (Swoboda et al. 1994) were germinated and grown on solid MS media modified with various concentrations of potassium chloride (Table 1). Our results demonstrated that the presence of potassium chloride in the growth medium significantly increased recombination rate (RR) (Fig. 1a). The presence of 18.8 mM potassium chloride resulted in a 9.3-fold increase in RR as compared to control MS medium (Student's *t* test, $\alpha = 0.01$) (Fig. 1a). Consistently, the supplementation of the growth medium with 47 and 94 mM potassium chloride led to a 15.4- and 19.2-fold increase in RR, as compared to plants grown on MS medium (Student's *t* test, $\alpha = 0.01$) (Fig. 1a). Overall, a strong positive correlation between the

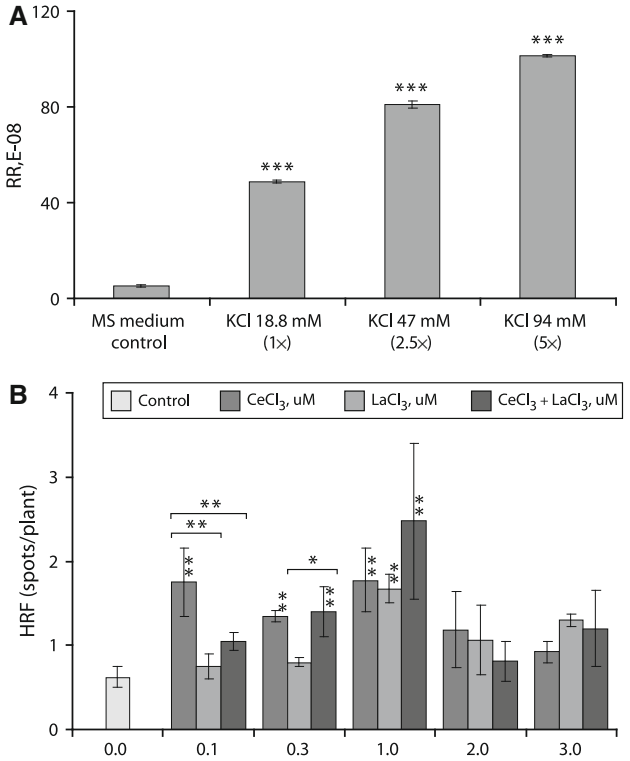

Fig. 1 The effect of growth media supplemented with various amounts of potassium chloride (**a**) and rare earth elements (**b**) on recombination frequencies and recombination rates in plants. **a** Recombination rates (as an average of three experiments with standard error) were measured in the population of ~100 (per each experimental group) *Arabidopsis* plants germinated and grown for 3 weeks on solid MS medium (control) or modified medium containing 18.8 (1×), 47 (2.5×) and 94 (5×) mM potassium chloride. × stands for the concentration of potassium in standard MS medium. Asterisks show significant difference (Student's *t* test) from the control ($P < 0.01$). **b** Homologous recombination frequencies (as an average of three experiments with standard deviation) were measured in the population of ~50 (per each experimental group) *N. tabacum* plants germinated and grown for 4 weeks on solid MS medium (control) or MS medium supplemented with 0.1, 0.3, 1.0, 2.0 and 3.0 μM of either cerium (III) or lanthanum (III) chlorides, or in the presence of both elements. *Asterisks* show significant difference (Student's *t* test) from the control (*vertical asterisks*) or other treatment (*horizontal asterisks*), where *one asterisk* is $P < 0.1$ and *two asterisks* are $P < 0.05$

amount of potassium chloride present in growth media and RR was observed ($r = 0.93$, $P < 0.05$).

Rare earth elements increase the frequency of homologous recombination in *N. tabacum*

Effects of rare earth elements on induction of HR was analyzed using transgenic *N. tabacum* line #LU2 plants (Ilnytskyy et al. 2004). In the pilot experiments we found that 0–3 μM of $LaCl_3$ and $CeCl_3$ was preferable concentration range as higher concentrations resulted in the growth inhibition (data not shown). It should be noted that although we used chloride salts of cerium and lanthanum, the concentration of chloride were the fraction of those used in potassium chloride salts, 0.1–3.0 μM for $LaCl_3$ or $CeCl_3$ versus 18.8–94.0 mM for KCl. Thus, the effects observed in the experiments with cerium and lanthanum salts were due to the rare earth elements, rather than chloride ions.

Our results demonstrated that supplementation of growth medium with 0, 0.1, 0.3, 1.0, 2.0 and 3.0 μM of cerium (III) or lanthanum (III) chlorides resulted in an increase of HRF (Fig. 1b). Strikingly, while two elements have very similar chemical properties, they had a different influence on HRF. Cerium (III) chloride resulted in a greater than twofold increase in HRF while present in the medium in the lowest concentration of 0.1 μM (Student's *t* test, $\alpha = 0.05$) (Fig. 1b). In contrast, lanthanum (III) chloride increased the HRF only when it was present in the medium at 1.0 μM (Student's *t* test, $\alpha = 0.05$) (Fig. 1b). Consistently, there was no additive positive effect observed when the growth medium was supplemented with both cerium (III) and lanthanum (III) chlorides at 0.1 or 0.3 μM each. Only when 1.0 μM of each element was added to the growth medium, a statistically insignificant 1.4- and 1.5-fold increase of HRF was observed as compared to the media supplemented with 1.0 μM of either cerium (III) or lanthanum (III) chlorides, respectively (Fig. 1b). Finally, neither cerium (III) nor lanthanum (III) chlorides had a significant effect on HRF when present in medium in concentrations higher than 1.0 μM.

Rare earth elements increase the root growth and rooting in *N. tabacum*

While analyzing the influence of rare earth elements on HRF, we observed a strong positive effect on root growth from presence of cerium (III) and lanthanum (III) chlorides in media (Fig. 2). Similar to the HRF response discussed above, a broad range of cerium (III) chloride concentration from 0.3 to 2.0 μM had a positive effect on root growth (Kolmogorov–Smirnov test, $P < 0.01$). In contrast, a strong positive response to lanthanum (III) chloride was observed only at 2.0 μM, where the average root length was increased by 2.4-fold compared to the control MS medium (Kolmogorov–Smirnov test, $P < 0.01$) (Fig. 2a). Presence of 0.3 μM of cerium (III) chloride increased the average root length by 2.7-fold as compared to the control MS medium (Kolmogorov–Smirnov test, $P < 0.01$). Increasing cerium (III) chloride concentration to 1.0 and 2.0 μM resulted in a somewhat less pronounced response, as the average root length was increased only by 2.3- and 1.7-fold, respectively (Kolmogorov–Smirnov test, $P < 0.01$) (Fig. 2a). Finally, a weak 1.5-fold increase in root length was observed when lanthanum (III) chloride was used at 3.0 μM (Kolmogorov–Smirnov test, $P < 0.1$).

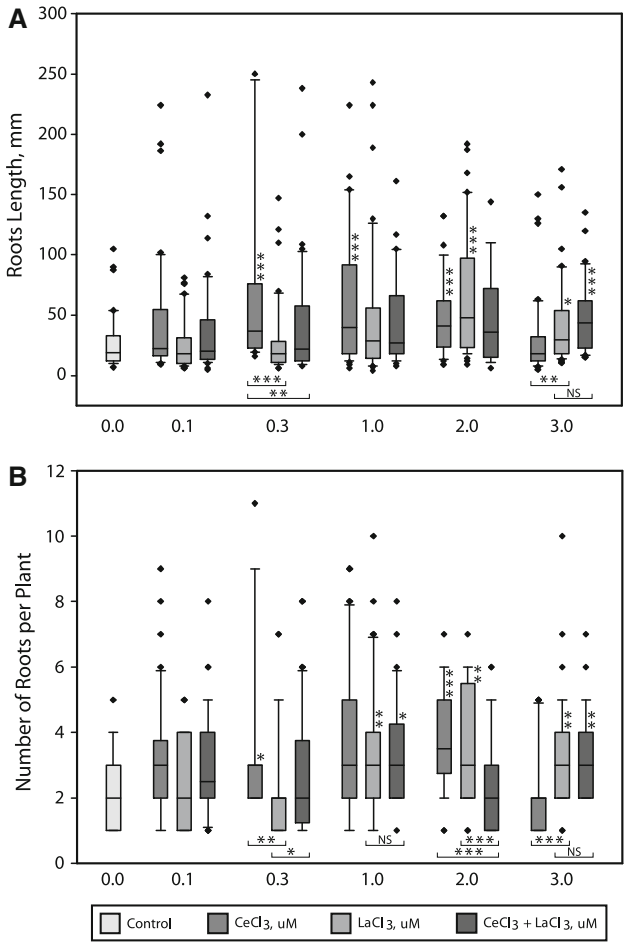

Fig. 2 The effect of supplementation of growth media with various amounts of cerium (III) and lanthanum (III) chlorides on root growth in *N. tabacum*. *N. tabacum* plants were germinated and grown for 4 weeks on solid MS medium (control) or MS medium supplemented with 0.1, 0.3, 1.0, 2.0 and 3.0 μM of either cerium (III) or lanthanum (III) chlorides, or in the presence of both elements. To evaluate the effect of rare earth elements on plant growth, the roots length (**a**) was measured, and a number of roots formed on each single plant (**b**) was scored in the population of 10 plantlets. Data are shown as an average from five independent measurements with standard deviation. *Asterisks* show significant difference of the roots length or roots number distributions (Kolmogorov–Smirnov test) from the control (*vertical asterisks*) or other treatments (*horizontal asterisks*), where *one asterisk* is $P < 0.1$, *two asterisks* are $P < 0.05$ and *three asterisks* are $P < 0.01$. *NS* not significant difference

The increase in root growth was paralleled by enhanced rooting capacities. While the plants grown on the control MS medium had on average 2.2 roots per plant, the plants grown on media supplemented with 0.3 and 2.0 μM of cerium (III) chloride had 3.5 and 3.7 roots per plant, respectively (Kolmogorov–Smirnov test, $P < 0.1$ for 0.3 μM, $P < 0.01$ for 2.0 μM) (Fig. 2b). In general, 70% of control plants analyzed in this study had from 1 to 2 roots formed per plant (Table S2). In contrast, about 75% of plants grown in presence of 2.0 μM of cerium (III) chloride formed from 3 to 7 roots per plant. Similar response was observed when lanthanum (III) chloride was used at 2.0 μM: plants developed on average 3.5 roots per plant, and 66% of all analyzed plants formed from 3 to 7 roots per plant (Table S2). Moderate correlation between the number of roots and root length was found for the plants grown on the medium containing cerium (III) chloride in concentrations of 0.3, 1.0 and 2.0 μM, lanthanum (III) in the concentration of 2.0 μM and for plants grown on both salts as concentrations of 2.0 and 3.0 μM (in all cases $r > 0.5$ but < 0.75).

Finally, the positive effect of rare earth elements on plant growth was supported by an increase in dry mass of plants grown at optimal concentrations of cerium (III) and lanthanum (III) chlorides (Figure S2). The significant 2.4- and 1.9-folds difference was observed, when plants were grown at 0.3 μM of cerium (III) and 2.0 μM of lanthanum (III) chlorides, respectively (Student's *t* test, $\alpha = 0.05$).

It is noteworthy that no added positive effect on the growth of roots from simultaneous presence of two rare earth elements in the media was found. The significant difference in root length and root number, observed in several cases between the control and the various treatments with a simultaneous application of cerium (III) and lanthanum (III) chlorides, can be fully attributed to a presence of only one of the two analyzed elements in the media (Fig. 2).

The influence of potassium and cerium (III) chlorides on callus regeneration and the frequency of stable T-DNA integrations in *N. tabacum*

To analyze whether potassium chloride and rare earth elements have any effect on the frequency of stable plant transformation, we performed an experiment on genetic transformation of plants grown in media supplemented with potassium or cerium (III) chloride. Previous experiments showed that active concentration of lanthanum (III) chloride is higher than of cerium (III) chloride and the observed effects are smaller so we decided to test cerium (III) chloride only. To compare the effects of these salts on calli regeneration efficiency and stable transformation frequency with those previously described for ammonium nitrate, we also included growth media modified in their ammonium nitrate content (Table 2). The concentration of ammonium nitrate of 20.6 mM was used as a control.

Consistent with previous experiments, growing plants on the medium supplemented with 41.2 and 61.8 mM ammonium nitrate increased the total number of regenerated calli by 3.9- and 7.5-fold, respectively (Fig. 3a). The presence of 50 mM potassium chloride also resulted in 6.0-fold increase in callus regeneration. At the same time, the presence of 0.1–1.0 μM cerium (III) improved callus

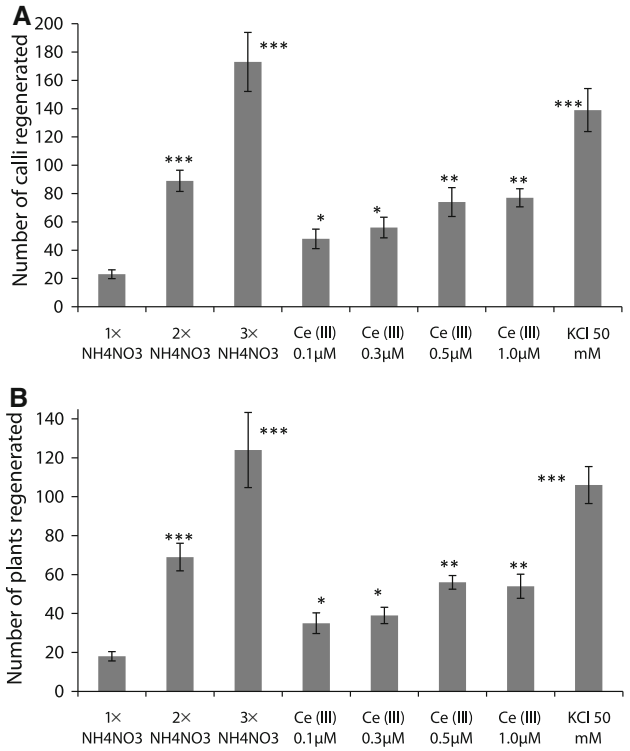

Fig. 3 Number of calli (**a**) and transgenic plants (**b**) regenerated from *Agrobacterium*-mediated transformation of tobacco leaves pre-exposed to ammonium nitrate, potassium chloride and cerium (III) chloride. Plants were grown in the presence of different amounts of ammonium nitrate [20.6 (1×), 41.2 (2×) and 61.8 (3×) Mm], potassium chloride (50 mM) or cerium (III) chloride (0.1, 0.3, 0.5 and 1.0 μM) were used for *Agrobacterium*-mediated transformation with the construct carrying the luciferase gene. × stands for the concentration of ammonium nitrate in standard MS medium. **a** Calli were regenerated under selective conditions (hygromycin, 25 mg/L). The data are shown as an average number of calli regenerated per each experimental group. **b** Stable transformation events were confirmed via *luciferase* gene expression. The data are shown as an average number of transgenic plants regenerated per each experimental group. *Asterisks* show significant differences between treatment group and a control group, where *one asterisk* is $P < 0.1$, *two asterisks* are $P < 0.05$ and *three asterisks* are $P < 0.01$

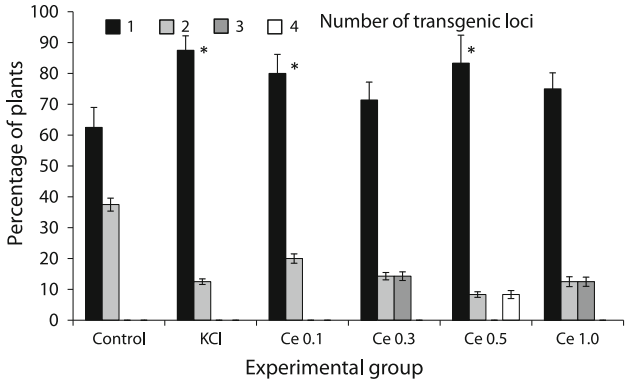

Fig. 4 Segregation analysis of regenerated transgenic plants. Regenerated transgenic plants were subjected to segregation analysis using 25 mg/L of hygromycin. Typically 300–400 seeds were used for the analysis of segregation of each individual line. KCl group is the group of plants regenerated from exposure to 50 mM KCl, whereas Ce 0.1, Ce 0.3, Ce 0.5 and Ce 1.0 represent groups of plants regenerated from exposure to 0.1, 0.3, 0.5 and 1.0 μM of $CeCl_3$. *Y* axis shows the average percentage of plants with one, two, three or four transgenic loci. *Asterisks* show significant differences between treatment and control groups ($P < 0.05$)

regeneration by 2.1- to 3.3-fold (Fig. 3a). In all cases we observed significant difference in the number of calli regenerated from media supplemented with various amount of $CeCl_3$ or KCl as compared to control media containing 20.6 mM NH_4NO_3 ($P < 0.05$).

Next, we analyzed the frequency of the appearance of stable transformants. The presence of 41.2 and 61.8 mM ammonium nitrate in media increased the total number of stable transformants by 3.8- and 6.7-fold, respectively (Fig. 3b). Exposure to media containing 50 mM potassium resulted in a 5.9-fold increase, whereas exposure to 0.1–1.0 μM cerium (III) chloride lead to 1.9- to 3.0-fold increase in the number of transformants, respectively (Fig. 3b). In all cases we observed significant difference in the number of plants regenerated from media supplemented with various amount of $CeCl_3$ or KCl as compared to control media containing 20.6 mM NH_4NO_3 ($P < 0.05$). The difference between media containing 0.1 and 0.3 μM cerium (III) chloride was insignificant ($P > 0.05$), as well as the difference between 0.5 and 1.0 μM ($P > 0.05$) (Fig. 3b). Overall, our data supports the idea that the application of potassium chloride and rare earth elements in growth media improves plant transformation efficiency.

Segregation analysis

Increased frequency of transformation may also cause the transgene to integrate in multiple genomic locations. Since single-locus insertions are usually preferable, we analyzed whether the use of KCl, $CeCl_3$ and $LaCl_3$ resulted in the increase in the number of transgenic loci. For this, the segregation analysis of the T1 progeny of self-pollinated T0 plants was performed (Fig. 4). Segregation analysis showed that most integration events are single-locus only (Fig. 4). It was found that plants regenerated from media supplemented with 50 mM KCl, 0.1, 0.5 and 1.0 μM of $CeCl_3$ had more frequent occurrence of single-locus integration events, as compared to control plants (Fig. 4). This suggests that increased transformation efficiency found in plans regenerated from media supplemented with KCl and cerium (III) chloride was achieved without compromising the quality of transgenic plants produced.

Exposure to KCl and $CeCl_3$ salts results in more intact integrations at the right T-DNA border

We found that exposure to KCl and $CeCl_3$ resulted in increased RR and transformation frequency. HR is a more precise mechanism of strand break repair as compared to NHEJ, so we hypothesized that exposure to these salts could also influence the transgene integration pattern.

As the VirD2 protein nicks the T-DNA right border between nucleotides 3 and 4 (Kim et al. 2007), the intact integration events should contain the T-DNA right border sequence starting from nucleotide 4. The left border is typically less preserved. To test for the right and left border integrity, we used a nested PCR approach for the amplification of the T-DNA sequence. The presence of 3 fragments of 323, 296 and 251 nt in the PCR product from the right border would indicate that annealing sequence for all 3 primers was preserved, suggesting no deletion or a deletion smaller than 13 nt at the right border (Fig. 5a, b). If less than 3 fragments were present, there could be truncations at the right border; the presence of 2, 1 or 0 fragments would suggest truncations of 13–40, 40–85, or larger than 85 nt, respectively. Similar to the results with right border, the presence of 3 fragments of 332, 315 and 153 nt in the PCR product from the left border would indicate that there was no deletion or a deletion at the left border was smaller than 9 nt (Fig. 5a, c). The presence of 2, 1 or 0 fragments would suggest truncations of 9–36,

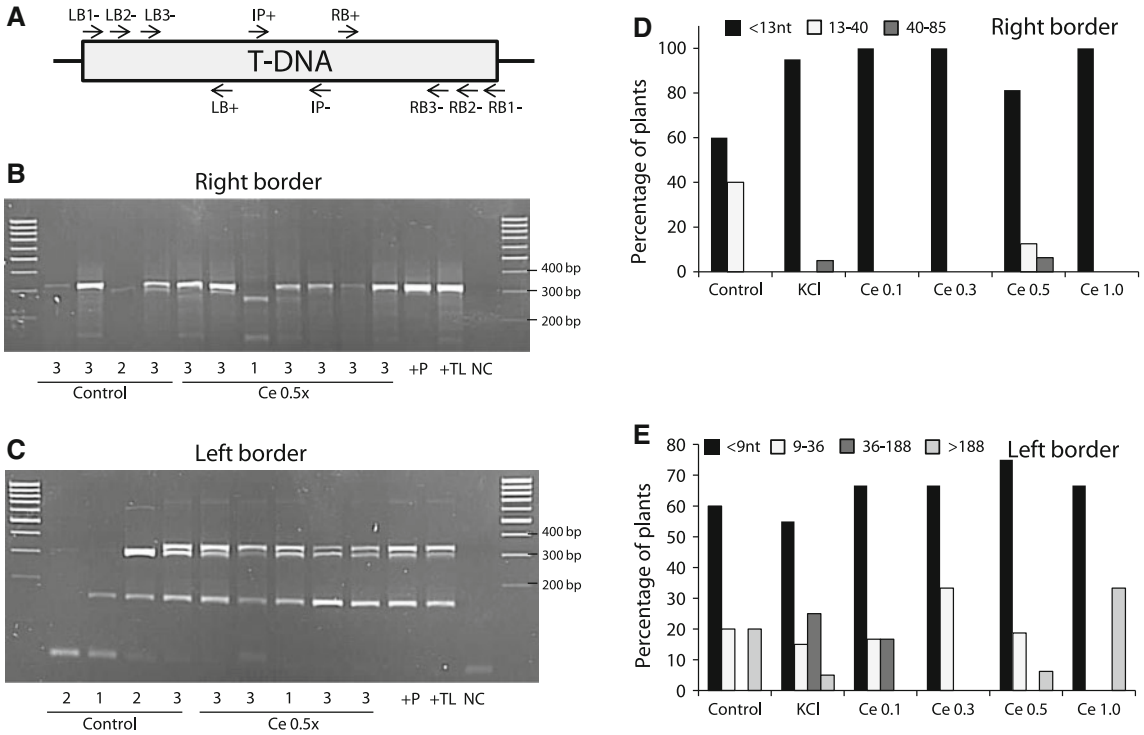

Fig. 5 Analysis of the intactness of the right and left T-DNA borders in regenerated plants. **a** Schematic presentation of the PCRs for the analysis of T-DNA intactness. The intactness of each border was analyzed using four primers, one primer going outward from T-DNA (RB+ and LB+) and 3 primers going inward (RB1−, RB2− and RB3− and LB1−, LB2−, LB3−). In the case when no fragments were observed upon PCR, the PCR was redone with internal primers, IP+ and IP−, each annealing to sequence located in over 2,000 nt from the border. **b** Analysis of the right border intactness. *First and last lanes* show the ladder. *Lines* marked as '+', show a positive control, from a plasmid containing T-DNA (+P) and from genomic DNA of a transgenic line (+TL), whereas lane, marked as *NC*, shows the negative control—PCR from genomic DNA extracted from wild type. All the other lanes represent PCR amplifications from genomic DNA of individual transgenic lines. *Numbers under the image* show the number of PCR fragments, *1* (251 bp), *2* (251 and 296 bp) or *3* (323, 296 and 251 bp in size). **b** Analysis of the left border intactness. *First and last lanes* show the ladder. Lanes marked as '+', show a positive control, from a plasmid containing T-DNA (+P) and from genomic DNA of a transgenic line (+TL), whereas lane, marked as *NC*, shows the negative control—PCR from genomic DNA extracted from wild type. All the other lanes represent PCR amplifications from genomic DNA of individual transgenic lines. *Numbers under the image* show the number of PCR fragments, *1* (153 bp), *2* (153 and 315 bp) or *3* (153, 315 and 332 bp in size). **c** Summary of the right border intactness analysis. Percentage of plants having T-DNA truncations of various sizes found in various experimental media groups. KCl group is the group of plants regenerated from exposure to 50 mM KCl, whereas Ce 0.1, Ce 0.3, Ce 0.5 and Ce 1.0 represent groups of plants regenerated from exposure to 0.1, 0.3, 0.5 and 1.0 μM of $CeCl_3$. **d** Summary of the left border intactness analysis. Percentage of plants having T-DNA truncations of various sizes found in various experimental media groups. KCl group is the group of plants regenerated from exposure to 50 mM KCl, whereas Ce 0.1, Ce 0.3, Ce 0.5 and Ce 1.0 represent groups of plants regenerated from exposure to 0.1, 0.3, 0.5 and 1.0 μM of $CeCl_3$

36–188, or larger than 188 nt, respectively. In the cases when we were not able to amplify any fragment, a pair of 'internal' primers was used to confirm the transgene presence.

About 60% of plants regenerated from the control medium containing 1× NH_4NO_3 had no deletions or deletions were smaller than 13 nt, whereas 40% had deletions between 13 and 40 nt. In contrast, plants regenerated from the media supplemented with either KCl or various amounts of $CeCl_3$ had at least 80% of plants without deletions at the right border. Moreover, 100% of plants regenerated from media containing 0.1, 0.3 and 1.0 μM of $CeCl_3$ had no deletions at the right border (Fig. 5d). In contrast, approximately 2/3 of all tested plants had deletions smaller than 9 nt at the left border. There was no significant difference observed between the control 1× NH_4NO_3 group and any of the groups with supplemented amount of KCl or $CeCl_3$ ($P > 0.05$; Fig. 5e). This experiment showed that exposure to KCl and $CeCl_3$ results in less frequent T-DNA truncations at the right border.

Southern blot analysis of selected transgenic lines

To evaluate the complexity of integration events the total number of the inserted T-DNA copies was determined for the selected transgenic lines. Based on the previously discussed segregation analysis, four plants with a single transgenic locus, one plant with two and one plant with three transgenic loci were chosen for Southern blot analysis. As we used a restriction enzyme (NdeI) that cuts T-DNA only at a single position and does not cut through region of probe hybridization (see Fig. 6a), the number of fragments observed on Southern blot would correspond to the number of loci carrying the transgene(s). In all lines analyzed in this experiment, the number of fragments on the Southern blot corresponded to the number of transgenic loci as identified by segregation analysis (Fig. 6).

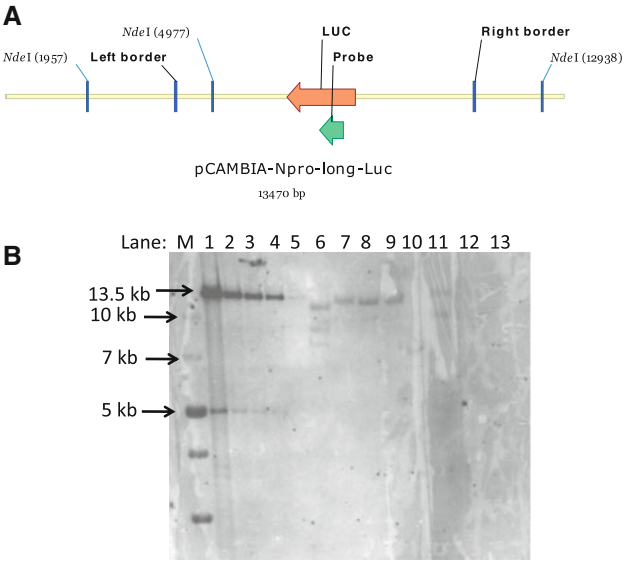

Fig. 6 Southern blot analysis of selected transgenic lines. **a** Structure of pCAMBIA–Npro-long-Luc vector and T-DNA. Position of LUC gene and the probe is shown. Genomic DNA was digested with *Nde*I; thus it is expected that the number of fragments found on Southern blot should correspond to the number of genomic loci carrying the transgene. **b** Southern blot. Lane description: *M* marker, 1 kb DNA Ladder Plus (Fermentas). *1* pCAMBIA NLUC digested with *Pst*I, 500 pico grams; *2* pCAMBIA NLUC digested with *Pst*I, 100 pico grams; *3* pCAMBIA NLUC digested with *Pst*I, 50 pico grams; *4* pCAMBIA NLUC digested with *Pst*I, 25 pg; *5*. pCAMBIA NLUC digested with *Pst*I, 1 pg; *6* line Ce 0.3 × 1 (segregation analysis, 3 loci; Southern blot, 3 fragments); *7* line Ce 0.5 × 3 (segregation analysis, 1 locus; Southern blot, 1 fragment); *8* line Ce 0.5 × 7 (segregation analysis, 1 locus; Southern blot, 1 fragment); *9* line Ce 0.5 × 8 (segregation analysis, 1 locus; Southern blot, 1 fragment); *10* line Ce 0.5 × 9 (segregation analysis, 1 locus; Southern blot, 1 fragment); *11* control 1× NH_4NO_3 (segregation analysis, 2 loci; Southern blot, 2 fragments); *12* no sample; *13* genomic DNA of the wild-type Havana plant

Discussion

In this study we analyzed the effect of high concentrations of potassium chloride and the presence of rare earth elements in growth media on the activity of HR. We found that: (a) supplementation of plant growth media with potassium chloride results in a dose-dependent increase of RR; (b) application of two rare earth elements, cerium and lanthanum, can increase HR frequency; (c) cerium (III) and lanthanum (III) chlorides promote root growth and increase rooting in tobacco plants; (d) growing plants in media supplemented with potassium or cerium (III) chloride before transformation can increase the frequency of T-DNA integration in the plant genome; (e) plants regenerated from potassium and cerium (III) chlorides supplemented medium exhibit normal segregation pattern and have better protected T-DNA right borders.

High concentrations of potassium chloride increase recombination rates in *Arabidopsis*

Our previous studies of the effects of salt stress on the plant genome demonstrated that the presence of sodium chloride in growth media can significantly increase RR in plants (Boyko et al. 2010a, b). This suggests the possibility of using this chemical for increasing HRF in plants in order to influence the outcomes of transformation with *Agrobacterium*. Unfortunately, high concentrations of sodium chloride result in a significant increase in the level of DNA double strand breaks (Boyko et al. 2010a, b), therefore limiting the application of this chemical to relatively low concentrations. Furthermore, elevated levels of Na^+ ions in media are toxic to plants and result in ionic stress

(Hasegawa et al. 2000; Zhu 2000, 2002). The role of Na^+ ions in HR induction is negligible. In contrast, Cl^- ions mediate an increase in RR under salt stress conditions (Boyko et al. 2010b). We hypothesized that delivering Cl^- ions with other cations to growth media could increase HRF without triggering an ionic stress response associated with Na^+. K^+ is a natural candidate to use, since it is normally present in high concentrations in growth media in the form of potassium nitrate (Murashige and Skoog 1962).

Our data supported a hypothesis regarding the important role of Cl^- ions in increasing RR. Exposure to potassium chloride resulted in a dose-dependent increase in RR. Unfortunately, the mechanism behind the effect of Cl^- ions on RR is unclear and remains to be specified. Since both control and "KCl 1×" modified media contained the same concentration of K^+ ions, it could be suggested that the effect of potassium chloride on HR was mediated by Cl^- ions. Indeed, it was previously reported that a genotoxic aspect of salt stress is Cl^--dependent (Boyko et al. 2010b).

Previously, we have shown that the increase in RR in response to stress is much higher in tobacco as compared to Arabidopsis (Filkowski et al. 2004b). Thus, although we have not analyzed the response of tobacco plants to KCl, we assume that the RR increase will be at least equal to the one observed in Arabidopsis.

These data strongly support the use of potassium chloride in growth media for enhancing HRF and improving plant transformation. Importantly, the effectiveness of such approach was successfully demonstrated for ammonium nitrate, one of five MS macrosalts that are normally present in standard MS medium (Boyko et al. 2009).

The presence of rare earth elements increases the frequency of homologous recombination in *N. tabacum*

Our previous studies demonstrated that enrichment of growth media with ammonium nitrate has a significant positive influence on HRF and can improve the efficiency of *Agrobacterium*-mediated plant transformation (Boyko et al. 2009). We believe that these influences can at least be partially mediated by the positive effect of enhanced nitrogen metabolism on plant growth, development and differentiation (Forde 2002; Cao et al. 2007; Weiping et al. 2003; Yin et al. 2009).

Our data demonstrate a new finding that the presence of rare earth elements in plant growth environment can increase HRF. Furthermore, these findings are consistent with our hypothesis and support a hypothetical link between nitrogen metabolism and HR activity. Both rare earth elements analyzed in this study had significant inducing effect on HRF. However, in contrast to previously described effects of ammonium nitrate (Boyko et al. 2009) and potassium chloride, the effect of cerium (III) and lanthanum (III) on HR activity was not dose-dependent and was observed only at well-defined concentration range: 0.1–1.0 μM for cerium (III) and 1.0 μM for lanthanum (III). The simultaneous presence of these two elements in media did not increase the activity of HR, as compared to media where only one of two analyzed elements was present. This response may be explained by a small overlap between the optimal concentration ranges of cerium (III) and lanthanum (III).

The presence of rare earth elements promotes growth in *N. tabacum*

The observed positive effects of cerium (III) and lanthanum (III) chlorides on root growth and rooting are interesting findings. Unfortunately, published data that describe the effects of cerium (III) and lanthanum (III) on plant growth are contradictory and require further clarifications. While some authors reported no or little effect on root growth (Diatloff et al. 2008; Hu et al. 2002), others observed a significant increase of root length and root number in response to cerium (III) and lanthanum (III) treatments (Weiping et al. 2003). The discrepancy between the published data can be attributed to the fact that the spectrum of plant species and concentrations of cerium (III) and lanthanum (III) tested differed among the studies.

Plant roots are the primary bioaccumulation site for cerium (III) and lanthanum (III), and the uptake of these two elements positively correlates with their concentration in growth media (Diatloff et al. 2008; Hu et al. 2002). Both cerium (III) and lanthanum (III) were shown to increase chlorophyll content, and to accelerate photosynthetic light reactions (Chen et al. 2001; Yuguan et al. 2009). Consistently, supplementing growth media with cerium (III) under conditions of magnesium (Yuguan et al. 2009) and calcium (Hao et al. 2008; Huang et al. 2008) deficiency was sufficient to alleviate the negative effects on photosynthesis.

The strong positive impact of rare earth elements on plant growth observed in our study can also be explained by the accelerated nitrogen metabolism. A number of independent studies have showed that the presence of cerium (III) and lanthanum (III) in growth media promoted protein synthesis and ammonia assimilation, and increased activities of nitrate reductase, glutamine synthetase, and glutamate dehydrogenase enzymes (Cao et al. 2007; Weiping et al. 2003; Yin et al. 2009). Indeed, we observed a significant gain of biomass in treatments, where cerium (III) and lanthanum (III) were used at their optimal concentrations.

The presence of potassium and cerium (III) chlorides improves the frequency of calli regeneration and of stable T-DNA integrations

It was previously demonstrated that the activity of host DNA repair proteins plays a critical role during T-DNA integration. In fact, it has been shown that simultaneous inactivation of HR and NHEJ pathways by mutations in *RAD52* and *KU70* genes completely inhibits T-DNA integration in yeast (van Attikum and Hooykaas 2003). We hypothesized that an increase of HRF in plants subjected to tissue culture and transgene delivery should result in the increase in the frequency of transformation events. This hypothesis was consistent with our previous data demonstrating that high ammonium nitrate concentrations in growth media could increase HRF and significantly improve the transformation yield (Boyko et al. 2009). Our current results confirmed the hypothesis and demonstrated a positive effect of KCl and $CeCl_3$ on plant transformation. The best results by far were obtained when KCl was used. KCl in the concentration of 50 mM was nearly as effective as ammonium nitrate in the concentration of 61.8 mM.

As to cerium (III) chloride, we showed here that exposure even to the very low concentration of 0.1 μM improved transformation frequency. This implies that cerium can be used simultaneously with other chemicals, like ammonium nitrate, to potentiate their effect on plant transformation. Manipulation of the amount of macrosalts in growth media has its own physical and physiological limit: very high salt concentrations depress the water potential of media and are usually toxic to plants. Since cerium (III) chloride is active at such low concentration, it can be an ideal additive for improvement of transformation frequency. In contrast, KCl should probably be used with caution, because the concentration higher than 50 mM results in some visible physiological changes in plants. The individual range of KCl to be used for improvement of transformation should be estimated for each plant species separately.

It is noteworthy that previous reports demonstrated that even very small quantities of different microelements in growth media could significantly improve SE. Supplementing standard MS medium with higher quantities of cupric sulphate promoted tissue regeneration on callus induction and plant regeneration media (Kothari et al. 2004). Similarly, zinc sulphate was shown to be essential for regeneration and proper growth of shoots (Kothari et al. 2004). In contrast, media devoid of zinc sulphate enhanced regenerative callus formation (He et al. 1991; Kothari et al. 2004). Silver nitrate in media improved the total embryo yield in anther culture in *Brassica* (Dias and Martins 1999; Sahrawat et al. 2003).

Exposure to KCl and $CeCl_3$ results in the less frequent deletions at the 5′ of the transgene

Our data showed that exposure to KCl and $CeCl_3$ resulted in the increased intactness of the right border of T-DNA. Substantial amount of data exists on the analysis of T-DNA integration sites; insertions and deletions of various sizes were observed (Fladung 1999; Kumar and Fladung 2002; Meza et al. 2002; Tinland et al. 1995; Windels et al. 2003). According to published literature, the 5′-end (the right border) of the T-DNA seems to be better preserved than the 3′-end (the left border) (Brunaud et al. 2002; Rossi et al. 1996; Tinland et al. 1995). This indeed was the case in our current work (Fig. 5) as well as in the case of our recently published paper (Boyko et al. 2009).

The difference between changes at the right and left T-DNA is probably due to the mechanism(s) of T-DNA integration. Three different mutually exclusive models that explain T-DNA integration were incorporated to a hypothetical model (reviewed in Tzfira et al. 2004). This model suggests that the intactness of the 5′-end is explained by attachment of VirD2. At the same time, invading T-strand undergoes minor degradation at the unprotected 3′-end. Additional loss of nucleotides from the 3′-end could occur upon conversion of a single stranded T-DNA to a double stranded T-DNA form. Converted dsT-DNA undergoes integration into the host DNA via NHEJ or HR pathways. It is suggested that VirD2 alone or together with host protein searches for microhomology and anneals to the DNA at available transient strand breaks. Then, the 5′-end overhangs are filled-in, and the dsT-DNA is ligated into DNA break in the genome.

We observed the exposure to KCl and $CeCl_3$ to increase the rate of protection at the right border. Since these compounds increase the HRF as well, it is possible that there is a more frequent or perhaps more efficient involvement of recombination proteins in the transgene integration, and thus better ability either to search for microhomology or to form the protein/DNA microfilaments. This could allow for better protection of the invading 5′ T-DNA strand. The exact mechanism remains to be uncovered.

Conclusion

In this study we described the effect of potassium chloride and salts of rare earth elements on the frequency of HR, plant growth rate and plant transformation efficiency. The data demonstrated that all tested chemicals have the potential for improving the efficiency of plant transformation. We also think that the simultaneous application of different transformation-enhancing chemicals in growth media could result in potentiating their effect on

transformation efficiency. From this viewpoint, rare earth elements represent the most likely candidates for potentiating the influence of other macrosalts on the effectiveness of *Agrobacterium*-mediated genetic transformation.

Acknowledgments We would like to thank Zoë Migicovsky for proofreading the manuscript. The authors acknowledge NSERC Discovery, NSERC Strategic, Alberta Agriculture Research Institute and HFSP grant to I.K.

References

Boyko A, Hudson D, Bhomkar P, Kathiria P, Kovalchuk I (2006a) Increase of homologous recombination frequency in vascular tissue of Arabidopsis plants exposed to salt stress. Plant Cell Physiol 47:736–742

Boyko A, Zemp F, Filkowski J, Kovalchuk I (2006b) Double-strand break repair in plants is developmentally regulated. Plant Physiol 141:488–497

Boyko A, Matsuoka A, Kovalchuk I (2009) High frequency *Agrobacterium tumefaciens*-mediated plant transformation induced by ammonium nitrate. Plant Cell Rep 28:737–757

Boyko A, Blevins T, Yao Y, Golubov A, Bilichak A, Ilnytskyy Y, Hollander J, Meins F Jr, Kovalchuk I (2010a) Transgenerational adaptation of Arabidopsis to stress requires DNA methylation and the function of Dicer-like proteins. PLoS One 5:e9514

Boyko A, Golubov A, Bilichak A, Kovalchuk I (2010b) Chlorine ions but not sodium ions alter genome stability of *Arabidopsis thaliana*. Plant Cell Physiol 51(6):1066–1078

Brunaud V, Balzergue S, Dubreucq B, Aubourg S, Samson F, Chauvin S, Bechtold N, Cruaud C, DeRose R, Pelletier G, Lepiniec L, Caboche M, Lecharny A (2002) T-DNA integration into the Arabidopsis genome depends on sequences of pre-insertion sites. EMBO Rep 3:1152–1157

Cao R, Huang XH, Zhou Q, Cheng XY (2007) Effects of lanthanum(III) on nitrogen metabolism of soybean seedlings under elevated UV-B radiation. J Environ Sci (China) 19:1361–1366

Chen WJ, Tao Y, Gu YH, Zhao GW (2001) Effect of lanthanide chloride on photosynthesis and dry matter accumulation in tobacco seedlings. Biol Trace Elem Res 79:169–176

Choi Y-E, Yang D-C, Choi K-T (1998) Induction of somatic embryos by macrosalt stress from mature zygotic embryos of *Panax ginseng*. Plant Cell, Tissue Cult Organ Cult 52:117–181

Chugh A, Khurana P (2002) Gene expression during somatic embryogenesis—recent advances. Curr Sci 83:715–729

Dias JS, Martins MG (1999) Effect of silver nitrate on anther culture embryo production of different *Brassica oleracea* morphotypes. Sci Hortic 82:299–307

Diatloff E, Smith FW, Asher CJ (2008) Effects of lanthanum and cerium on the growth and mineral nutrition of corn and mungbean. Ann Bot 101:971–982

Filkowski J, Kovalchuk O, Kovalchuk I (2004a) Genome stability of vtc1, tt4, and tt5 Arabidopsis thaliana mutants impaired in protection against oxidative stress. Plant J 38:60–69

Filkowski J, Kovalchuk O, Kovalchuk I (2004b) Dissimilar mutation and recombination rates in Arabidopsis and tobacco. Plant Science 166:265–272

Fladung M (1999) Gene stability in transgenic aspen (*Populus*). I. Flanking DNA sequences and T-DNA structure. Mol Gen Genet 260:574–581

Forde BG (2002) Local and long-range signaling pathways regulating plant responses to nitrate. Annu Rev Plant Biol 53:203–224

Gorbunova VV, Levy AA (1999) How plants make ends meet: DNA double-strand break repair. Trends Plant Sci 4:263–269

Greer MS, Kovalchuk I, Eudes F (2009) Ammonium nitrate improves direct somatic embryogenesis and biolistic transformation of *Triticum aestivum*. N Biotechnol 26:44–52

Hao H, Ling C, Xiaoqing L, Chao L, Weiqian C, Yun L, Fashui H (2008) Absorption and transfer of light and photoreduction activities of spinach chloroplasts under calcium deficiency: promotion by cerium. Biol Trace Elem Res 122:157–167

Hasegawa PM, Bressan RA, Zhu JK, Bohnert HJ (2000) Plant cellular and molecular responses to high salinity. Annu Rev Plant Physiol Plant Mol Biol 51:463–499

He D-G, Yang Y-M, Scott KJ (1989) The effect of macroelements in the induction of embryogenic callus from immature embryos of wheat (*Triticum aestivum* L.). Plant Sci 64:251–258

He D-G, Yang Y-M, Scott KJ (1991) Zinc deficiency and the formation of white structures in immature embryo cultures of wheat (*Triticum aestivum* L.). Plant Cell Tissue Organ Cult 24(1):9–12

Hu X, Ding Z, Chen Y, Wang X, Dai L (2002) Bioaccumulation of lanthanum and cerium and their effects on the growth of wheat (*Triticum aestivum* L.) seedlings. Chemosphere 48:621–629

Huang H, Liu X, Qu C, Liu C, Chen L, Hong F (2008) Influences of calcium deficiency and cerium on the conversion efficiency of light energy of spinach. Biometals 21:553–561

Ilnytskyy Y, Boyko A, Kovalchuk I (2004) Luciferase-based transgenic recombination assay is more sensitive than beta-glucoronidase-based. Mutat Res 559:189–197

Immonen AST (1996) Influence of media and growth regulators on somatic embryogenesis and plant regeneration for production of primary triticales. Plant Cell Tissue Organ Cult 44(1):45–52

Jiménez JM (2001) Regulation of in vitro somatic embryogenesis with emphasis on the role of endogenous hormones. Revista Brasileira de Fisiologia Vegetal 13(2):196–223

Khanna HK, Daggard GE (2003) *Agrobacterium tumefaciens*-mediated transformation of wheat using a superbinary vector and a polyamine-supplemented regeneration medium. Plant Cell Rep 21(5):429–436

Kim SI, Veena, Gelvin SB (2007) Genome-wide analysis of *Agrobacterium* T-DNA integration sites in the Arabidopsis genome generated under non-selective conditions. Plant J 51:779–791

Kothari SL, Agarwal K, Kumar S (2004) Inorganic nutrient manipulation for highly improved in vitro plant regeneration in finger millet—*Eleusine coracana* (L.) Gaertn. In Vitro Cell Dev Biol - Plant 40:515–519

Kovalchuk I, Kovalchuk O, Hohn B (2000) Genome-wide variation of the somatic mutation frequency in transgenic plants. EMBO J 19:4431–4438

Kumar S, Fladung M (2002) Transgene integration in aspen: structures of integration sites and mechanism of T-DNA integration. Plant J 31:543–551

Li DD, Shi W, Deng XX (2003) Factors influencing *Agrobacterium*-mediated embryogenic callus transformation of Valencia sweet orange (*Citrus sinensis*) containing the pTA29-barnase gene. Tree Physiol 23:1209–1215

Liu Y, Stasiak AZ, Masson JY, McIlwraith MJ, Stasiak A, West SC (2004) Conformational changes modulate the activity of human RAD51 protein. J Mol Biol 337:817–827

Maës OC, Chibbar RN, Caswell K, Leung N, Kartha KK (1996) Somatic embryogenesis from isolated scutella of wheat: effects of physical, physiological and genetic factors. Plant Sci 121:75–84

Malabadi RB, Staden JV (2006) Cold enhanced somatic embryogenesis in *Pinus patula* is mediated by calcium. S Afr J Bot 72(4):613–618

Meza TJ, Stangeland B, Mercy IS, Skarn M, Nymoen DA, Berg A, Butenko MA, Hakelien AM, Haslekas C, Meza-Zepeda LA, Aalen RB (2002) Analyses of single-copy Arabidopsis T-DNA-transformed lines show that the presence of vector backbone sequences, short inverted repeats and DNA methylation is not sufficient or necessary for the induction of transgene silencing. Nucleic Acids Res 30:4556–4566

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Perl A, Kless H, Blumenthal A, Galili G, Galun E (1992) Improvement of plant regeneration and GUS expression in scutellar wheat calli by optimization of culture conditions and DNA-microprojectile delivery procedures. Mol Gen Genet 235:279–284

Racusen RH, Schiavone FM (1990) Positional cues and differential gene expression in somatic embryos of higher plants. Cell Differ Dev 30:159–169

Rossi L, Hohn B, Tinland B (1996) Integration of complete transferred DNA units is dependent on the activity of virulence E2 protein of *Agrobacterium tumefaciens*. Proc Natl Acad Sci USA 93:126–130

Sahrawat AK, Becker D, Lütticke S, Lörz H (2003) Genetic improvement of wheat via alien gene transfer, an assessment. Plant Sci 165:1147–1168

Shim KS, Schmutte C, Yoder K, Fishel R (2006) Defining the salt effect on human RAD51 activities. DNA Repair (Amst) 5:718–730

Shrivastav M, De Haro LP, Nickoloff JA (2008) Regulation of DNA double-strand break repair pathway choice. Cell Res 18:134–147

Sigurdsson S, Trujillo K, Song B, Stratton S, Sung P (2001) Basis for avid homologous DNA strand exchange by human Rad51 and RPA. J Biol Chem 276:8798–8806

Swoboda P, Gal S, Hohn B, Puchta H (1994) Intrachromosomal homologous recombination in whole plants. EMBO J 13:484–489

Takumi S, Shimada T (1997) Variation in transformation frequencies among six common wheat cultivars through particle bombardment of scutellar tissues. Genes Genet Syst 72:63–69

Tinland B, Schoumacher F, Gloeckler V, Bravo-Angel AM, Hohn B (1995) The *Agrobacterium tumefaciens* virulence D2 protein is responsible for precise integration of T-DNA into the plant genome. EMBO J 14:3585–3595

Tzfira T, Li J, Lacroix B, Citovsky V (2004) *Agrobacterium* T-DNA integration: molecules and models. Trends Genet 20:375–383

van Attikum H, Hooykaas PJ (2003) Genetic requirements for the targeted integration of *Agrobacterium* T-DNA in *Saccharomyces cerevisiae*. Nucleic Acids Res 31:826–832

Weiping S, Fashui H, Zhigang W, Yuzhen Z, Fugen G, Hongoing X, Mingliang Y, Youhong C, Mizhen Z, Jiale S (2003) Effects of cerium on nitrogen metabolism of peach plantlet in vitro. Biol Trace Elem Res 95:259–268

Windels P, De Buck S, Van Bockstaele E, De Loose M, Depicker A (2003) T-DNA integration in Arabidopsis chromosomes. Presence and origin of filler DNA sequences. Plant Physiol 133:2061–2068

Yin S, Ze Y, Liu C, Li N, Zhou M, Duan Y, Hong F (2009) Cerium relieves the inhibition of nitrogen metabolism of spinach caused by magnesium deficiency. Biol Trace Elem Res 132(1–3): 247–258

Yuguan Z, Min Z, Luyang L, Zhe J, Chao L, Sitao Y, Yanmei D, Na L, Fashui H (2009) Effects of cerium on key enzymes of carbon assimilation of spinach under magnesium deficiency. Biol Trace Elem Res 131:154–164

Zhu JK (2000) Genetic analysis of plant salt tolerance using Arabidopsis. Plant Physiol 124:941–948

Zhu JK (2002) Salt and drought stress signal transduction in plants. Annu Rev Plant Biol 53:247–273