Rapid report

Generation of nonvernal-obligate, faster-cycling *Noccaea caerulescens* lines through fast neutron mutagenesis

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Summary

• *Noccaea caerulescens* (formerly *Thlaspi caerulescens*) is a widely studied metal hyperaccumulator. However, molecular genetic studies are challenging in this species because of its vernal-obligate biennial life cycle of 7–9 months. Here, we describe the development of genetically stable, faster cycling lines of *N. caerulescens* which are nonvernal-obligate.

• A total of 5500 M_0 seeds from Saint Laurent Le Minier (France) were subjected to fast neutron mutagenesis. Following vernalization of young plants, 79% of plants survived to maturity. In all, 80 000 M_2 lines were screened for flowering in the absence of vernalization. Floral initials were observed in 35 lines, with nine flowering in < 12 wk. Two lines (A2 and A7) were selfed to the M_4 generation.

• Floral initials were observed 66 and 87 d after sowing (DAS) in A2 and A7, respectively. Silicle development occurred for all A2 and for most A7 at 92 and 123 DAS, respectively. Floral or silicle development was not observed in wild-type (WT) plants. Leaf zinc (Zn) concentration was similar in WT, A2 and A7 lines.

• These lines should facilitate future genetic studies of this remarkable species. Seed is publicly available through the European Arabidopsis Stock Centre (NASC).

Introduction

Noccaea caerulescens (formerly *Thlaspi caerulescens*) is a highly metal-tolerant plant species which hyperaccumulates nickel (Ni), cadmium (Cd) and zinc (Zn) (reviewed by Broadley *et al.*, 2007; Krämer, 2010). It is a short-lived, self-compatible biennial/perennial species of Brassicaceae which is functionally nonmycorrhizal (Regvar *et al.*, 2003). It occurs on calamine, serpentine and nonmineral soils, with a wide distribution in central, northern and western Europe (Reeves & Brooks, 1983; Baker & Brooks, 1989; Reeves *et al.*, 2001). Current evidence indicates that within the Brassicaceae, hyperaccumulation of Ni has evolved independently at least six times, whereas that of Zn and Cd has occurred only twice (Broadley *et al.*, 2007; Krämer, 2010), once at the base of the *Noccaea/Raparia* clade, and once in *Arabidopsis halleri*, a species which is also the focus of intense recent study (e.g. Hanikenne *et al.*, 2008).

Significant advances have been made in understanding the genetics of metal tolerance and accumulation in *N. caerulescens*. These include numerous studies of natural genetic variation in metal tolerance and accumulation (e.g. Ingrouille & Smirnoff, 1986; Baker *et al.*, 1994; Roosens *et al.*, 2003), expression analysis of metal transporter genes (Assunção *et al.*, 2001), cloning and functional characterization of metal transporter genes in heterologous expression

systems (e.g. Papoyan & Kochian, 2004), the development of structured populations used for mapping quantitative trait loci (QTL) (e.g. Assunção *et al.*, 2006), global transcriptome analysis (Hammond *et al.*, 2006; van de Mortel *et al.*, 2006, 2008), and the development of protocols for *Agrobacterium tumefaciens*-mediated transformation (Peer *et al.*, 2003; Guan *et al.*, 2008). However, dissecting the genetic basis and molecular mechanisms of hyperaccumulation in *N. caerulescens* is challenging as a result of the length of its life cycle and obligate vernalization requirement. Thus, ecotypes cultivated to date require up to 32 wk to flower, including a 7–12 wk period of short-day vernalization (5°C and 8 h photoperiod), with an additional 4 wk for seed ripening (Peer *et al.*, 2003, 2006). In addition to the length of time *per se*, growing plants for up to 9 months in controlled environments poses a significant challenge (and cost) in terms of husbandry, and increases the potential for genotype × environment interactions, including those associated with maintaining plants in a disease-free state.

The removal of vernalization requirements to induce flowering has led to the development of rapid-cycling populations in several important model Brassicaceae species, including crop *Brassica* ssp., and this has facilitated molecular genetic analyses (Williams & Hill, 1986; Iniguez-Luy *et al.*, 2009). In late-flowering ecotypes of *Arabidopsis thaliana*, the vernalization requirement has been removed through fast neutron-induced mutations in either *FLOWERING LOCUS C* (*FLC*) or *FRIGIDA* (*FRI*), which interact synergistically to repress flowering (Michaels & Amasino, 1999; Sung & Amasino, 2004). Recent expression analysis has identified conserved roles for *FLC* homologues in vernalization responses in *Brassica rapa* (Zhao *et al.*, 2010) and *Beta vulgaris* (Reeves *et al.*, 2007), as well as in the perennial species *Arabis alpina* (Wang *et al.*, 2009).

The aim of this study was to produce genetically stable fast cycling lines of *N. caerulescens* using fast neutron mutagenesis, to support future forward and reverse molecular genetic studies. Mutation breeding using fast neutron bombardment of seeds creates random deletions, ranging from one base to > 100 kb, and is commonly employed in mutating plant genomes, representing a rapid approach to obtain large mutant pools (Kodym & Afza, 2003; Salt *et al.*, 2008; Bruce *et al.*, 2009). This technique is a relatively inexpensive method for producing large mutant populations in species whose genomes are not amenable to T-DNA transformation, generating genome-wide saturation in relatively small populations.

Materials and Methods

Plant material

Noccaea caerulescens (J.&C. Presl) F.K.Mey. (formerly *Thlaspi caerulescens* J.&C. Presl) was obtained from Saint Laurent le Minier, France, in 2005 (kindly provided by Guy Delmot, Saint Laurent le Minier, France, 43°55′49″N, 3°39′51″E). Seeds were bulked in a single pool for three generations. Plants were grown in Levington M3 high nutrient peat-based compost under glasshouse conditions (GC) for 12 wk (22.3 and 13.3°C mean day and night temperatures, respectively, at 16 h photoperiod), followed by 10 wk vernalization (5°C, 8 h photoperiod) and returned to 12 wk GC for flowering at Sutton Bonington Campus, University of Nottingham, UK.

Fast neutron mutagenesis

In February 2007, 5500 M_0 seed were irradiated with fast neutrons for 137 min, at a dose rate of 60 Gy (Biological Irradiation Facility, Budapest Research Reactor (BRR), Budapest Neutron Centre, Budapest, Hungary). The mean water kerma dose rate was monitored in real time by U-235 and Th-232 fission chambers and a Geiger–Müller counter and was 438 mGy min^{-1} (± 3.0%). The BRR reactor is a tank-type reactor with a nominal thermal power of 10 MW, which is moderated and cooled by light water. Filters of different densities were arranged in order to decrease gamma and neutron intensity and to modify the neutron spectrum and the neutron : gamma ratio. The filters were set at the following distances from the core towards the irradiation cavity: 143.6 mm Al + 18 mm Pb + 15 mm Al. At the beam stop behind the sample, shielding comprised 30 mm Fe + 45 mm Pb + 8 mm Al + 20 mm B_4C. Seeds were irradiated inside a rotating Cd capsule (16 rpm) of 2 mm wall thickness at a temperature < 30°C and humidity < 70%, at normal air pressure. Following irradiation, seed were repackaged to avoid contamination of surface packaging. The measured surface gamma dose from the seeds was 130 × background dose. Upon dispatch, this had decreased to < 2 × background dose, where (background dose is *c.* 90 nGy h^{-1}).

Selecting early-flowering, rapid-cycling mutants

M_1 plants were grown under GC set to 16 h photoperiod using supplementary sodium lighting, with 22.3 and 13.3°C mean day and night temperatures, respectively. Seeds were sown in plug trays (2 cm^2 plugs) containing Levington M3 high nutrient peat-based compost (pH 5.3–5.7) (Monro Group, Wisbech, Cambridgeshire, UK). Two-week-old seedlings were then transplanted into 0.32 l pots (height 7.9 cm; diameter 9 cm) containing a compost mix (Levington M3, sand (< 1 mm) and grit (1–3 mm) at a ratio of 2 : 1 : 1, respectively, v : v : v) (Monro Group). After 12 wk, plants were vernalized at 5°C with an 8 h photoperiod for 10 wk, before returning to GC. Subsequently, 80 000 M_2 lines were grown under GC in 2 cm^2 plugs and after 2 wk transplanted into 0.32 l pots to select for nonvernalized early flowering (within 16 wk) individuals.

Subsequent faster-cycling M_3 progeny were selfed and selected under similar conditions as M_2 lines. Two M_4 lines, observed to have consistently early-flowering phenotypes (A2 and A7), were selected for further characterization.

Characterizing early flowering mutants

Seeds of two M_4 early-flowering lines (A2 and A7) and one S_2 WT were grown for 123 d under controlled-environment (CE) conditions. Two seed were germinated in 1.05 l pots (height 11.3 cm, diameter 13 cm) containing 1 l of compost mix 2 : 1 : 1 (v : v : v) Levington M3 high nutrient compost : perlite (2–5 mm) : vermiculite (2–5 mm) (Monro Group). One plant from each pot was later harvested for mineral analysis, and the remainder left to fruit. A randomized block design comprising six replicates was used, with six individual pots of each of three lines allocated at random within each replicate within a growth tray (length 97 cm, width 38 cm, height 5 cm) (Giant Plant Grobag Tray, Sankey, UK). A single Zn solution was applied to all pots on a single occasion after 1 month of growth, supplying 455 mg Zn kg^{-1} compost. The CE conditions were 16 h photoperiod 19°C (± 2°C), lighting intensities of 137–147 μmol m^{-2} s^{-1} from eight metal halide lamps (Osram Powerstar HQI-BT 400W/D Osram, Berkshire, UK) and relative humidity (RH) 83% (± 10%). Phenotypic development was recorded daily while temperature and RH were monitored every 10 min using a data logger (Tinytag Plus 2; Gemini Data Loggers Ltd, Chichester, UK).

Characterizing mineral composition of early-flowering mutants

Aerial tissue was harvested, dried at 60°C for 2 d and homogenized manually to ensure particle size uniformity. Approximately 300 mg of dried tissue was digested under closed-vessel microwave heating (45 min, 20 bar) in 2 ml of 70% trace analysis grade (TAG) HNO_3, 1 ml H_2O_2 (Fisher Scientific UK Ltd, Loughborough, Leicestershire, UK) and 1 ml milli-Q water (18.2 MΩ cm). The microwave system comprised a Multiwave 3000 platform with a 48-vessel 48MF50 rotor (Anton Paar GmbH, Graz, Austria). Samples were digested in perfluoroalkoxy (PFA) liners inserted into polyethylethylketone (PEEK) pressure jackets (Anton Paar GmbH). Digested samples were diluted to 15 ml with milli-Q water and stored at room temperature. Mineral analysis was conducted using inductively coupled plasma-mass spectrometry (ICP-MS) as described previously (Broadley *et al.*, 2010). Briefly, samples were further diluted 1-in-10 with milli-Q water and analysed using an ICP-MS (X-SeriesII; Thermo Fisher Scientific Inc., Waltham, MA, USA). Internal standards included Sc (50 ng ml^{-1}), Rh (10 ng ml^{-1}) and Ir (5 ng ml^{-1}) in 2% TAG HNO_3. External multi-element calibration standards (Claritas-PPT grade CLMS-2; SPEX Certi-Prep Ltd, Stanmore, Middlesex, UK) included Al, As, Ba, Bi, Cd, Co, Cr, Cs, Cu, Fe, Mn, Mo, Ni, Pb, Rb, Se, Sr, U, V and Zn, in the range 0–100 μg l^{-1}, and Ca, Mg, K and Na in the range 0–100 mg l^{-1}. Data were corrected using blank digestions.

Results and Discussion

We generated genetically stable faster-cycling lines of *N. caerulescens* which are nonvernal-obligate. From 5500 M_0 seeds irradiated at 60 Gy, M_1 plants were grown using standard procedures for *N. caerulescens*, including a 10 wk period of vernalization. Approximately 80% of M_1 seeds germinated, with 2% showing signs of leaf colour variegation; 79% of plants survived to maturity. Approximately 80 000 M_2 seeds were maintained in a single pool at an average of 25 seeds per M_1 plant. M_2 seeds were grown initially in modules (Fig. 1a) and transplanted to pots under GC (Fig. 1b). The M_2 plants were screened for early-flowering phenotypes with no vernalization requirement (Fig. 1c). A total of 0.49% M_2 seedlings demonstrated lethal albinism (Fig. 1a). Floral initials were observed in 35 individuals in the absence of vernalization. Of these, nine individual plants flowered within 12 wk, producing an average of 100 M_3 seeds per selfed plant (Fig. 1c). One selfed M_2 individual, 'A2', produced *c.* 800 M_3 seeds ($A2M_3$). Two of these nine plants ($A2M_3$ and $A7M_3$) were selfed, again without vernalization, to produce $A2M_4$ and $A7M_4$ seeds, respectively. These two lines were compared for flowering and mineral uptake traits with an S_2 WT line from the original population.

Lines of $A2M_4$, $A7M_4$ and the S_2 WT were transplanted to pots under CE conditions at 7 DAS; germination in module trays was > 98% for all lines by 7 DAS. By 66 DAS, all $A2M_4$ plants had developed floral initials, by 71 DAS all $A2M_4$ plants had unopened flower buds, and by 79 DAS all $A2M_4$ plants had fully opened flowers (Fig. 2). The $A7M_4$ flowering was *c.* 3 wk slower than $A2M_4$. Thus, by 87 DAS, all $A7M_4$ plants developed floral initials, by 97 DAS all had unopened flower buds, and by 104 DAS all had fully open flowers. Silicle development was well established for all $A2M_4$ and the majority of $A7M_4$ individuals by 92 and 123 DAS, respectively. No floral or silicle development was observed in any of the S_2 WT plants at these dates.

Wild-type *N. caerulescens* produced 32 and 23% more leaf biomass than the $A2M_4$ and $A7M_4$ lines, respectively. However, there was no significant difference in leaf tissue DW between the two faster-cycling lines (data not shown). Mineral analysis of dried leaf tissue demonstrated that both $A2M_4$ and $A7M_4$ rapid-cycling mutant lines contained similar leaf Zn concentrations to the WT, which were in the range > 0.3% Zn on a DW basis. This indicates that the

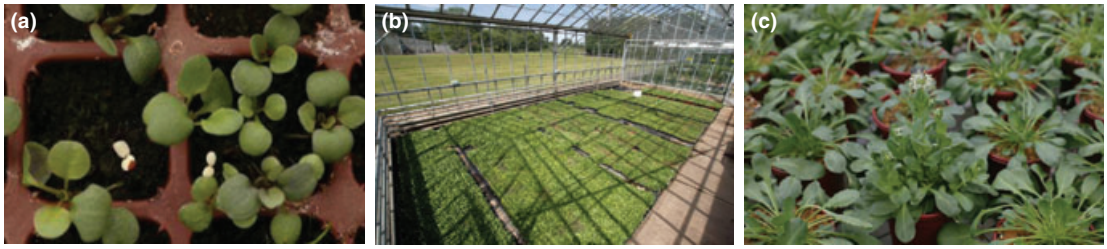

Fig. 1 M_2 fast neutron mutagenized *Noccaea caerulescens*: (a) Seedlings growing in 2 cm^2 plug trays, showing lethal albinism; (b) a subset of 80 000 M_2 is screened for nonvernal-obligate phenotypes under glasshouse conditions; (c) 12-wk-old, prevernal early-flowering M_2 plant in the glasshouse. Bar, 10 cm.

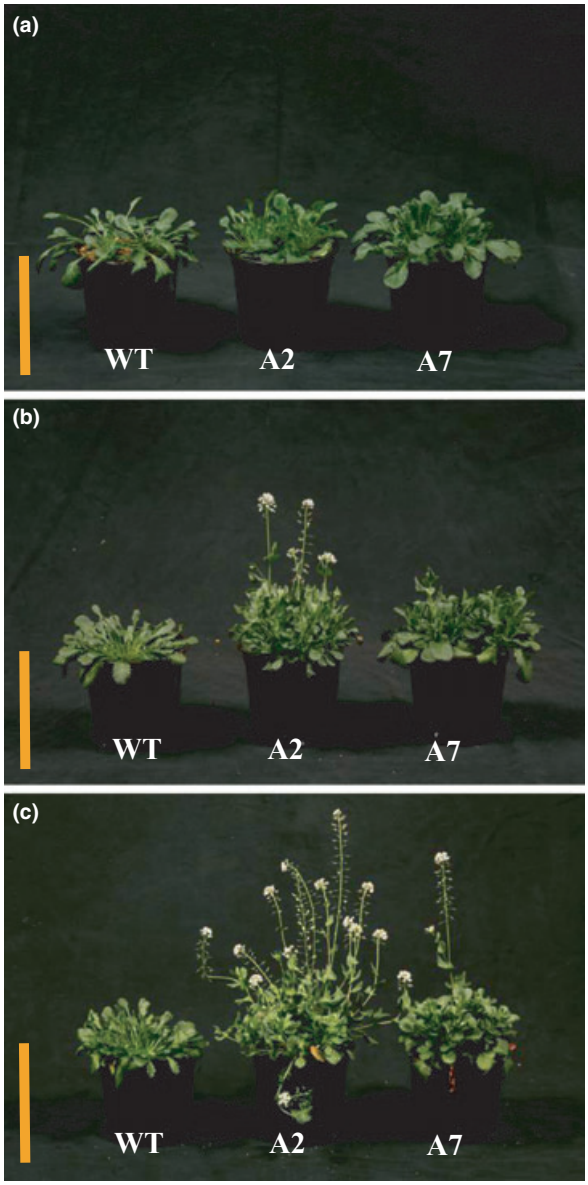

Fig. 2 *Noccaea caerulescens* lines. S_2 wild-type (WT), $A2M_4$ (A2) and $A7M_4$ (A7) growing under controlled-environment conditions (16 h photoperiod, 19°C (± 2°C), and relative humidity 83% (± 10%)). Photographs taken at 70 (a), 96 (b) and 112 (c) d after sowing. Bars, 15 cm.

hyperaccumulation phenotype (Reeves & Brooks, 1983; Broadley *et al.*, 2007) was retained. Thus, when grown with the addition of 455 mg Zn kg^{-1} to the compost, WT, $A2M_4$ and $A7M_4$ plants accumulated 0.34, 0.33 and 0.35% Zn on a DW basis, respectively (Fig. 3a). However, WT, $A2M_4$ and $A7M_4$ plants differed in leaf concentrations of other minerals, including the macronutrients Mg, Ca and K, the micronutrient Fe and Cd, which were all typically higher in the WT. Leaf Mg concentrations (in WT, $A2M_4$ and $A7M_4$ plants, respectively) were 0.42, 0.31 and 0.37%, leaf Ca concentrations were 0.80, 0.61 and 0.69%, leaf K concentrations were 2.86, 2.19 and 2.86% on a DW basis, leaf Fe concentrations were 140, 48 and 103 mg kg^{-1} DW, and leaf Cd concentrations were 1.1, 0.5 and 0.8 mg kg^{-1} DW (Fig. 3b–f). These variations might be the result of phenological differences between lines (Nord & Lynch, 2009). In this study, no exogenous Cd was supplied to soil and therefore it is not known if the Cd-hyperaccumulating phenotype has been retained. This requires further study.

Phenotypically, both mutant lines were stable and there was no evidence of significant intraline variability in flowering, growth or Zn accumulation. Neither A2 nor A7 displayed seed dormancy or altered germination, and all seeds germinated within 7 d. However, there is a significant reproductive cost of accelerated life cycle in terms of decreased fertility. Wild-type plants can typically produce between 500 and 3000 seeds, whereas the mean number of seeds per plant for A2 was 109, and for A7 was 19.

The A2 and A7 lines had clearly lost the requirement for vernalization to initiate flowering whilst remaining self-fertile. From a preliminary backcross experiment to WT *Noccaea* lines, there is no evidence to date that this nonvernalization trait is dominant. Both mutant lines exhibited much more rapid flowering and seed maturation phenotypes than any WT grown under our conditions. It is likely that these lines will significantly reduce the period currently required to cultivate vernal-obligate WT *N. caerulescens* (Peer *et al.*, 2003, 2006), enabling production of up to four generations of seed in a single year. Both lines appear to have retained the Zn hyperaccumulator phenotype, and so these lines have potential for establishing further molecular

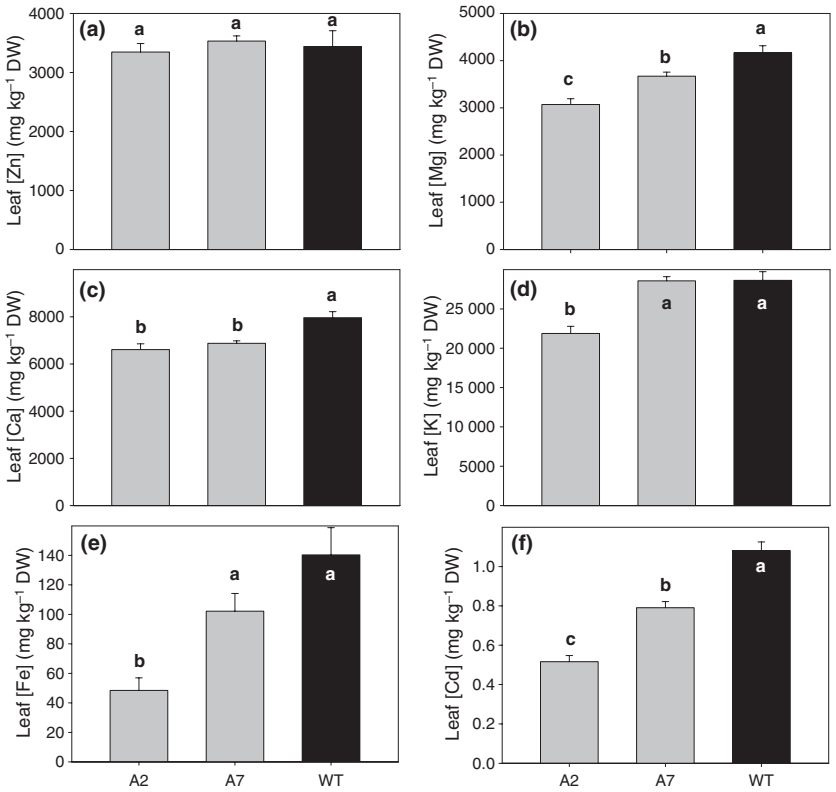

Fig. 3 Mean leaf concentrations of zinc (Zn; a), magnesium (Mg; b), calcium (Ca; c), potassium (K; d), iron (Fe; e) and cadmium (Cd; f) in rosette leaves of *Noccaea caerulescens* lines M_4A2 (A2), M_4A7 (A7) and S_2 wild-type (WT) growing under controlled-environment conditions for 123 d. Bars sharing a lower case letter are statistically indistinguishable based on ANOVA and least-significant-difference test ($P = 0.05$).

genetic insights, especially when efficient transformation systems and full genome sequence become available. If faster-cycling lines of other *N. caerulescens* ecotypes can be similarly developed, there is scope for establishing additional mapping populations and introgression lines to facilitate locus resolution. As we have found with rapid-cycling *Brassica*, the elimination of a vernalization requirement greatly accelerates the ability to resolve traits introgressed from a wide range of germplasm, including subsequent selection for reduced time to flowering and seed maturation. It may also be possible to further mutate lines A2 and A7 to produce even faster-cycling lines in the future. Lines A2 and A7 are available as a community resource from the European Arabidopsis Stock Centre (NASC; http://arabidopsis.info).

We have not yet investigated the molecular basis for rapid cycling in the *N. caerulescens* A2 and A7 lines. In the first instance, it will be interesting to test if functional homologues of *FLOWERING LOCUS C* (*FLC*) and *FRIGIDA* (*FRI*) (Michaels & Amasino, 1999; Sung & Amasino, 2004; Reeves *et al.*, 2007; Wang *et al.*, 2009; Zhao *et al.*, 2010) have been affected. It may be possible to test this hypothesis using high-throughput transcriptome sequencing or DNA hybridizations to tiling or exon arrays designed for *Arabidopsis* (Mockler *et al.*, 2005) or *Brassica* (Love *et al.*, 2010) using heterologous- (cross-) species-based approaches (Broadley *et al.*, 2008). However, further selfing, backcrossing and complementation will most likely still be required since the mutational load is not yet known.

Our results demonstrate that fast neutron mutagenesis is a viable approach to develop nonvernal-obligate, faster-cycling *N. caerulescens* lines. It is anticipated that these lines will become a valuable community resource for future molecular genetic investigations into metal tolerance and hyperaccumulation.

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