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GENETIC TRANSFORMATION AND HYBRIDIZATION

High frequency *Agrobacterium tumefaciens*-mediated plant transformation induced by ammonium nitrate

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Abstract Success in plant genetic transformation depends on the efficiency of explant regeneration and transgene integration. Whereas the former one depends on explant totipotency, the latter depends on the activity of host DNA repair and chromatin organisation factors. We analyzed whether factors that result in an increase in recombination frequency can also increase transformation efficiency. Here, we report that a threefold increase in the concentration of NH_4NO_3 in the growth medium results in more than a threefold increase in the *Agrobacterium tumefaciens*-mediated transformation frequency of *Nicotiana tabacum* plants. Regeneration of calli without selection showed that the increase in transformation frequency was primarily due to the increase in transgene integration efficiency rather than in tissue regeneration efficiency. PCR analysis of insertion sites showed a decrease in the frequency of truncations of the T-DNA right border and an increase on the left border. We hypothesize that exposure to ammonium nitrate modifies the activity of host factors leading to higher frequency of transgene integrations and possibly to the shift in the mechanism of transgene integrations.

Keywords Plant transformation efficiency · T-DNA integration · *Agrobacterium tumefaciens* · *Nicotiana tabacum* · Homologous recombination · Ammonium nitrate

Abbreviations

HR	Homologous recombination
NHEJ	Non-homologous end-joining
DSB	Double strand break
ssDNA	Single stranded DNA
ssT-DNA	Single stranded T-DNA
dsT-DNA	Double stranded T-DNA
HRF	Homologous recombination frequency
RR	Recombination rate
CRE	Callus regeneration efficiency
STF	Stable transformation frequency
GUS	*uidA* (β-glucuronidase) gene
LUC	Luciferase gene
DSBR	DSB repair (model)
SSGR	Single-strand-gap repair (model)
Dpg	Days post germination
MS	Murashige-Skoog (media)

Introduction

Sustainable agriculture relies on the ability of industry to quickly adjust to the needs of growers and society by deploying new plants with improved/modified traits and enhanced value. Conventional agriculture relying on selection and traditional breeding programs is incapable of meeting these needs rapidly. New systems are essential for the delivery of next-generation plants with novel traits to exploit the potential of a crops-based and bioproducts industry to meet societal and environmental needs. The application of transgenic approaches to crop improvement

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is an essential element of short- and long-term strategies to exploit plant genomics for overcoming yield impediments faced by producers and to develop novel bioproducts of interest to industry (Martino-Catt and Sachs 2008). As such, transgene-centered approaches provide new economic opportunities that will promote a strong agricultural industry and ensure sustainable agriculture through the provision of novel germplasm capable of withstanding abiotic and biotic environmental stresses.

Success in generation of transgenic plants depends on two major factors: regeneration capacity of explants and transgene integration. Whereas the former one depends on totipotency, the ability of a cell to dedifferentiate and give rise to a new plantlet, the latter depends on multiple factors, including transgene delivery into the host cell cytoplasm, transport through cytoplasm and nucleus, and integration into the host genome (van Attikum and Hooykaas 2003; Citovsky et al. 2007). Lack of success in regeneration of transgenic plants from certain plant species could be explained either by poor regeneration of explants and poor acceptance of transgenes or, frequently, by both. It can be hypothesized that successful transgenesis occurs when regeneration capacity of a cell co-insides with its ability to accept a transgene.

Successful transgene integration into the recipient genome depends on the activity of host DNA repair proteins (van Attikum and Hooykaas 2003; Citovsky et al. 2007). It is noteworthy that at various stages of *Agrobacterium tumefaciens*-mediated T-DNA delivery and integration, including delivery of the T-complex to the host cell cytoplasm, transport through the cytoplasm and nuclear import, intracellular transport, T-DNA uncoating and integration, the activity and factors of various host cellular mechanisms are actively utilized by *Agrobacterium* to complete transformation (reviewed in Citovsky et al. 2007). In fact, transgene integration is preferentially targeted to double strand breaks (DSBs) in the host genome (Chilton and Que 2003; Tzfira et al. 2003). DSBs are created as a result of direct DNA damage or as a transient step during DNA repair.

There are two major evolutionarily conserved DNA repair pathways available in a cell that can repair DSBs. These are homologous recombination (HR) and non-homologous end-joining (NHEJ). They both compete for the DSB substrate, and as their names suggest, they have different requirements with respect to a repair template (high homology vs. microhomology), thereby offering a different fidelity of DNA repair (Salomon and Puchta 1998; Gorbunova and Levy 1999; Orel et al. 2003; Dudas and Chovanec 2004; Puchta 2005; Bleuyard et al. 2006; Shrivastav et al. 2008). In general, the balance between these two competitors is tightly controlled, and it depends on the availability of repair templates, cell cycle phase, proliferation rate, and functions of specific cell types (reviewed in Shrivastav et al. 2008).

RAD51 plays a critical role in HR via the formation of the Rad51/ssDNA nucleofilament based on the 3′ overhang of DSBs, and it also promotes strand exchange reactions (Bleuyard et al. 2006). The KU heterodimer that consists of Ku70 and Ku80 mediates NHEJ and represents a part of DNA–PK. KU binds to various types of DNA ends including single-stranded gaps, helps protect them and forms a bridge between two ends of a break, thereby contributing to their juxtaposition (Bleuyard et al. 2006 and references therein).

Since T-DNA integration requires open chromatin and strand breaks in particular, it is possible to assume that T-DNA has to interact with NHEJ or HR factors during the last step of *Agrobacterium*-mediated transformation. Such an interaction was indeed suggested recently; Li et al. (2005) showed the physical interaction between Ku80 and ds-T-DNA prior to integration.

Different fidelity of DNA repair by HR and NHEJ is reflected in the precision and intactness of transgene integration via either of these two pathways. While the involvement of NHEJ usually results in multiple transgene insertions containing deletions and filling sequences (Salomon and Puchta 1998; Chilton and Que 2003), HR can mediate precise site-specific transgene integrations, which makes it a highly desired tool for gene targeting (Vergunst and Hooykaas 1999; Puchta 2002; Reiss 2003). Consequently, factors that could promote HR and/or inhibit NHEJ can considerably facilitate the improvement of *Agrobacterium*-mediated genetic transformation and gene targeting in particular.

Previous studies indeed showed that expression of the yeast *RAD54* gene, a member of the SWI2/SNF2 superfamily of chromatin remodelling genes in *Arabidopsis,* increased the gene targeting frequency by one to two orders of magnitude (Shaked et al. 2005). This specific induction is consistent with reduced rates of gene targeting previously reported in yeast, mouse stem cells, and chicken DT40 cells with the inactivated *RAD54* gene or its homologs (Bezzubova et al. 1997; Essers et al. 1997). An important role of chromatin remodelling factors in controlling the frequency of HR was also supported by early studies of Hanin et al. (2000) who identified the *Arabidopsis MIM* gene encoding for a protein belonging to the SMS (structure maintenance of chromosomes) family. Overexpression of *MIM* gene in plant cells resulted in a twofold increase in the intrachromosomal HR frequency as compared with the wild type (Hanin et al. 2000). Mysore et al. (2000) demonstrated that overexpression of another factor regulating the chromatin structure, the *RAT5* histone H2A gene in wild-type *Arabidopsis* plants also increases transformation efficiency.

Overexpression of DNA repair genes derived from different organisms in plant cells may increase the frequency of site-specific integration events. In fact, expression of the *E. coli RecA* gene in transgenic tobacco plants increased the frequency of HR between sister chromatids by 2.4-fold as compared with wild type plants (Reiss et al. 2000). In a similar way, overproduction of the bacterial resolvase RuvC resulted in more than a tenfold increase of somatic crossover and intrachromosomal recombination in transgenic tobacco plants (Shalev et al. 1999). Nevertheless, despite its positive effect on HR, overproduction of RecA and RuvC in plant cells had no significant effect on the gene targeting frequency.

Inactivation of host genes that negatively influence T-DNA transformation and the HR pathway may represent an alternative strategy. In fact, mutation in the *Arabidopsis AtRad50* gene that encodes a protein involved in the NHEJ pathway stimulated HR and caused a hyper-recombinant phenotype (Gherbi et al. 2001). Using *Neurospora* strains deficient in *KU70* and *KU80* homologs, the studies of Ninomiya et al. (2004) demonstrated that the rate of HR and gene targeting can be greatly increased by suppressing or blocking the NHEJ repair pathway. Similarly, *Arabidopsis fas1* and *fas2* mutant plants deficient in the activity of a CAF-1 protein involved in nucleosome assembly and chromatin condensation were hypersusceptible to *Agrobacterium* transformation (Endo et al. 2006). Moreover, *fas* mutants displayed a hyper-recombinant phenotype which was consistent with enhanced transcription of *AtRad51* and *AtRad54* genes (Endo et al. 2006).

We hypothesised that using factors that increase HRF could influence the transformation efficiency and possibly the quality of transgene integrations. In several pilot experiments, we identified ammonium nitrate as a macro-salt component capable of increasing HRF. We analyzed the ability of high concentrations of ammonium nitrate to influence the *Agrobacterium*-mediated transformation efficiency of tobacco and found more than a threefold increase in the number of regenerated transgenic plants. We found that the primary mechanism responsible for the increase was the improvement of transgene integration rather than tissue regeneration capacity.

Materials and methods

Plants used in the experiment

In the current work, *Nicotiana tabacum* cultivar Big Havana wild type plants and transgenic *A. thaliana* line #11 plants were used. *A. thaliana* plants that carry in the genome two truncated non-functional overlapping copies of the *uid*A (encoding β-glucuronidase, GUS) gene as a homologous recombination substrate (Supplementary Fig. 1) have been previously described (Swoboda et al. 1994; Ilnytskyy et al. 2004). DSB repair by HR in the region of homology results in a recombination event that restores the reporter gene, thereby activating β-glucuronidase (GUS) (Supplementary Fig. 1). In all cases, plants were grown in high light conditions (32.8 $\mu Em^{-2} s^{-1}$) at 22°C under the 16 h light regime and at 18°C under the 8 h dark regime at a constant humidity of 65%.

Analysis of the influence of modifications of MS medium on homologous recombination frequency

The effect of various chemicals on HRF was tested using *Arabidopsis* line #11 plants that carried a GUS-based recombination substrate in the genome (Swoboda et al. 1994; Ilnytskyy et al. 2004). *Arabidopsis* seeds were surface-sterilized and plated on MS medium deprived of one of the following components: sucrose, EDTA, NT micro, $FeCl_3 \cdot 6H_2O$, B5 vitmains, 2-(*N*-morpholino) ethanesulfonic acid (MES), MS macro (Supplementary Table 1; Experiment #II). HRF was analyzed at 21 days post germination (dpg). Three independent experiments were performed.

After identification of MS macro as a mixture of components having the most dramatic influence on HRF, *Arabidopsis* seeds were plated on MS medium deprived of one of MS macro components (Supplementary Table 1; Experiment #II).

After identification of ammonium nitrate as a component having the most substantial influence on HRF, *Arabidopsis* seeds were plated on MS medium supplemented with various concentrations of ammonium nitrate. To get ammonium nitrate as a single source of nitrogen in the medium, potassium nitrate originally present in MS medium was substituted with potassium sulfate (Table 1). The modified MS medium containing 20.6 mM ammonium nitrate (an amount naturally present in the standard MS medium) (Murashige and Skoog 1962) was used as a control. Then, *Arabidopsis* seeds were germinated and grown on the MS medium supplemented with different concentrations of NH_4NO_3 (Table 1), and HRF was again analyzed. Three independent experiments were performed.

Analysis of homologous recombination events in *Arabidopsis*

Analysis of HRF was performed as previously described (Boyko et al. 2006). Plantlets were histochemically stained as described before (Boyko et al. 2006). Recombination events were observed as sectors of blue. The frequency of HR was calculated by counting the number of HR events (sectors) in each plant separately, summing it up and then

Table 1 Medium compositions for the analysis of the effect of ammonium nitrate on transformation efficiency in *N. tabacum*

Control: MS macro (mM)		Experimental media compositions, all final concentrations listed in mM				
		NH_4NO_3 0.3×	NH_4NO_3 1×	NH_4NO_3 2.5×	NH_4NO_3 3×	NH_4NO_3 5×
NH_4NO_3	20.6	6.18	20.6	51.5	61.8	103
KNO_3	18.8	–	–	–	–	–
$CaCl_2$	3	3	3	3	3	3
$MgSO_4$	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
K_2SO_4	–	9.4	9.4	9.4	9.4	9.4

Composition of the media used for the experiments. To have ammonium nitrate as the only source of nitrate in MS medium, potassium nitrate was substituted with potassium sulphate

relating it to the number of plants in the tested population of 200 plants.

The recombination rate (RR) was calculated by relating HRF to the number of haploid genomes per plant. It represented the number of HR events per haploid genome. 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 (Swoboda et al. 1993) and the number of plants used for DNA preparation. To avoid a bias during DNA preparation, DNA was extracted using two different methods.

DSB measurement (the ROPS assay)

Quantification of 3′OH DNA breaks was performed as previously described (Basnakian and James 1996; Boyko et al. 2007). Two independent measurements from each of three independent experiments were done.

Preparation of *N. tabacum* plants for Agro-mediated transformation

Seeds of the wild type *N. tabacum* cultivar Big Havana were surface-sterilized and plated on MS medium containing various quantities of ammonium nitrate (Table 1). For transient transformation testing, one week-old seedlings were harvested from Petri dishes for transformation with *Agrobacterium*.

For stable integration, three to five 1-week-old *N. tabacum* wild type plants were transferred to a single sterile 250 ml glass flask containing 15 ml of sterile control or modified liquid MS medium. Then, flasks were installed on an orbital shaker, and plants were continuously grown under the aforementioned growth conditions at 50–75 rpm. The growth medium was replaced weekly with 25 ml of fresh medium. Three weeks later, plants were removed from flasks, and 2–3 pairs of fully developed 2–4 cm long fresh leaves were harvested for transformation with *Agrobacterium*. Following transformation, regenerated transgenic plants were grown and propagated on soil at 22°C/18°C, under the 16/8 h light/dark regime. Transformation experiments were repeated three times, with 3–4 months period of time between repetitions.

Agrobacterium strains and constructs used in the experiments

Two *Agrobacterium* GV3101 strains containing pPM6000 helper plasmid were used for transformation. One strain carried T-DNA containing the active *uid*A gene driven by the 35S CaMV promoter and the *barnase* gene as an herbicide resistance marker. Another strain carried a T-DNA cassette containing the active *luciferase* (LUC) gene driven by the *N-gene* promoter and the *hph* gene that confers resistance to antibiotic hygromycin as a selection marker. The ‘35S::GUS’ T-DNA construct was used to analyze efficiency of transient and stable transformation, whereas the ‘N::LUC’ was used for stable transformation analysis only.

Agrobacterial strains were grown to a final optical density of 0.6 measured at 600 nm and prepared for transformation as previously described (Kovalchuk et al. 2000).

For the analysis of transient transformation efficiency, cotyledons were submersed into the ‘35S::GUS’ *Agrobacterium* culture and vacuum-infiltrated for 10 min. Once infiltrated, they were blotted dry, placed on solid MS medium and incubated in the dark at 22°C for 3 days. Next, cotyledons were stained with X-gluc for the evaluation of transient transformation efficiency (Kovalchuk et al. 2000).

For the stable transformation efficiency, leaves were blotted on sterile filter paper and then submersed into a Petri dish laid out with Whatman paper socked with *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. The main vein and leaf margins were left intact. When cutting

was completed, leaves were allowed to be submersed for 10 min, and 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 a combination of ticarcillin (100 mg/L) with potassium clavulanate (3 mg/L) to control *Agrobacterium* growth. Selection conditions were obtained by supplementing a regeneration medium with hygromycin (25 mg/L). Non-selective conditions were used for studying effects of ammonium nitrate on the callus regeneration efficiency.

Following 3–4 weeks on callus-inducing medium, the developed shoots were excised from calli and transferred to the root-inducing solid standard MS medium containing NAA (0.5 mg/L), ticarcillin (100 mg/L) and potassium clavulanate (3 mg/L). After 1–2 weeks of root induction, plantlets were transplanted to soil and checked for *luciferase* gene expression.

Luciferase gene-expressing plants were counted as stable transformants, and their number was related to the total number of incisions made on the leaf surface during transformation to obtain a stable transformation frequency (STF). Shoots produced on callus-inducing medium were scored, and their number was related to the total number of incisions made on the leaf surface during transformation to obtain callus regeneration efficiency (CRE).

Visualization of the *luciferase* reporter gene activity in *N. tabacum*

Constitutive expression of the *luciferase* gene in stable transformants regenerated from tobacco leaves was visualized using a CCD camera (Gloor Instruments; Basel, Switzerland). When transplanted to soil, regenerated plants were topically treated with a 0.5 mM beetle luciferine (Promega), a 0.05% Tween-80 solution and incubated in the dark for 30–45 min. Following incubation, plants were photographed using a CCD camera.

T-DNA segregation analysis

Seeds of self-pollinated transgenic *N. tabacum* plants regenerated after *Agrobacterium* transformation with a N::LUC T-DNA-containing construct were germinated and grown for 3 weeks in solid standard MS medium containing hygromycin (25 mg/L). Following 3 weeks, plants showing an antibiotic resistance phenotype were scored, and a segregation ratio was calculated. For the lines were the results were not clear, the segregation analysis was repeated two more times. Statistical significance of the calculated segregation ratios was confirmed using the Chi square test statistic with $\alpha = 0.05$.

Total DNA and RNA preparation

Total genomic DNA was prepared using the *Gene Elute Plant Genomic DNA Miniprep Kit* (Sigma) according to the manufacturer's protocol.

Total RNA was prepared using the *Trizol* reagent (Invitrogen) accordingly to the manufacturer's protocol. cDNA was prepared using the *Revertaid H-Minus First Strand cDNA Synthesis Kit* according to the manufacturer's protocol (Fermentas).

PCR amplification of left and right borders

To detect the intactness of the left and right borders of integrated T-DNA, several sets of primers were used. Each PCR was performed with a set of four primers, one in the outward (from integrated T-DNA) direction and 3 in the inward direction (Fig. 9). PCR amplification of the left border should result in 3, 2, 1 or 0 fragments of 332, 315 and 153 nt, depending on whether a deletion involves the binding site for primer 1, primer 2 or primer 3, respectively. Similarly, PCR amplification of the right border should result in fragments of 323, 296, 251 nt. Each reaction contained: 0.6 units of *Takara Ex Taq™ DNA Polymerase* (Takara Bio USA), *1x Ex Taq™ Buffer* (contains 2 mM $MgCl_2$) (Takara Bio USA), *dNTP Mixture* (2.5 mM each dNTP) (Takara Bio USA), 10 μM of each primer and 0.5 μg of genomic DNA in a final volume of 25 μl. PCR conditions: 95°C 5 min; 35 cycles of 95°C 30 s min, 57.5 (left border) or 60 (right border) °C 30 s, 72°C 35 s; 72°C 10 min.

The following primers were used. For the left border: 'LB+', 5′-CAC TCG TCC GAG GGC AAA GAA ATA-3′, 'LB1-', 5′-ATT GTG GTG TAA ACA AAT TGA CGC T-3′, 'LB2-', 5′-GAC AAC TTA ATA ACA CAT TGC GGA CG-3′, 'LB3-', 5′-GGG TTT CTT ATA TGC TCA ACA CAT GAG CG-3′. For the right border: 'RB+', 5′-AGG CGC CAC CTT TCT AAT ACC TGT-3′, 'RB1-', 5′-GTT TAA ACT GAA GGC GGG AAA CGA C-3′, 'RB2-', 5′-TCT GAT CCA AGC TCA AGC TGC TCT-3′, 'RB3-', 5′-GCA ACT GTT GGG AAG GGC GAT-3′. The PCR was repeated twice for ambiguous results.

Statistical treatment of data

In all cases, the mean and standard error or standard deviation was calculated. Statistical significance of the experiment was confirmed either by the two-tailed paired Student's *t*-test with $\alpha = 0.05$ or $\alpha = 0.1$ (comparing data from two treatments), or by the single factor ANOVA (comparing data

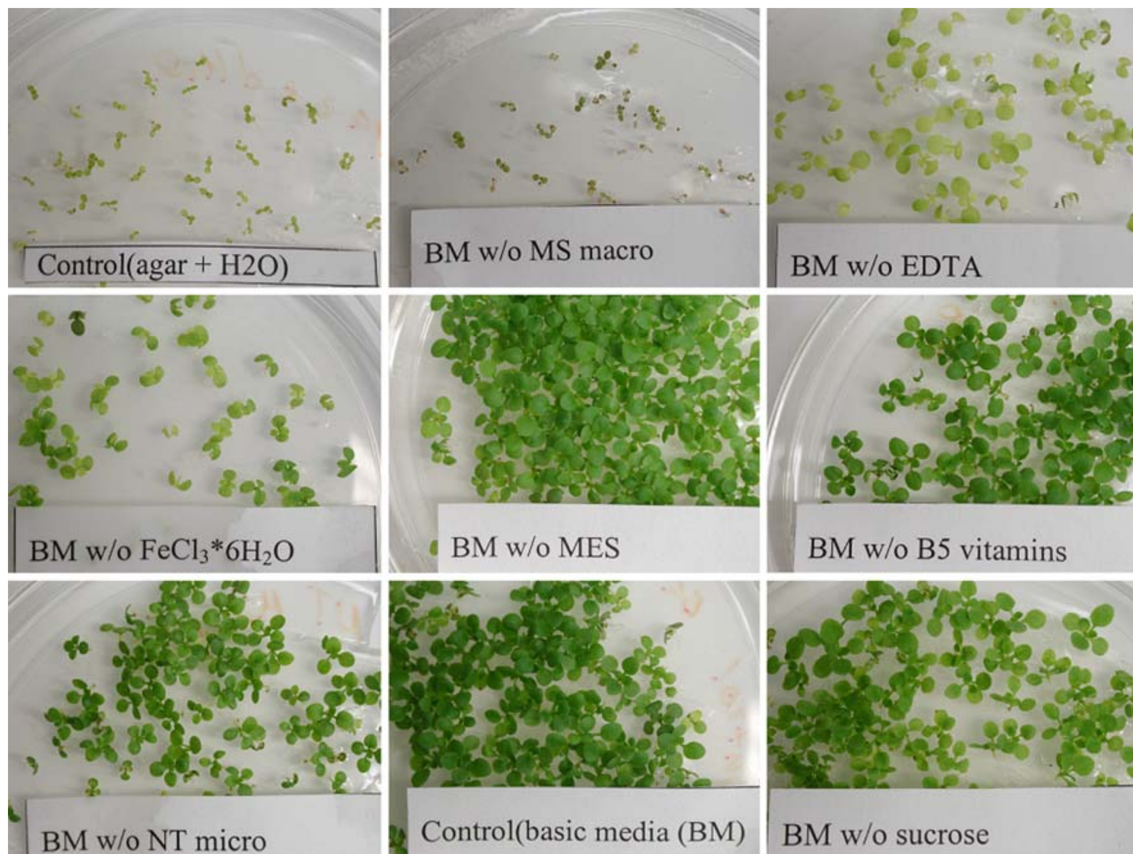

Fig. 1 Physiological appearance of plants grown on the media lacking certain MS components. Plants were germinated on sterile media lacking one of the components or group of components. Pictures were taken at 21 dpg

from three or more treatments). Statistical analysis was performed using the *JMP 5.0* software (SAS Institute Inc).

Results

Selection of growth medium components that influence homologous recombination frequency

While analyzing the homologous recombination frequency (HRF) in *Arabidopsis* and tobacco plants grown in soil and on sterile MS medium, we noted a substantial difference (data not shown). HRF was three to fivefold higher in plants germinated and grown in sterile conditions (data not shown). We hypothesized that MS medium either presents a stress to plants or contains components that increase HRF.

To test the hypothesis, we generated several media compositions based on MS that lack certain components. First, we prepared media that lack one of the following components: sucrose, EDTA, NT micro, $FeCl_3 \cdot 6H_2O$, B5 vitamins, MES, and MS macro. B5 vitamins, MES and MS macro, each represents a complex mixture of chemicals (see the details in Supplementary Table 1). Plants that were germinated and grown for 2 weeks on media lacking sucrose showed a twofold higher level of HRF as compared to control plants. Plants that were germinated and grown on media lacking MS macro components were very small (Fig. 1) and showed extremely low levels of HRF (Fig. 2a). Plants grown without several other components also showed a statistically significant decrease, although changes were not as dramatic as for MS macro (Fig. 2a).

Since plants grown without MS macro components were substantially smaller, it was important to prorate HRF to the number of cells/genomes. To do that, we also calculated the recombination rate (RR), the occurrence of recombination events per single-copy transgene per single cell division. RR was calculated by relating HRF to the total number of genomes present in the cell (see "Materials and methods"). This analysis showed that plants grown without MS macro components have lower RR (Fig. 2b).

Then, we prepared media lacking one of MS macro components. Growing plants on such media showed that NH_4NO_3 is the main chemical compound that contributes to higher RR in plants grown on MS media (Fig. 2c).

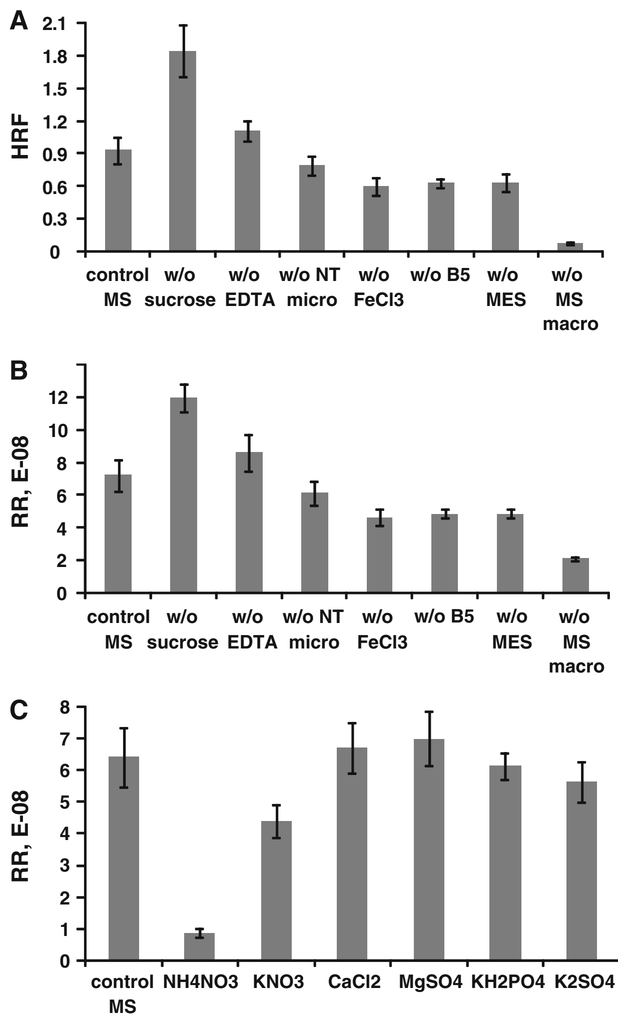

Fig. 2 HRF and RR in plants grown on the media lacking certain MS components. **a** HRF was measured in *Arabidopsis* plants germinated and grown in the media lacking one of the MS components (see "Materials and methods" for details). '*Y*' axis shows the average HRF. Values represent the mean (from three experiments) ± sd. '*X*' axis shows the experimental group. **b** '*Y*' axis shows RR, calculated by relating the HRF (shown in **a**) to the average number of haploid genomes in each experimental group. Values represent the mean (from three experiments) ± sd. **c** '*Y*' axis shows RR in *Arabidopsis* plants grown on media depleted of one of MS macro components. Values represent the mean (from three experiments) ± sd

High concentrations of ammonium nitrate increase recombination rates in *Arabidopsis*

To further analyze the influence of ammonium nitrate on recombination rates, we germinated and grew *Arabidopsis* plants in MS media supplemented with various quantities of ammonium nitrate. To have ammonium nitrate as a single source of nitrogen in all modified media, we substituted potassium nitrate for potassium sulfate (Table 1). The control medium composition was not changed. The following experimental groups were made: control (20.6 mM of NH_4NO_3 and 18.8 mM of KNO_3), 0.3× (6.18 mM of NH_4NO_3), 1× (20.6 mM of NH_4NO_3), 2.5× (51.5 mM of NH_4NO_3) and 5× (103.0 mM of NH_4NO_3), representing unmodified MS and MS media with various content of ammonium nitrate (Table 1).

It is noteworthy that plants grown in the medium containing 2.5× ammonium nitrate looked healthier, grew better and had higher biomass (data not shown) as compared to plants grown in 1.0× ammonium nitrate (Fig. 3). In contrast, plants grown in the medium containing 5× ammonium nitrate displayed growth inhibition and appeared pale green, which possibly indicated the lower chlorophyll content (Fig. 3). This demonstrated a negative effect of 103 mM ammonium nitrate on plant physiology.

Our results confirmed that increased ammonium nitrate concentration leads to the increase in RR in *Arabidopsis*. Plants germinated and grown in 2.5× and 5× ammonium nitrate showed 2.2- and 4.1-fold increases of RR, respectively, as compared to plants grown in the control unmodified MS medium (Student's t test, $\alpha = 0.05$) (Fig. 4a). Consistently, a depletion of ammonium nitrate in the growth medium to 30% of its concentration in the control medium decreased RR by a factor of 1.3 (Fig. 4a). Importantly, only a minor difference of 9% was observed during a comparison of RRs between plants grown in the modified medium containing 20.6 mM of ammonium nitrate and control plants grown in standard unmodified MS (Fig. 4a) This suggests that a substitution of potassium nitrate for potassium sulfate in all modified media did not significantly affect RR. Overall, a strong positive correlation between RR and the amount of ammonium nitrate present in the growth medium was found ($r = 0.99$, $P < 0.05$).

In order to define the best concentration of ammonium nitrate that would increase RR but would not change a plant's appearance, we exposed plants to 1×, 2×, 3×, 4× and 5× ammonium nitrate. We found 3× to be the maximum concentration that increases RR and does not change a plant's appearance (data not shown).

High concentrations of ammonium nitrate do not increase DNA double strand breaks

Ammonium nitrate-induced changes in RR could be due to a higher level of DNA DSBs. Measurement of DSB levels, however, showed a significant (Student's t-test, $\alpha = 0.05$) 1.2-fold decrease in plants grown in 5× and no change in plants grown in 2.5× ammonium nitrate (Fig. 4b), suggesting that the increase in RR was not due to an increase in DSB levels.

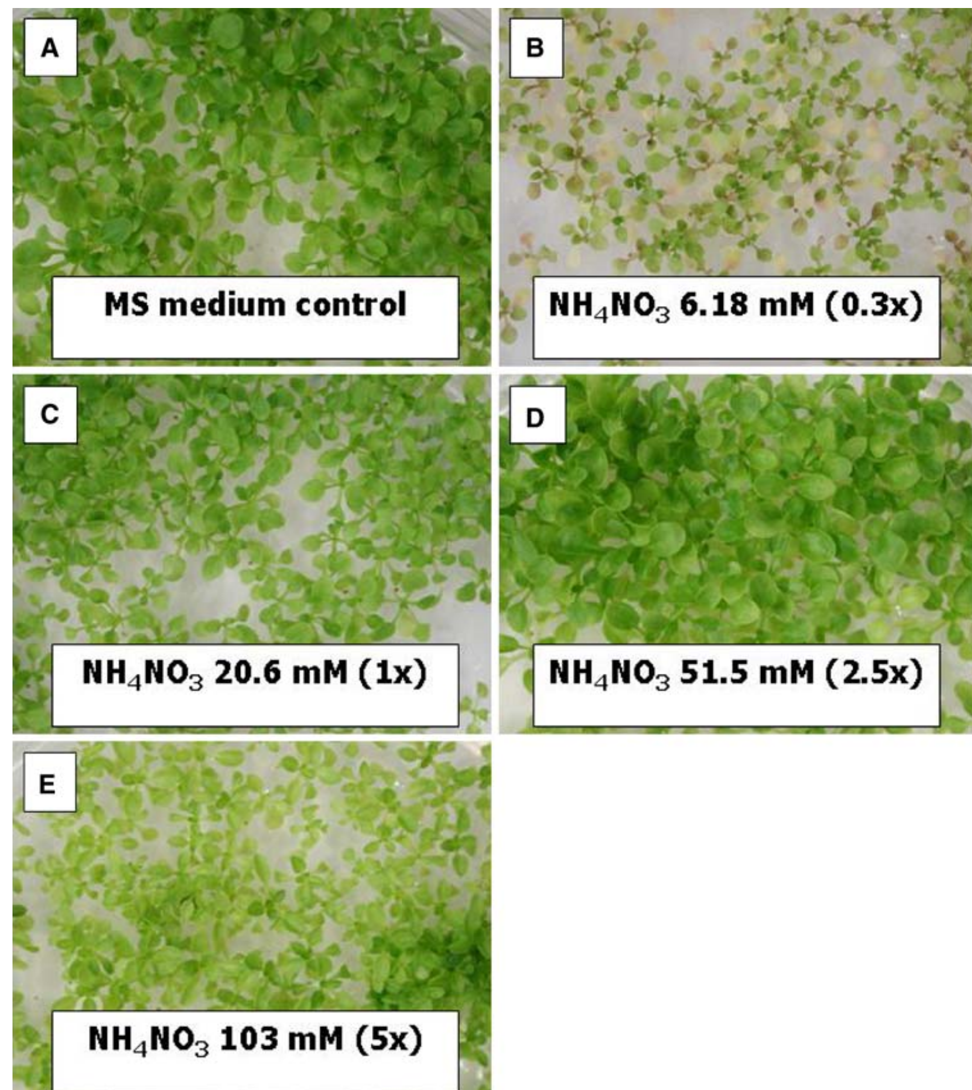

Fig. 3 Phenotypic appearance of *Arabidopsis* plants grown on solid MS medium supplemented with various amounts of ammonium nitrate. **a–e** *Arabidopsis* plants germinated and grown for 3 weeks on MS medium (control) and modified medium containing 6.18 (0.3×), 20.6 (1×), 51.5 (2.5×) and 103 (5×) mM of ammonium nitrate. '1×' stands for a concentration of ammonium nitrate in standard MS medium

Ammonium nitrate improves *Agrobacterium*-mediated transient transformation of *N. tabacum*

Previous experiments showed that enrichment of a plant growth medium with ammonium nitrate to a level of 200 or 300% of its original concentration has a positive effect on recombination rate and does not exert a negative influence on physiology of *Arabidopsis* plants.

The efficiency of plant transformation depends on plant health and responsiveness of a plant cell to transgene delivery (Malik et al. 2008). Transgene integration depends on the activity of host factors, such as repair proteins, chromatin modifiers, etc.

We hypothesized that increased concentrations of ammonium nitrate could also have a positive effect on transformation. Transformation of *Arabidopsis* plants is a simple task only in the case of floral-dip method. The analysis of influence of ammonium nitrate on regeneration and transformation of somatic tissue is much more efficient in *Nicotiana tabacum* (tobacco). In order to prove that exposure of tobacco plants to ammonium nitrate also results in the HRF increase, we exposed tobacco plants to the range of ammonium nitrate concentrations, from 6.18 mM (0.3×) to 103 mM (5×). We confirmed that these concentrations indeed result in recombination increase and that 1×–3× is the ideal range of concentration for tobacco (data not shown).

First, we analyzed the efficiency of transient *Agrobacterium*-mediated transformation of tobacco cotyledons germinated and grown in liquid MS supplemented with 1×, 2× and 3× ammonium nitrate. We performed co-cultivation of these seedlings with *Agrobacteria* carrying the 35S::GUS construct.

The highest GUS expression was observed in plants grown in the medium containing 61.8 mM (3×) ammonium nitrate (Fig. 5c). Consistently, the second highest

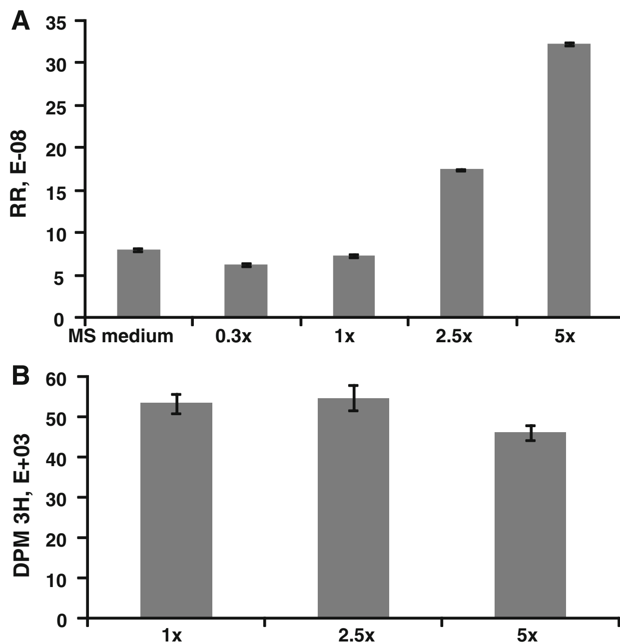

Fig. 4 High concentrations of ammonium nitrate increases recombination rates and slightly decreases the DSBs in *Arabidopsis*. **a** RRs were measured in plants germinated and grown for 3 weeks on MS medium (control) and modified medium containing 6.18 (0.3×), 20.6 (1×), 51.5 (2.5×) and 103 (5×) mM ammonium nitrate. '1×'stands for a concentration of ammonium nitrate in standard MS medium. Two independent experiments were performed. Values represent the mean ± SD. *Asterisks* show a statistically significant difference as compared with control MS medium. Student's *t*-test: $\alpha = 0.05$, $t = 2.57$. ANOVA: $P < 0.05$. **b** The figure shows DSB levels (radioactive counts, DPM ^{3}H, see "Materials and methods") in plants germinated and grown for 4 weeks in the presence of 20.6 (1×), 51.5 (2.5×) and 103 (5×) mM ammonium nitrate. The higher the radioactive count, the more DSBs are present in the genome. '1×' stands for a concentration of ammonium nitrate in standard MS medium. Two independent experiments were performed, and radioactivity of each sample was counted twice. Values represent the mean ± SD. *Asterisks* show a statistically significant difference compared with control 20.6 mM (1×) medium. Student's *t* test, $\alpha = 0.05$, $t = 2.05$

GUS expression was found in plants grown in the presence of 41.2 mM (2x) ammonium nitrate (Figure 5b). Finally, plants grown in the presence of 20.6 mM (1×) ammonium nitrate displayed the lowest transgene expression (Fig. 5a).

Transient expression analysis shows the efficiency of T-DNA delivery to the plant nucleus. Transgene activity tested at 3 days after co-cultivation shows the activity stemming from stable integration events and, most predominantly, from un-integrated T-DNA molecules. Thus, this experiment suggested that enrichment of a growth medium with ammonium nitrate could improve transformation efficiency.

Ammonium nitrate improves *Agrobacterium*-mediated transformation efficiency of *N. tabacum*

Since a high content of ammonium nitrate resulted in an increase in transient plant transformation, we hypothesized that it could also increase stable transformation. Tobacco plants were germinated and grown in media supplemented with 0.3×, 1×, 2×, 3× and 5× ammonium nitrate. Leaves of 1-month-old plants were transformed via leaf incision technique with Agrobacteria carrying *N::LUC* and *hph* genes in T-DNA. This construct was used instead of *35::GUS* to allow non-destructive analysis of marker gene expression. Transformation efficiency was calculated as the number of calli regenerated in the presence of hygromycin from single incision area and was named callus regeneration efficiency (CRE).

We found that enrichment of growth media with ammonium nitrate significantly increased CRE (Fig. 6a–c). Transformation of 2× and 3× groups showed 1.9- and 2.7-fold higher CRE, respectively, as compared to 1× group (Student's *t*-test, $\alpha = 0.05$) (Fig. 6). In contrast to its positive effect on RR, the presence of 103 mM (5×) ammonium nitrate in the growth medium inhibited CRE which constituted 46% of CRE in plant tissues obtained from the control 1× medium (Student's *t*-test, $\alpha = 0.05$) (Fig. 6a). Inhibition of CRE was consistent with a previously observed negative effect that very high concentrations of this chemical had on plant physiology. Finally, a depletion of ammonium nitrate in the growth medium had an insignificant effect on CRE, as it was shown for 0.3× group (Fig. 6a). On the whole, a strong positive correlation between CRE and ammonium nitrate concentrations in the medium was observed ($r = 0.96$, $P < 0.05$).

Next, we regenerated plants from callus material and calculated stable transformation frequency (STF) reflected as the average number of transgenic plants that are resistant to hygromycin and express LUC regenerated from a single-leaf incision. Consistent with its positive effect on CRE, a high content of ammonium nitrate also stimulated STF (Fig. 7a–c). A 1.7- (Student's *t* test, $\alpha = 0.1$) and 2.5-fold (Student's *t*-test, $\alpha = 0.05$) increase in STF was observed for 2× and 3× groups, respectively, as compared to the 1× group (Fig. 7a). Similarly to its effect on CRE, exposure to 5× ammonium nitrate drastically reduced STF: it comprised 38% of STF observed in plant tissues obtained from the control 1× group (Student's *t*-test, $\alpha = 0.05$) (Fig. 7a). A decrease in ammonium nitrate concentrations to 6.18 mM (0.3×) had no significant effect on STF (Fig. 7a). Overall, a strong positive correlation between STF and ammonium nitrate concentrations in the medium was observed ($r = 0.97$, $P < 0.05$).

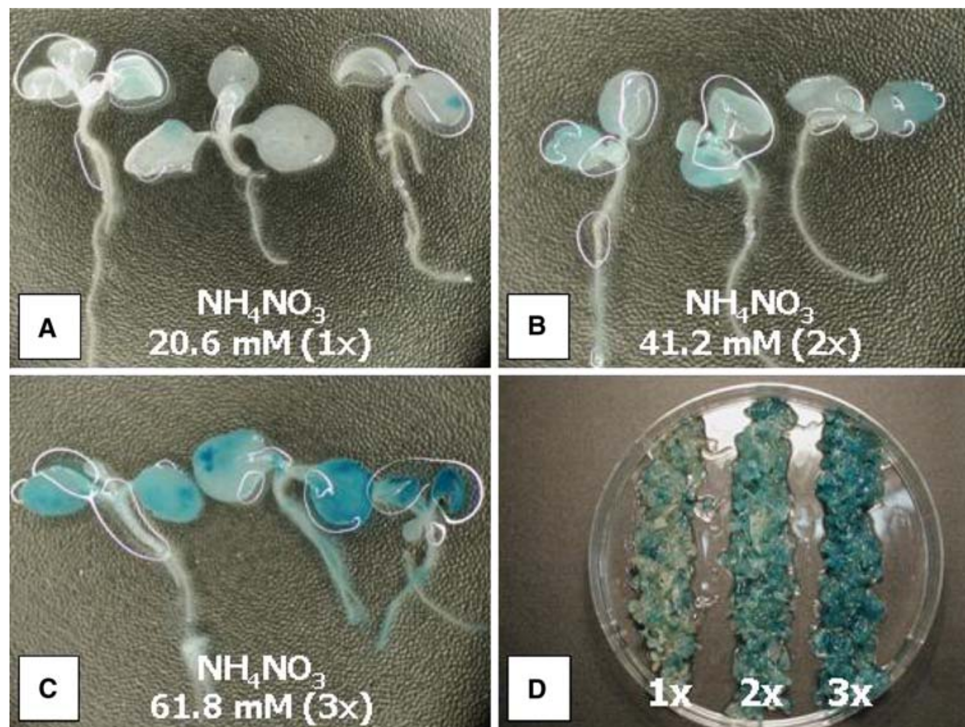

Fig. 5 The efficiency of transient transformation of *N. tabacum* plants grown in liquid MS medium supplemented with various quantities of ammonium nitrate. **a–c** GUS expression in transiently transformed tobacco seedlings germinated and grown in the medium supplemented with 20.6 (1×), 41.2 (2×) and 61.8 (3×) mM ammonium nitrate. '1×' stands for a concentration of ammonium nitrate in standard MS medium. *N. tabacum* plants were transformed with *Agrobacterium* T-DNA containing the GUS reporter gene under control of the 35S CaMV promoter. Transient transformation efficiency was evaluated on the third day after a transformation event. Three independent experiments were performed. **d** Head-to-head comparison of GUS expression in transformed plants grown in the presence of different quantities of ammonium nitrate before transformation

These experiments showed that ammonium nitrate has a significant positive effect on transformation efficiency.

A positive effect of ammonium nitrate on transformation efficiency is primarily due to an increase in the frequency of transgene integration rather than improvement of tissue regeneration

A positive effect of ammonium nitrate on the total number of stable transformants could be either due to an enhanced callus regeneration capacity or a higher frequency of transgene integration. Previous experiments did not allow us to distinguish between these two effects, since active selection with hygromycin was used. To differentiate between the effects of ammonium nitrate on the callus regeneration capacity and transgene integration frequency, we regenerated transformed plants under non-selective conditions. This allowed an equal survival of both transgenic and non-transgenic calli.

To our surprise, no statistically significant differences in CRE were observed in 0.3–3× groups (Table 2). Growth media containing 2× and 3× ammonium nitrate yielded CRE that was 1.09- and 1.18-fold higher than that observed in the 1× group (Table 2). Lowering ammonium nitrate concentration to 0.3× decreased CRE by 1.11 fold only (Table 2). These results suggested a negligible influence of ammonium nitrate on a callus regeneration capacity of plant material used for transformation.

In contrast to a negligible effect on CRE, high concentrations of ammonium nitrate greatly increased the frequency of transgene integration events, as reflected by the increased STF (Table 2). The presence of 2× and 3× ammonium nitrate resulted in STF that was 2.12 and 3.16-fold higher than that obtained from plants grown in the presence of 1× (Table 2). Consistently with previous experiments, a depletion of ammonium nitrate to 6.18 mM (0.3×) led to a minor reduction of STF (Table 2).

These experiments suggest that a positive role ammonium nitrate plays in transformation is primarily due to its effect on transgene integration.

Segregation analysis

Higher transformation efficiency could be associated with an increase in the number of simultaneous integration events targeted to various genomic loci. In general, single-copy

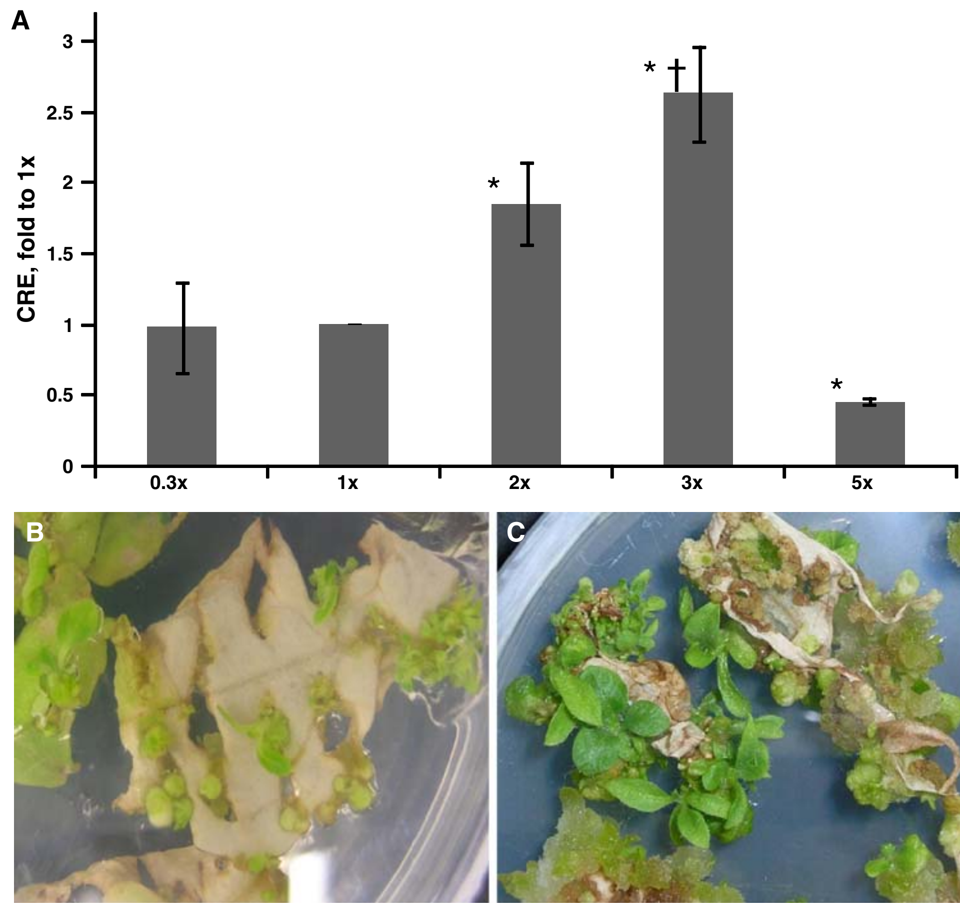

Fig. 6 Callus regeneration efficiency in *N. tabacum* plants grown in liquid MS medium supplemented with various amounts of ammonium nitrate. **a** '1×' stands for a concentration of ammonium nitrate in standard MS medium. Calli were regenerated under selective conditions (hygromycin, 25 mg/L). '*Y*' axis shows CRE as the mean ± SD, calculated from five independent experiments. CRE of plants grown in the presence of 20.6 mM (1×) ammonium nitrate was standardized to 1.0. CRE of plants grown in other types of growth medium shows fold changes in CRE as related to the medium containing 20.6 mM (1×) ammonium nitrate. *Asterisks* show statistically significant difference as compared to control 20.6 mM (1×) medium. Student's *t*-test, $\alpha = 0.05$, $t = 2.26$. † shows statistically significant difference compared with 41.2 mM (2×) ammonium nitrate containing medium. Student's *t* test, $\alpha = 0.05$, $t = 2.26$. ANOVA: $P < 0.01$. **b** and **c** Representative pictures showing a difference in callus regeneration efficiency in host tissues grown in the presence of 20.6 (1×) and 61.8 mM (3×) ammonium nitrate

and single-locus T-DNA integration events can be considered as a perfect transformation outcome. To compare the number of genomic loci targeted by integration events in plants grown in the presence of a standard and increased amount of ammonium nitrate, we performed segregation analysis of the T1 progeny of self-pollinated T0 plants (Fig. 8). Over 100 individual transgenic lines per each experimental group were used for the analysis.

Segregation analysis showed that most integration events are single-locus integration events (Fig. 8a). A comparison between 1x and higher concentrations of ammonium nitrate did not show any changes in the percentage of plants with single-locus T-DNA integration events (Fig. 8a). This suggests that increased transformation efficiency was achieved without compromising the quality of transgenic plants produced.

Exposure to ammonium nitrate results in more intact integrations at the right T-DNA border and less intact integrations at the left border

Since an increase in ammonium nitrate concentrations leads to an increase in both recombination rate and transformation efficiency, we hypothesized that ammonium nitrate could have an effect on the intactness of integrated transgenes. In T-DNA processing, the VirD2 protein nicks the T-DNA right border between nucleotides 3 and 4 (Kim et al., 2007). Thus, intact integration events should contain the T-DNA right border sequence starting from nucleotide 4. We used a nested PCR approach for the amplification of left and right borders of the T-DNA sequence. The presence of three fragments of 153, 315 and 332 nt in the PCR product from the left border would indicate that annealing

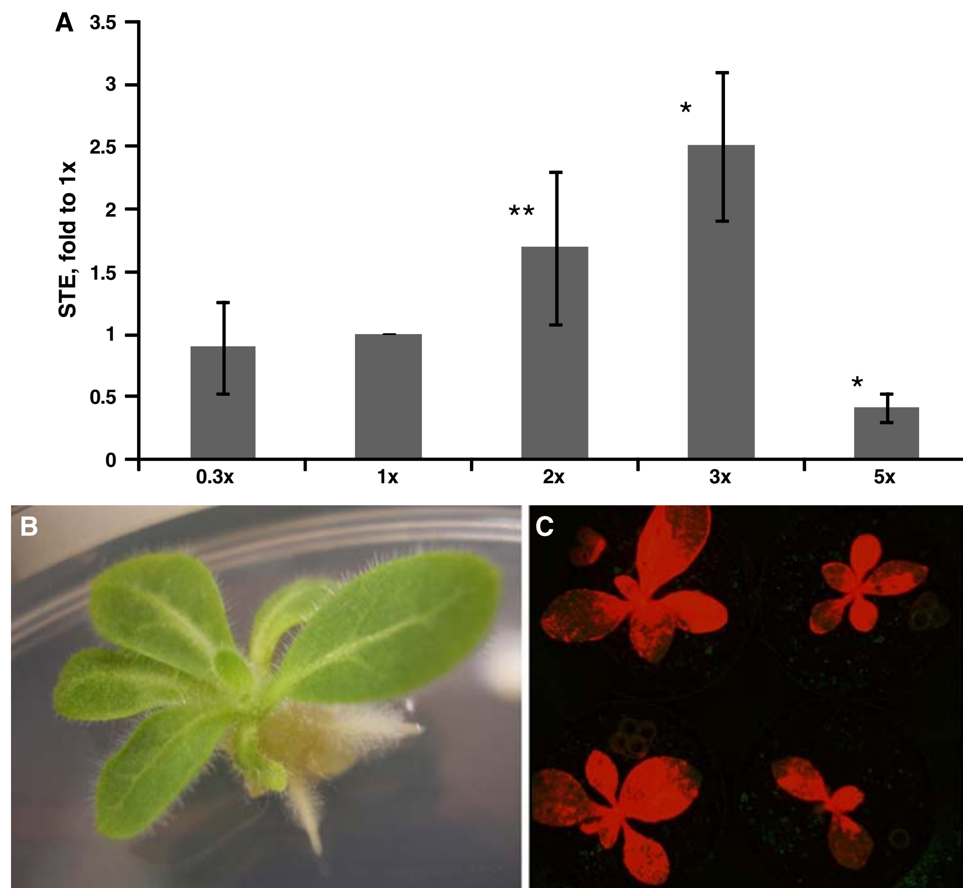

Fig. 7 Stable transformation frequency in *N. tabacum* plants grown in liquid MS medium supplemented with various concentrations of ammonium nitrate**. a** Leaf tissues for transformation with *Agrobacterium* were harvested from *N. tabacum* plants grown in modified liquid MS medium in the presence of various concentrations of ammonium nitrate. '1×' stands for a concentration of ammonium nitrate in standard MS medium. Stable transformation frequency (STF) represents the total number of transgenic plants (expressing *luciferase* gene) regenerated per single incision made in a leaf during transformation. STF in plants grown in the presence of 20.6 mM (1×) ammonium nitrate was standardized to 1.0. STF in plants grown in other types of growth medium shows fold changes as related to the medium containing 20.6 mM (1×) ammonium nitrate. Five independent experiments were performed. Values represent the mean ± SD. *Asterisks* show a statistically significant difference compared with control 20.6 mM (1×) medium. *Single asterisk*—Student's t-test, $\alpha = 0.05$: for the medium containing 61.8 mM (3×) and 103 mM (5×) ammonium nitrate, $t = 2.26$. *Double asterisks*—Student's t-test, $\alpha = 0.1$: for the medium containing 41.2 mM (2×) ammonium nitrate, $t = 1.83$. † shows a statistically significant difference compared with the medium containing 41.2 mM (2×) ammonium nitrate. Student's t-test, $\alpha = 0.1$, $t = 1.83$. ANOVA: $P < 0.01$. **b** Regenerated plants grown in the root inducing medium before being transplanted to soil, and before conducting a reporter gene expression test. **c** A *luciferase* gene expression test in regenerated plants transplanted to soil (red color indicates transgene expression)

sequence for all three primers was preserved, and that there was no deletion or a deletion at the left border was smaller than 9 nt (Fig. 9). The presence of 2, 1 or 0 fragments would suggest truncations of 9–36 nt, 36–188 nt, or larger than 188 nt, respectively. Similarly, the presence of 3 fragments of 251, 296 and 323 nt in the PCR product from the right border would suggest no deletion or a deletion smaller than 9 nt (Fig. 9). The presence of 2, 1 or 0 fragments would suggest truncations of 13–40 nt, 40–85 nt, or larger than 85 nt, respectively. In all cases, if no fragment was observed, a pair of 'internal' primers was used to confirm that a PCR reaction worked.

Almost three-fourth of plants from the 1x group had no deletions or deletions were smaller than 9 nt at the left border, and only 16.3% had deletions that were larger than 188 nt. In contrast, approximately half of plants from groups 2× and 3× had deletions smaller than 9 nt, and 20–26.9% had deletions larger than 188 nt (Table 3).

For the right border, three-fourth of plants from the 1x group had deletions smaller than 13 nt, 12% had deletions larger than 85 nt. Groups 2× and 3× had 89.7 and 84.2% of plants with deletions smaller than 13 nt. Remarkably, none of 58 plants from the 2× group had deletions larger than 85 nt at the right border (Table 3).

Table 2 CRE and STF in *N. tabacum* plants regenerated under non-selective conditions

NH_4NO_3	Number of incisions	Number of calli	Number and % of LUC "+" plants	CRE/fold to 1×	STF/fold to 1×
0.3×	205	483	11/2.28	2.36/0.89	0.05/0.73
1×	190	503	14/2.78	2.65/1.00	0.07/1.00
2×	173	498	27/5.42	2.88/1.09	0.16/2.12
3×	193	602	45/7.48	3.12/1.18	0.23/3.16

'×' stands for a concentration of ammonium nitrate in standard MS medium, where 0.3× is 6.18, 1× is 20.6, 2× is 41.2 and 3× is 61.8 mM. 'Number of incisions' stands for the total number of incisions made. 'Number of calli' total number of regenerated calli. 'Number and % of LUC "+" plants" the first value shows the number of LUC + plants, and the second number shows the percent of LUC + out of all regenerated calli. 'CRE/fold to 1×' the first number shows CRE, whereas the second number shows the fold difference to 1×. 'STF/fold to 1×' the first number shows STF, whereas the second number shows the fold difference to 1×

The analysis also showed that 63.53% of all 1× plants had either no deletion or deletions smaller than 9 nt and 13 nt at the left and right borders, respectively. In contrast, 2× and 3× groups contained 53.85 and 50% of such plants, respectively (Table 4).

This experiment showed that exposure to ammonium nitrate results in less frequent T-DNA truncations at the right border and more frequent T-DNA truncations at the left border.

Discussion

In this study, we analyzed the effects of high ammonium nitrate concentrations in a growth medium on the HR activity and evaluated the influence of this chemical on stable *Agrobacterium*- mediated transformation efficiency. We have found that (a) the presence of ammonium nitrate results in a dose-dependent increase in RR; (b) elevated concentrations of ammonium nitrate have a positive effect on plant growth and phenotypic appearance; (c) exposure to high concentrations of ammonium nitrate does not increase DSB levels in plants; (d) exposure of plants to high concentrations of ammonium nitrate increases the frequency of Agrobacterium-mediated transient transformation; (e) plants grown on media enriched with ammonium nitrate exhibit the significantly increased frequency of stable transformation; (f) a positive effect of ammonium nitrate on plant transformation is mainly mediated via the increased frequency of transgene integrations in the host genome; (g) plants regenerated from ammonium nitrate-rich media exhibit normal segregation and transgene integration patterns.

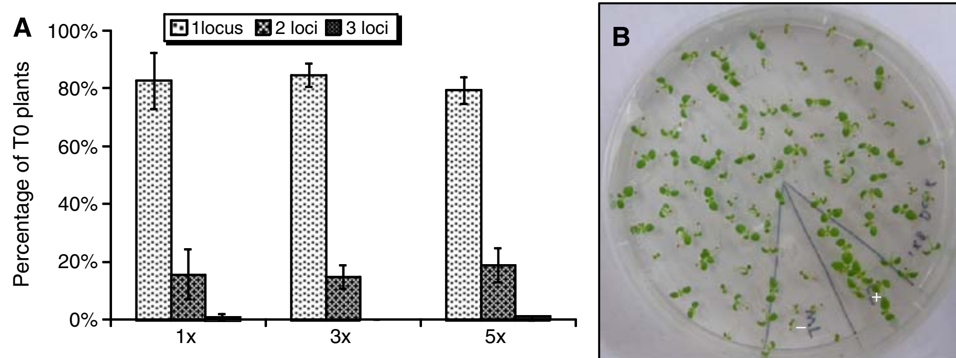

Fig. 8 The number of genomic loci containing integrated T-DNA, as determined using segregation analysis of the T1 progeny of self-pollinated tobacco plants. **a** The number of genomic loci containing integrated T-DNA, as determined by segregation analysis of the T1 progeny of transformed plants grown in medium containing 20.6 (1×), 61.8 (2×) and 103 (3×) mM ammonium nitrate before transformation. '*x*' Stands for a concentration of ammonium nitrate in standard MS medium. The total number of integration events (transgenic plants) obtained from each treatment was taken to be 100%. Segregation analysis was performed on solid MS medium supplemented with hygromycin (25 mg/L). Each plate contained wild type and hygromycin positive plants for a negative and positive control, respectively. A segregation ratio was calculated based on antibiotic resistant phenotypes at 3 weeks post germination. Three independent experiments were performed. Values represent the mean ± se. The statistical significance of segregation ratios calculated was confirmed using the Chi square statistic for $\alpha = 0.05$. **b** Antibiotic resistant and sensitive phenotypes of T1 plants grown in MS medium containing hygromycin (25 mg/L); "+" and "−" stand for positive (hygromycin resistant) and negative (wild type) selection controls, respectively

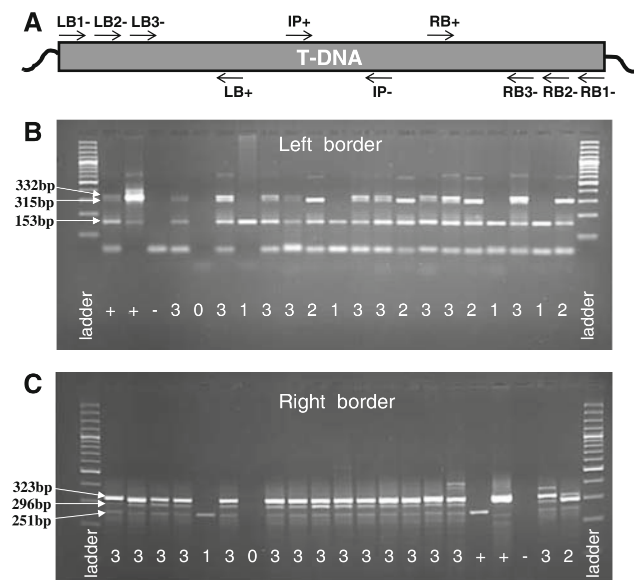

Fig. 9 PCR analysis of the intactness of the left and right T-DNA borders. **a** Schematic presentation of the PCRs for the analysis of T-DNA intactness. Four primers were used for the analysis of each border. For each individual reaction, we used one primer going outward from T-DNA (LB+ and RB+) and 3 primers going inward (LB1−, LB2−, LB3− and RB1−, RB2− and RB3−). PCR on intact T-DNA borders would result in 3 fragments, whereas PCR on truncated T-DNA would result in 2, 1 or even 0 fragments, depending on the size of the truncation. 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 left border intactness. First and last lines show the ladder. Second and third lines marked as '+', show a positive control, from genomic DNA of a transgenic line and from a plasmid containing T-DNA, respectively. Fourth line, marked as '−', shows the negative control—PCR from genomic DNA extracted from wild type. All the other lines show the number of PCR fragments of 332, 315 and 153 bp in size. **c** Analysis of the right border intactness. First and last lines show the ladder. Lines marked as '+', show a positive control, from genomic DNA of a transgenic line and from a plasmid containing T-DNA, whereas line, marked as '−', shows the negative control—PCR from genomic DNA extracted from wild type. All the other lines show the number of PCR fragments of 323, 296 and 251 bp in size

Ammonium nitrate increases the recombination rate and decreases the level of DNA double strand breaks

One of the ways to improve transformation efficiency is to make the host more susceptible to T-DNA integrations. Since T-DNA integration in the plant genome is highly dependent on the activity of host DNA repair factors (Citovsky et al. 2007), we hypothesized that growing plants under conditions that induce the HR activity before transformation could improve transformation efficiency. The main emphasis was made on the selection of growth medium compositions and factors that could significantly increase HRF without negatively affecting host physiology.

Nitrogen is one of the major macronutrients necessary for plant growth and development. Plants can uptake nitrogen from media predominantly in the form of NH_4^+ and NO_3^- ions. The uptake of these two ions is under control of systemic signals related to the total nitrogen status in a whole plant (Ruffel et al. 2008). However, the presence of NO_3^- ions usually results in higher levels of nitrogen intake and consequently higher total nitrogen content in plants, compared to the presence of NH_4^+ ions (Ruffel et al. 2008). Our preliminary experiments demonstrated that a depletion of ammonium nitrate in a growth medium has the most pronounced effect on HRF.

To study the effects of high ammonium nitrate concentrations in plant growth media on the HR activity, we supplemented MS medium with various amounts of this chemical. Our data revealed a strong positive correlation between the amount of ammonium nitrate present in the medium and RR in plants. Furthermore, consistently with an important role ammonium nitrate plays in plant metabolism, development and nutrition intake (Crawford 1995; Stitt 1999; Miller et al. 2007), increased concentrations of ammonium nitrate improved plant growth. It is also possible that a positive effect on plant physiology was mainly mediated by NO_3^- ions that are known to stimulate lateral root growth, root initiation and elongation (Forde 2002a, b). Similarly, NO_3^- ions not only induce factors involved in NO_3^- assimilation but also positively regulate enzymes of the pentose phosphate pathway and carboxylic acid metabolism (Ruffel et al. 2008).

The effects of inorganic nitrogen sources on the DNA repair activity still remain unclear in contrast to their well-documented influence on plant physiology. It is noteworthy that recent studies demonstrated a link between the activity of human Rad51 and ammonium-sulphate concentrations at a molecular level (Sigurdsson et al. 2001; Liu et al. 2004; Shim et al. 2006). Ammonium-based salts were shown to induce conformational changes in hRad51 leading to an increase in its activity and therefore promoting recombination (Sigurdsson et al. 2001; Liu et al. 2004; Shim et al. 2006). In this study, we hypothesised that ions of both NH_4^+ and NO_3^- positively influence the HR activity and plant growth, thus leading to growth- and recombination-stimulating effects. Consistently with this idea, the transition from moderate to high concentrations of ammonium nitrate in media significantly inhibited plant growth, but it still resulted in an increase in HRF. A depletion of ammonium nitrate in growth media led to drastic changes in plant appearance, but in contrast, only a minor decline in HRF was observed.

A positive dose-dependent effect of increased ammonium nitrate concentrations on HRF could result from elevated DNA damage caused by high salt concentrations. To check this possibility, we measured DNA DSB levels in

Table 3 Summary of the data showing the intactness of the right and left borders of T-DNA in plants regenerated from media supplemented with various level of ammonium nitrate

	0.3×		1×		2×		3×		5×	
	No. of lines	%	No. of lines	%	No. of lines	%	No. of lines	%	No. of lines	%
Right border										
<13 nt	16	72.73	69	75	52	89.66	112	84.21	35	76.09
13–40 nt	2	9.09	9	9.78	2	3.45	3	2.26	0	0
40–85 nt	3	13.64	3	3.26	4	6.9	6	4.51	1	2.17
>85 nt	1	4.55	11	11.96	0	0	12	9.02	10	21.74
Total	22		92		58		133		46	
Left border										
<9 nt	16	72.73	68	73.91	30	52.63	67	51.54	30	66.67
9–36 nt	0	0	4	4.35	5	8.77	23	17.69	4	8.89
36–188 nt	3	13.64	5	5.43	7	12.28	14	10.77	3	6.67
>188 nt	3	13.64	15	16.3	15	26.32	26	20	8	17.78
Total	22		92		57		130		45	

The columns show the size of deletions at the right and left border; for the right border, less than 13 nt (or no deletion at all), 13–40 nt, 40–85 nt, and more than 85 nt; for the left border, less than 9 nt (or no deletion at all), 9–36 nt, 36–188 nt, and more than 188 nt. '# of lines' shows the number of lines with particular deletion size. '%' shows percent of lines with certain deletion out of all lines tested

Table 4 Percent of lines having deletions at both borders

RB\LB	<9 nt	9–36 nt	36–188 nt	>188 nt
0.3×				
<13 nt	72.7	0	0	0
13–40 nt	0	4.55	0	0
40–85 nt	0	0	9.1	0
>85 nt	0	0	9.1	4.55
1×				
< 13 nt	63.53	3.53	3.53	2.35
13–40 nt	5.88	1.18	0	3.53
40–85 nt	2.35	0	0	1.18
>85 nt	2.35	0	1.18	9.41
2×				
<13 nt	53.85	7.69	0	28.12
13–40 nt	1.72	1.72	0	0
40–85 nt	6.9	0	0	0
>85 nt	0	0	0	0
3×				
<13 nt	50	1.54	8.46	6.15
13–40 nt	0	13.85	1.54	0.77
40–85 nt	1.54	1.54	0.77	0.77
>85 nt	0	0.77	0	12.31
5×				
<13 nt	62	4	6	2
13–40 nt	0	0	0	0
40–85 nt	2	0	0	0
>85 nt	6	4	0	14

The table shows the percent of lines with various types of deletions: rows show the right border and columns show the left border

plants grown in media enriched with ammonium nitrate. No significant increase in the DSB level was found, suggesting that it was not DNA damage that increased HR. Intriguingly the a medium containing 61.8 mM (3×) ammonium nitrate yielded larger plants with a significantly lower level of DNA DSBs as compared to plants obtained from the control medium. These combined data allow us to speculate that ammonium nitrate can directly stimulate the activity of plant factors involved in the HR DNA repair pathway.

Effects of ammonium nitrate on callus regeneration and transgene integration

The efficiency of T-DNA integration largely depends on the activity of host replication, transcription and DNA repair factors. A majority of DSBs and T-DNA integration events are processed via NHEJ which affects the intactness of integrated transgene sequences (Gorbunova and Levy 1999; Puchta 2005). Increasing the typical low activity of HR could possibly allow a better quality of integration events as well as generation of site-specific insertions and gene targeting (Vergunst and Hooykaas 1999; Puchta 2002; Reiss 2003).

Our studies showed that enrichment of growth medium with ammonium nitrate could increase the frequency of stable transformation events. Our findings were consistent with recent reports indicating a positive effect of increased HRF on plant transformation (Shaked et al. 2005). Indeed, expression of the yeast *RAD54* gene involved in the HR

repair pathway increased the gene targeting frequency in plants by one to two orders of magnitude (Shaked et al. 2005). Similarly, hypersusceptibility of *Arabidopsis fas1* and *fas2* mutants to *Agrobacterium* transformation was in part attributed to enhanced transcription of the *AtRad51* and *AtRad54* genes (Endo et al. 2006). In addition, growing wheat calli in a medium containing niacinamide, a PARP inhibitor, for 4 days just before bombardment significantly increased the number of low-copy transgene integration events (De Block et al. 1997). Since PARP facilitates the NHEJ repair pathway, an inhibitor promotes the HR pathway.

An increased number of transgenic plants obtained from modified media can be due to the increased regeneration capacity of plant tissues derived from growth media enriched with ammonium nitrate. Alternatively, it is possible that ammonium nitrate promotes transgene integration. To compare the effects of ammonium nitrate on these two parameters separately, we regenerated plants under non-selective conditions. This experiment allowed us to conclude that ammonium nitrate basically enhanced the frequency of transgene integrations. Its effect on tissue regeneration was minor.

It is noteworthy that a number of reports suggest a positive effect of elevated concentrations of ammonium nitrate on somatic embryogenesis. Similarly to our study, Choi et al. (1998) compared the effects of five different macrosalts present in a standard MS medium (Murashige and Skoog 1962) on the frequency of somatic embryo formation, and they found that an increased concentration of ammonium nitrate was the most effective for somatic embryogenesis. In fact, the concentration of ammonium nitrate (Choi et al. 1998) that resulted in the best embryo response was similar to that which resulted in the highest STF in our experiment. Consistently, Menke-Milczarek and Zimny (2001) proposed a similar concentration of ammonium nitrate as an optimal one for enhancing wheat somatic embryogenesis. Furthermore, He et al. (1989) compared the effects of concentrations of each of five MS macrosalts on the induction and morphology of embryogenic callus from immature embryo of wheat and found that elevated levels of ammonium nitrate were very effective for callus induction. Similarly, doubling the concentration of all MS salts in the induction medium led to a significant enhancement of regeneration frequency in wheat scutella (Maës et al. 1996). Importantly, using compounds containing ammonium (Rijven 1958; Halperin 1966; Walker and Sato 1981; He et al. 1989; Choi et al. 1998; Menke-Milczarek and Zimny 2001) and nitrate (Choi et al. 1998; Kothari et al. 2004; Menke-Milczarek and Zimny 2001) as a source of inorganic nitrogen in the medium appeared to be highly effective for somatic embryogenesis.

In our studies, we applied a different approach. We used ammonium nitrate only before transformation rather than for regeneration of embryos. A minor stimulating influence of ammonium nitrate on CRE was observed in the experiment in which calli were regenerated under non-selective conditions. This may be due to the residual effects caused by the presence of an increased amount of ammonium nitrate in growth media used before transformation.

There could be several possible explanations of a positive influence of ammonium nitrate on *Agrobacterium*-mediated plant transformation. The long-distance nitrogen signalling hypothesis of Forde (2002a) suggests that nitrogen is transduced to cytokinin via the enhanced isopentenyltransferase (*ipt*) activity in the roots and is translocated up the shoot with the subsequent promotion of leaf/bud outgrowth (Cline et al. 2006). Indeed, nitrogen-dependent accumulation of cytokinins and stimulating effects of nitrogen fertilization on plant growth are well-documented, and they are consistent with plant phenotypes observed in our study. Takei et al. (2001) demonstrated that after 4 h of nitrogen resupply, levels of cytokinins in maize leaves increased significantly and remained elevated for at least 24 h. Stimulation of cell division by cytokinins increases the number of cells entering the S/G2 phase of the cell cycle, thus promoting higher expression of HR proteins such as Rad51 (Chen et al. 1997). Moreover, it is still possible that elevated levels of ammonium nitrate can also directly stimulate the Rad51 activity in plants via an unknown mechanism in a similar manner to that previously described for human Rad51 (Sigurdsson et al. 2001; Liu et al. 2004; Shim et al. 2006).

Furthermore, cells undergoing active division are more likely to be targeted by a stable T-DNA integration event. An absolute requirement for the S-phase for transfer and/or T-DNA integration in *Petunia hybrida* was previously demonstrated (Villemont et al. 1997). Similarly, transformation of synchronized tobacco protoplasts during the S–M phase resulted in increased recovery of selection-resistant colonies (Meyer et al. 1985; Okada et al. 1986). An important role of active cell division in plant transformation was also supported by higher transformation efficiency observed in maize cells expressing a modified version of the viral replication-associated protein (RepA) that stimulates cell division (Gordon-Kamm et al. 2002). It can thus be hypothesized that the effects of ammonium nitrate on plant transformation are based on its combined influence on HR activity and cell division.

The influence of ammonium nitrate on the intactness of integrated T-DNA

All cloned T-DNA insertions were found within gene coding regions, which is consistent with the analysis of

T-DNA integration sites performed by Alonso et al. (2003). Since we used active selection conditions in our experiments, we can not exclude that plants with T-DNA insertions in loci with low transcriptional activity were lost during selection (Francis and Spiker 2005; Kim et al. 2007).

Our data showed that exposure to ammonium nitrate resulted in the increased intactness of the right border of T-DNA and decreased intactness of the left border. Analysis of T-DNA integration sites showed the existence of regions of microhomology, insertions of filler DNA and deletions of various size (Tinland et al. 1995; Fladung 1999; Kumar and Fladung 2002; Meza et al. 2002; Windels et al. 2003). Windels et al. (2003) showed 48 bp to be an average deletion size in the genome pre-insertion site. It is noteworthy that according to published literature, deletions at the 3′-end (the left border) occur more frequently than at the 5′-end (the right border) (Tinland et al. 1995; Tinland et al. 1996; Brunaud et al. 2002). This is in agreement with the data obtained from our studies.

Levels of changes that occur at the left and right T-DNA border could be explained by the mechanism(s) of T-DNA integration. Three different mutually exclusive models that explain T-DNA integration were originally proposed (reviewed in Tzfira et al. 2004). The first one, the DSB repair (DSBR) model, postulates that T-DNA preferentially integrates into DSB sites. Integration via the DSBR model requires conversion of ssT-DNA molecules into dsT-DNA molecules before integration. In contrast, the single-strand-gap repair (SSGR) model suggests that ssT-DNA integrates into a nicked DNA and then provides a template for complimentary DNA strand synthesis that substitutes the original host DNA sequence at the integration site. Finally, the microhomology-dependent model suggests that T-DNA initiates by annealing of the T-strand 3′-end to an area of microhomology in the target DNA. This is followed by microhomology-dependent annealing of the 5′-end and complementation of the T-strand to dsDNA.

According to the DSBR model, VirD2 detaches from ssT-DNA that gets processed into dsT-DNA. The initial step could potentially involve the interaction with Ku80 (Li et al. 2005). According to other models, it can not be excluded that VirD2 stays attached to the 5′-end immediately prior to integration.

Tzfira et al. (2004) summarized these three models, and based on additional recently published data, he suggested a new hypothetical model. According to this model, the invading T-strand can undergo minor degradation at the unprotected 3′-end, whereas the 5′-end remains intact due to the attachment of VirD2 to the 5′-end. After conversion to a dsDNA form, during which a further loss of nucleotides from the 3′-end occurs, dsT-DNA undergoes integration into the host DNA via NHEJ or HR pathways. Integration via the HR pathway could potentially include protection of the 5′-end by VirD2 followed by a search for microhomology and annealing of nucleoprotein filaments, the 5′-end overhangs are filled-in, and the dsDNA is ligated into DSB in the genome. This mechanism explains why the 5′-end of the T-DNA is less ‘processed’ than the 3′-end during integration.

Based on our observations that the 5′-end of the T-DNA is more intact and the 3′-end is less intact in plantlets regenerated from the 3× group, we suggest that there are at least two mechanisms of T-DNA integration. It can be proposed that exposure to ammonium nitrate shifts integration towards one of these mechanisms. We hypothesize that integration is indeed shifted towards the HR-mediated mechanism. According to Tzfira et al. (2004), HR-mediated integration of a dsT-DNA intermediate involves protection of the 5′-end and filling-in the 5′-end overhangs. If exposure to ammonium nitrate indeed activates the HR repair mechanism, we could expect to see more intact 5′-ends. However, it is not clear why there were more frequent deletions observed at the 3′-end. One would expect at least equal frequency or even less frequent deletions at the 3′-end due to less frequent involvement of the NHEJ pathway.

Alternative strategies for improving transformation efficiency via manipulation of the host

The current paper suggests that the increase in the frequency of transformations can be achieved via simple modifications of culture media. Several other reports showed that various chemicals as well as changes in the temperature induce transformation efficiency. It was demonstrated that supplementation of growth media with various chemicals can influence DNA repair. Waldman and Waldman (1991) exposed mammalian cells to 2 mM 3-methoxybenzamide, a competitive inhibitor of PARP (Waldman and Waldman 1990), and demonstrated a three to fourfold increase of intrachromosomal HR (Waldman and Waldman 1991). Similarly, the use of another PARP inhibitor, niacinamide, for biolistic-mediated wheat transformation increased the number of low-copy transgene integration events (De Block et al. 1997). Transient expression of a reporter gene was several-fold increased by performing bombardment in the presence of silver thiosulfate and calcium nitrate (Perl et al. 1992). Lowering temperatures during co-cultivation with *Agrobacterium* (Li et al. 2003) and chilling during a regeneration step (Immonen 1996) also improved transformation efficiency.

High transformation efficiency does not rely only on successful transformation *per se*; it also requires the establishment of optimal conditions for efficient

regeneration of transgenic material from transformed tissues. To date, a number of chemicals promoting somatic embryogenesis and diminishing negative effects during a tissue culture stage have been reported. The most interesting findings are the following: increasing cytosolic levels of calcium that can promote somatic embryogenesis (Racusen and Schiavone 1990; Chugh and Khurana 2002; Malabadi and Staden 2006); the application of silver nitrate 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 various nitrogen sources (Immonen 1996); promotion or prevention of shoot regeneration by cupric sulphate (Sahrawat et al. 2003; Kothari et al. 2004) and high EDTA (Kothari et al. 2004). 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 were reported to promote somatic embryogenesis in various plant species (He et al. 1989; Grimes and Hodges 1990; Mordhorst and Lorz 1993; Choi et al. 1998; Jiménez 2001; Kothari et al. 2004). High concentrations of ammonium nitrate in regeneration media greatly improved somatic embryogenesis and increased the yield of somatic embryos in ginseng (Choi et al. 1998). In rice, the relative ratio of ammonium to nitrate could affect the sensitivity of immature embryos to auxin; the ratio of 1:1 produced the greatest insensitivity (Grimes and Hodges 1990). In contrast, in barley microspore culture, it was shown that the ratio of two ions had no influence upon somatic embryogenesis yields (Mordhorst and Lorz 1993). However, a link between the total concentration of inorganic nitrogen and the yield of somatic embryos has been established (Mordhorst and Lorz 1993). In wheat, it was not possible to show a strong connection between either the ratio or the total content of nitrogen species and somatic embryogenesis yields (Menke-Milczarek and Zimny 2001).

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 somatic embryogenesis in various economically important plant species.

Finally, using conditions promoting HRF could substantially improve genetic engineering and allow the development of gene targeting technology in plants (Vergunst and Hooykaas 1999; Puchta 2002; Reiss 2003; Hanin and Paszkowski 2003; Lida and Terada 2004).

Summary

The low activity of HR in plant cells represents one of the major obstacles in the improvement of plant transformation quality and development of efficient gene targeting protocols. We hypothesized that activation of host DNA repair factors should increase the frequency of transgene integration. Our study demonstrated that manipulating the amount of ammonium nitrate present in growth media can improve the efficiency of *Agrobacterium*-mediated genetic transformation. Further studies should be directed to elucidating mechanisms that mediate the influence of ammonium nitrate on HR. This could help us gain a better understanding of plant physiological aspects necessary for efficient genetic transformation, thus indicating other factors or conditions that can be successfully applied for plant transgenesis.

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