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RESEARCH PAPER

Wheat (*Triticum aestivum*) NAM proteins regulate the translocation of iron, zinc, and nitrogen compounds from vegetative tissues to grain

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Abstract

The *NAM-B1* gene is a NAC transcription factor that affects grain nutrient concentrations in wheat (*Triticum aestivum*). An RNAi line with reduced expression of *NAM* genes has lower grain protein, iron (Fe), and zinc (Zn) concentrations. To determine whether decreased remobilization, lower plant uptake, or decreased partitioning to grain are responsible for this phenotype, mineral dynamics were quantified in wheat tissues throughout grain development. Control and RNAi wheat were grown in potting mix and hydroponics. Mineral (Ca, Cu, Fe, K, Mg, Mn, P, S, and Zn) and nitrogen (N) contents of organs were determined at regular intervals to quantify the net remobilization from vegetative tissues and the accumulation of nutrients in grain. Total nutrient accumulation was similar between lines, but grain Fe, Zn, and N were at lower concentrations in the *NAM* knockdown line. In potting mix, net remobilization of N, Fe, and Zn from vegetative tissues was impaired in the RNAi line. In hydroponics with ample nutrients, net remobilization was not observed, but grain Fe and Zn contents and concentrations remained lower in the RNAi line. When Fe or Zn was withheld post-anthesis, both lines demonstrated remobilization. These results suggest that a major effect of the *NAM* genes is an increased efflux of nutrients from the vegetative tissues and a higher partitioning of nutrients to grain.

Key words: Biofortification, grain protein content, iron, remobilization, senescence, zinc.

Introduction

Wheat is a crop of major importance and together with other staple cereals supply the bulk of calories and nutrients in the diets of a large proportion of the world population (Cakmak, 2008). Cereals are inherently low in protein and mineral micronutrients such as Fe and Zn (White and Broadley, 2005, 2009; Cakmak, 2008; Newell-McGloughlin, 2008). A major focus of wheat breeders has been grain protein concentration as it affects bread- and pasta-making quality, but micronutrient improvement has received less attention. Approximately half of the world's population suffers from Fe and/or Zn deficiencies (Cakmak, 2008) and millions of children suffer from protein-energy malnutrition

(de Onis *et al.*, 1993). As such, the improvement of nutritional quality of wheat could benefit the nutritional status of millions of people.

A common agronomic practice to increase grain protein concentration is the use of N fertilization. However, this practice is expensive and excess fertilizer run-off is a potential environmental contaminant (Masclaux-Daubresse *et al.*, 2008). A substantial percentage of the N in wheat grain is supplied by amino acids remobilized from vegetative tissue (Barneix, 2007; Gregersen *et al.*, 2008; Masclaux-Daubresse *et al.*, 2008). Much of this N content is derived from proteins that are disassembled and recycled during the leaf

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senescence stage of development (Hopkins *et al.*, 2007). Likewise, Fe and Zn have been shown to be remobilized from vegetative tissues in several plants (Hocking and Pate, 1977; Hocking, 1994; Miller *et al.*, 1994; Drossopoulos *et al.*, 1996; Waters and Grusak, 2008), although the specific sources are unknown. Zinc fertilization has been a successful strategy to improve wheat grain Zn concentration (Cakmak, 2008), and improvement in the partitioning or remobilization of Zn to grain could make fertilization efforts more efficient. Wheat grain with higher Zn concentration has been demonstrated to produce more vigorous crops (Cakmak, 2008; Yilmaz *et al.*, 1998). Thus, breeding or transgenic approaches that result in plants with increased partitioning of minerals to grain could be useful for both nutritional biofortification and reduced fertilizer application.

Chromosome 6B from wild emmer wheat (*Triticum turgidum* ssp. *dicoccoides*) was identified as a potential source of genetic variation for grain protein (Joppa and Cantrell, 1990), Zn, and Fe concentration (Cakmak *et al.*, 2004). A quantitative trait locus (QTL) for grain protein concentration was mapped on chromosome arm 6BS (Joppa *et al.*, 1997) and later mapped as a single Mendelian locus, *Gpc-B1* (Olmos *et al.*, 2003). In near-isogenic lines of this locus, increased grain protein was associated with the increased remobilization of amino acids from the flag leaf (Kade *et al.*, 2005), higher grain Fe and Zn concentrations (Cakmak *et al.*, 2004; Distelfeld *et al.*, 2007), and accelerated leaf yellowing, indicating accelerated senescence (Uauy *et al.*, 2006a). A NAC transcription factor, *NAM-B1*, was identified as the causal gene for *Gpc-B1* by positional cloning (Uauy *et al.*, 2006b). Other members of the NAC family are known to regulate developmental processes (Aida and Tasaka, 2006; Olsen *et al.*, 2005), including leaf senescence (Guo and Gan, 2006). In transgenic wheat *NAM* RNA interference (RNAi) lines in which *NAM-B1* and its homeologous genes had decreased expression, leaf yellowing was delayed, and grain protein, Fe, and Zn concentrations were greatly decreased (Uauy *et al.*, 2006b). These results, together with higher N, Fe, and Zn concentrations in RNAi line flag leaves at maturity, suggested a role for *NAM-B1* homeologues in the remobilization of N compounds, Fe, and Zn. However, without taking organ mass, nutrient concentrations at prior time points, and total nutrient accumulation of other organs into account, this model could not be confirmed. In addition, the body of literature does not contain sufficient data regarding sources of grain minerals (other than N) to support the idea that remobilization alone could account for the differences observed. Because a whole-plant partitioning profile has not been undertaken in plants differing in *NAM-B1* expression, it is currently unclear whether this gene directly affects remobilization (which is defined here as the net loss of stored mineral content from an organ over time), alters partitioning of nutrients within the plant, alters total plant uptake of these nutrients, or influences a combination of these processes. The current study uses multiple time point sampling of an expanded profile of mineral concentrations

and contents (which takes organ mass into account) of all shoot organs in *NAM* knockdown and control lines. This sampling allows the quantification of N and mineral remobilization as contributors to final grain protein and mineral content, and provides a better understanding of the physiological effects of the *NAM* genes. In addition, experimental treatments have been included to test the remobilization capacity of Fe and Zn in these lines, and radiolabelled Zn was used to quantify the short-term translocation to grain. The information presented here will inform future genomic and systems level studies designed to understand genes and processes that can be targeted to increase grain mineral concentrations for the biofortification of foods.

Materials and methods

Plant growth in potting mix

The lines used in this study were the *NAM* RNAi lines designed to reduce expression of all *NAM* family members (event L19-54) and its non-transgenic control (Uauy *et al.*, 2006b). The transformed line is Bobwhite, a semi-dwarf, hard, white, spring, common wheat (*T. aestivum*) variety. Wheat seeds were imbibed at 4 °C for 3 d and allowed to germinate in darkness at room temperature for 4 d. Seedlings were planted in commercial potting mix (Metro-Mix 300; Sun Gro Horticulture, Bellevue, WA, USA) and vermiculite at a 2:1 ratio in 17 cm diameter, 17 cm tall pots, three plants per pot, and placed in a growth chamber (16 h photoperiod; 350 $\mu\text{E m}^{-2} \text{s}^{-1}$ of photosynthetically active radiation at the top of the pots, 22.5/17.5 °C day/night, relative humidity set at 50%). Pots were placed in trays with two pots of each line per tray. Plants were watered as needed by sub-irrigation (usually twice per week) with a nutrient solution (2.0 l per tray) of the following composition: 1.2 mM KNO_3 , 0.8 mM $\text{Ca}(\text{NO}_3)_2$, 0.8 mM NH_4NO_3 , 0.3 mM KH_2PO_4 , and 0.2 mM MgSO_4 . Plants were sampled at anthesis of the first emerged head (d0), and at 14, 28, 35, 42, and 56 d after anthesis (DAA), with an additional harvest at 70 DAA for the RNAi line. At each sampling, the number of tillers was noted, and plants were cut with a scalpel into the following parts: heads, peduncles, stems, lower leaves, and flag leaves. For each plant, organs from all tillers were pooled and a total of 4–6 plants (replicates) from two separate pots were analysed per time point. Tissues were dried for 48 h in a drying oven at 60 °C, and dry weights were obtained. After drying, heads were separated into grain, rachis, and florets, grains were counted, and these parts were weighed.

Plant growth in hydroponics

Wheat seeds were imbibed as described above. Seedlings were planted in plastic cups with plastic beads for support, then placed in lids over 4.5 l containers, 10 plants per pot, in a growth chamber with the settings as described above. Plants were maintained in a complete nutrient solution of

the following composition: 3.0 mM KNO₃, 1.0 mM Ca(NO₃)₂, 0.5 mM KH₂PO₄, 0.5 mM MgSO₄, 0.75 mM K₂SO₄, 0.1 mM K₂SiO₃, 25 µM CaCl₂, 25 µM H₃BO₃, 0.5 µM MnSO₄, 0.5 µM ZnSO₄, 0.5 µM CuSO₄, 0.5 µM H₂MoO₄, 0.1 µM NiSO₄, and 10 µM Fe(III)-HEDTA (*N*-hydroxyethylethylene-diaminetriacetic acid; Sigma Chemical Co., St Louis, MO, USA). Solutions were buffered at pH 5.8 with 2.0 mM MES (2-[*N*-morpholino] ethane sulphonic acid, monohydrate; Research Organics, Cleveland, OH, USA), and changed twice weekly. For Fe or Zn deficiency treatments (0 Fe and 0 Zn, respectively), ZnSO₄ or Fe(III)-HEDTA were omitted from the solution after anthesis. Plants were sampled at anthesis of the first emerged head (d0), and at 42 DAA for the Fe deficiency and control treatments (+Fe+Zn). Since 0 Zn plants matured more rapidly than control or 0 Fe plants, plants were sampled at 35 DAA for Zn deficiency and control treatments. At each sampling, the number of tillers was noted, and plants were cut with a scalpel into the following parts: heads, peduncles, stems, lower leaves, and flag leaves. Usually, all tillers from a plant were collected, although the occasional late-emerging tiller was discarded. Thus, 2–5 tillers from a minimum of two plants per time point were collected. Organs from each tiller were analysed separately, then average values were calculated. Tissues were dried for 48 h in a drying oven at 60 °C, and dry weights were obtained. After drying, grain was removed from heads, and weighed separately.

Elemental analysis

For mineral analysis of potting mix-grown plants, above-ground organs were dissected and organs from all tillers of each plant were pooled as described above. All tissues except florets were ground in a stainless-steel coffee mill. Duplicate subsamples of approximately 250 mg were weighed into glass tubes, and digested in nitric:perchloric acid (4:1 v/v) for 1 h at 100 °C, then gradually to 200 °C until the sample was taken to dryness. Samples were then resuspended in 15 ml 2% nitric acid. All acids were trace metal grade (Fisher Scientific, Pittsburgh, PA, USA) and water was filtered through a MilliQ system (Millipore, Billerica, MA, USA) to 18 MΩ resistivity. Mineral concentrations were determined by ICP-OES (CIROS ICP model FCE12; Spectro, Kleve, Germany). The mineral content of the tissues was calculated by multiplying tissue DW by each mineral concentration.

For mineral analysis of hydroponically grown plants, a different digestion procedure was used (due to a laboratory decision to curtail the use of perchloric acid). Whole organs were weighed into glass tubes, and digested in 2.0 ml nitric acid overnight, then at 125 °C for 1.5 h. 1.5 ml 30% H₂O₂ was added and samples were digested for 1 h. A second 1.5 ml volume of H₂O₂ was added and samples were digested for 1 h. The temperature was then increased to 200 °C and samples were evaporated to dryness. Residues were dissolved in 15 ml 2% nitric acid. Mineral concen-

trations were determined by ICP-OES. The mineral content of the tissues was calculated by multiplying tissue DW by each mineral concentration.

For N analysis, tissue samples were dried to constant weight and ground to a fine powder using a ball mill. The samples were analysed for N concentration by a continuous-flow mass spectrometer (Europa Scientific, Cambridge, UK) at the University of California, Davis Stable Isotope Facility.

Radiotracer studies

Wheat plants were grown in hydroponics as described above. The dates of anthesis were noted, and at mid-grain fill (20–25 DAA), plants were moved from a complete nutrient solution to a complete solution spiked with ⁶⁵Zn (Brookhaven National Laboratory, Upton, NY, USA) at 1 µCi l⁻¹ for continuous labelling experiments, and at 4 µCi l⁻¹ for pulse labelling experiments. All labellings were initiated between 3–4 h into the photoperiod. Plants were not removed from the growth chamber during the labelling period. For continuous labelling, the plants remained in the labelling solution for up to 24 h. For pulse-labelling, the plants were removed from the labelling solution after 3 h and rinsed for 10 min in complete, unlabelled nutrient solution, then placed in fresh complete, unlabelled nutrient solution. At 12 h (at or near end of photoperiod) or 24 h (3–4 h into following photoperiod) after the commencement of labelling, shoots were excised and cut into lower leaves, stems, flag leaf, peduncle, and heads. Heads were oven-dried for 4–12 h, then the grains were removed. All tissues were quantified for ⁶⁵Zn by gamma counting.

Statistics

Analyses of variance were performed using the SAS Version 9.1 program (SAS Institute, Cary, NC, USA). The general linear model (PROC GLM) was used to assess the effect of the reduced *NAM* transcript levels in the RNAi lines as compared to the isogenic controls. Data were transformed when necessary using logarithmic and power transformations in order to meet the assumptions of the model. For comparisons over growth periods, each time point was analysed separately using orthogonal contrasts. Differences in mineral content and concentration between tissues at different time points were analysed using unpaired *t* tests. Values after the '±' sign are standard errors of the mean throughout the text.

Results

Potting mix experiment

Control and RNAi plants grew similarly in terms of appearance and total plant size (see Supplementary Fig. 1 at *JXB* online). This similarity was also true for individual

plant organs, although some tissues differed at some time points. Total grain weight, on a per head basis, was similar between the two lines. Weight of individual kernels was nearly identical and reached maximum values by 35–42 DAA, although total grain dry weight (DW) continued to increase as a result of increased seed numbers at later time points. Across sampling points, RNAi lines had a higher number of grains per head, although these differences were significant only at 14 DAA and 35 DAA ($P < 0.03$). It was observed that, as described previously by Uauy *et al.* (2006b), the most notable difference between the two lines was delayed leaf yellowing of the RNAi line.

The complete data set of mineral contents for the potting mix-grown plants is presented in Supplementary Table S1 at *JXB* online. At anthesis, Fe and the contents of most other minerals (in μg) were similar in the vegetative organs of both lines (see Supplementary Table S1 at *JXB* online), suggesting that *NAM* genes had no effect on the content of these minerals to this point in development. Total Zn content was slightly lower in the RNAi line at anthesis ($P = 0.04$). At grain maturity (56 DAA), the total shoot contents (vegetative tissues plus grain) of Fe, Zn, and N were similar in both lines ($P > 0.65$), indicating that total uptake and accumulation of each mineral was not significantly affected by the *NAM* genes. Total vegetative Fe content (the sum of all non-grain organs) decreased between anthesis and maturity (i.e. exhibited net remobilization) in the control line (14.7%, $P = 0.36$; Fig. 1; Table 1). Although the decrease in content between anthesis and maturity was not significant, the RNAi line total vegetative Fe content did not decrease, but rather increased significantly ($P < 0.02$). Total vegetative Zn decreased only in the control line (60.9%, $P < 0.001$). Comparing the quantity of mineral remobilized from all vegetative tissues to the quantity of mineral in the grain pool at maturity (56 DAA), the net remobilized Fe and Zn could account for between 13.0% of grain Fe and 42.6% of total grain Zn content in the control line, assuming that all of each mineral demonstrating net remobilization was translocated to the grain (Table 2). In the control line, Fe content decreased over time in lower and flag leaves, stems, peduncle, and rachis, indicating net remobilization. Contrary to this, Fe remained constant or accumulated over time in all tissues of the RNAi line (Table 1). This was especially marked in the peduncle of RNAi plants, which accumulated 286% of the initial Fe content. Zinc content decreased significantly in all vegetative tissues of the control line ($P < 0.01$; Fig. 1; Table 1) In the RNAi line, Zn was remobilized from both the flag leaf and lower leaves, but the percentage change was lower than the control (Table 1). Zinc decreased in the RNAi line until 35 DAA in stem, peduncle, and head tissues, after which the content increased (see Supplementary Table S1 at *JXB* online).

Total N was quantified in selected tissues and time points for the control and RNAi line. At anthesis and grain maturity, total N contents were similar between both lines ($P = 0.92$ and $P = 0.68$), suggesting that reduced *NAM* expression did not alter total N uptake and accumulation.

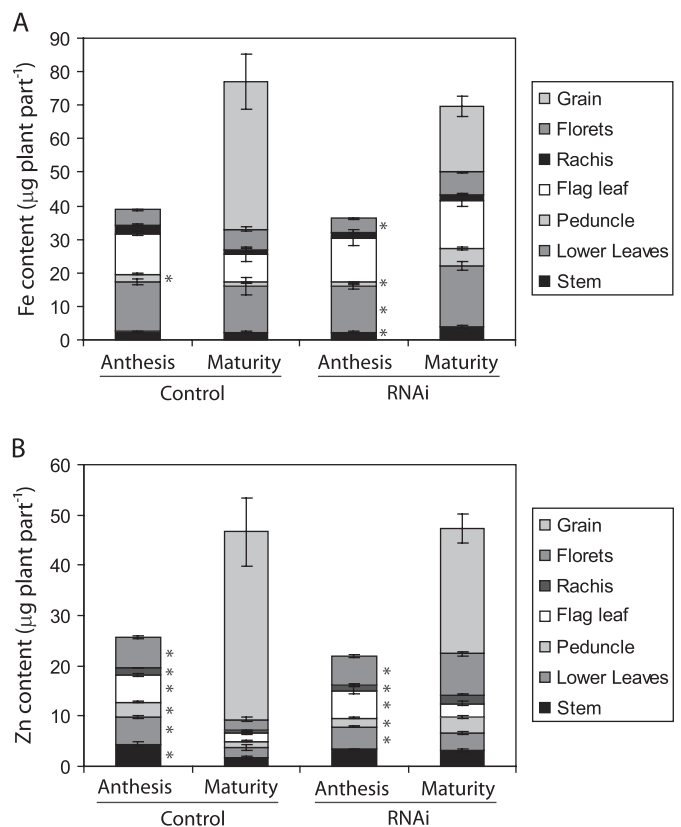


Fig. 1. Stacked bar graph representing mineral contents in individual vegetative tissues and grain in potting mix-grown control and RNAi wheat at anthesis and at maturity (56 DAA). (A) Fe content, (B) Zn content. Values \pm SE. Asterisk represents statistical difference ($P < 0.05$) between time points.

Throughout grain development, total and individual grain N content were significantly lower in the RNAi line than in the control line (Fig. 2; $P < 0.05$). As with Zn, RNAi plants remobilized N from flag leaves and lower leaves over the time-course, but to a lesser extent than in the control plants (29% RNAi versus 45% control flag leaf, 8.4% RNAi versus 33.7% control lower leaves). The greatest differences in flag leaf N remobilization occurred between 35 DAA and 56 DAA (Table 1). Comparing N content of all shoot vegetative organs (except the rachis, which was used entirely for mineral analysis) between these time points (Fig. 3), total shoot N (vegetative tissues plus grain) increased by similar quantities between the RNAi (8.0 mg) and control lines (7.5 mg). However, grain of the control line gained 14 mg of N, while grain of the RNAi line increased only by 2.7 mg. Vegetative tissues of control plants showed a net remobilization of 6.6 mg of N, while those of the RNAi line did not show a net remobilization of N, but rather N increased by 5.3 mg. From anthesis to maturity, N was remobilized from the control stem (18%) and peduncle (48%), while N content increased in these tissues of the RNAi line (+29% peduncle, +119% stem). This resulted in significant differences between control and RNAi lines for both grain N and vegetative N at maturity (56 DAA) ($P < 0.01$).

Table 1. Per cent change in Fe and Zn content from anthesis to maturity, and per cent change in N content from 35 DAA to 56 DAA

Experiment	Treatment	Nutrient	Flag leaf		Lower leaves		Peduncle		Stem		Florets		Rachis		Total vegetative	
			Control	RNAi	Control	RNAi	Control	RNAi	Control	RNAi	Control	RNAi	Control	RNAi	Control	RNAi
			Potting mix 56 DAA	Fe	-32.8	6.8	-7.4	33.1	-47.8	285.7	-8.0	77.3	32.6	53.4	-33.3	28.6
Potting mix 56 DAA	Zn	-71.7	-50.9	-61.3	-24.4	-61.3	82.4	-61.4	-2.9	-63.9	43.9	-60.0	54.5	-60.9	1.8	
Potting mix 56 DAA	N	-32.0	-5.0	-22.0	6.0	-26.0	17.0	-20.0	52.0	-6.0	20.0	nd ^a	nd	-21.0	17.0	

Experiment	Treatment	Nutrient	Flag Leaf		Lower Leaves		Peduncle		Stem		Head		Total vegetative	
			Control	RNAi	Control	RNAi	Control	RNAi	Control	RNAi	Control	RNAi	Control	RNAi
			Hydroponic 35 DAA	+Zn	Zn	-48.3	-14.0	15.5	20.8	0.0	-15.9	-34.9	-27.8	153.8
Hydroponic 35 DAA	0Zn	Zn	-80.6	-60.3	-69.9	-86.8	-92.8	-86.8	-90.1	-83.9	-49.2	-50.5	-74.8	-69.6
Hydroponic 42 DAA	+Fe	Fe	-54.0	-16.0	-18.0	-16.0	-37.0	13.0	-20.0	25.0	111.0	48.0	-7.9	-3.7
Hydroponic 42 DAA	0Fe	Fe	-74.0	-21.0	-57.0	-36.0	-77.0	-40.0	-56.0	-15.0	-59.0	-55.0	-65.6	-40.2

^a nd, Not determined.

Table 2. Total net remobilization from vegetative tissues as percentage of seed mineral content

Experiment	Treatment	Cu		Fe		K		Mn		P		S		Zn	
		Control	RNAi	Control	RNAi	Control	RNAi	Control	RNAi	Control	RNAi	Control	RNAi	Control	RNAi
		Potting mix 56 DAA		31.6	-	13.0	-	-	-	-	-	10.0*	-	-	-
Hydroponic 35 DAA	+Zn	34.2 ^{a,b}	66.4*	-	-	-	14.7	-	-	-	-	-	-	-	-
Hydroponic 35 DAA	0 Zn	- ^a	7.8	-	-	-	-	-	-	-	-	-	-	180.3*	185.5*
Hydroponic 42 DAA	+Fe	15.6*	64.9*	7.4	5.5	-	-	-	-	53.5*	-	-	-	-	-
Hydroponic 42 DAA	0Fe	5.0	-	165.2*	163.2*	17.8	0.5	74.9*	-	51.1*	-	33.5*	-	-	-

^a No net remobilization occurred.

^b Significant between anthesis and maturity at $P < 0.05$.

Hydroponic experiments

To characterize further the mineral remobilization in the RNAi lines under different levels of Fe and Zn, plants were grown using hydroponic conditions, either in a complete nutrient solution for the duration of the experiment, or in an Fe- or Zn-deficient solution from anthesis onwards. Withholding Fe and Zn forced plants to rely solely on stored Fe and Zn to supply the grain. This treatment was used to assess potential net remobilization from the vegetative tissue to the grain while preventing uptake and xylem translocation of these minerals during grain fill, although some root-associated Fe and Zn may have supplied a finite quantity of residual mineral content. If the RNAi line was capable of remobilizing minerals, a net loss of Fe or Zn content would be detected in vegetative tissues between anthesis and maturity. The full data set is presented as Supplementary Table S2 available at *JXB* online. Leaf yellowing was delayed in the RNAi line in hydroponics, similar to potting mix-grown plants. The control and RNAi lines had similar Fe and Zn vegetative contents at anthesis (Fig. 4; $P > 0.75$). For total shoots (less grain), neither line exhibited significant net remobilization of Fe or Zn when grown on complete nutrient solution, but both remobilized significant quantities of these minerals when grown on

deficient nutrient solutions ($P < 0.02$). Iron-deprived RNAi plants had significantly less net remobilization, with a decrease of 40.2% of vegetative Fe content as compared to 65.6% for the control line (Table 1; $P = 0.01$). Zinc-deprived RNAi plants showed a net remobilization of 69.6% of Zn content compared to 74.8% for the control line, but the difference between lines was not significant (Table 1; $P = 0.66$). The quantities of Fe and Zn remobilized were more than enough to account for grain mineral content (Table 2), although total Fe or Zn content in the grain for each line was significantly lower than when these minerals were supplied continuously (Fig. 4; see Supplementary Table S2 at *JXB* online; $P < 0.03$). Some shoot Fe and Zn may have been translocated to roots to maintain root growth. Roots of both lines grown in complete nutrient solution did not decrease in Fe or Zn content during grain fill (see Supplementary Table S3 at *JXB* online). In Fe- and Zn-withholding treatments, root mineral content and concentration decreased substantially in both lines. Roots of all lines and treatments continued to grow during grain fill, and were larger at grain maturity than at anthesis, making calculation of remobilization by subtraction (as done with shoot organs) impractical.

Despite the lack of significant net Fe and Zn remobilization from the total vegetative tissues of plants grown in

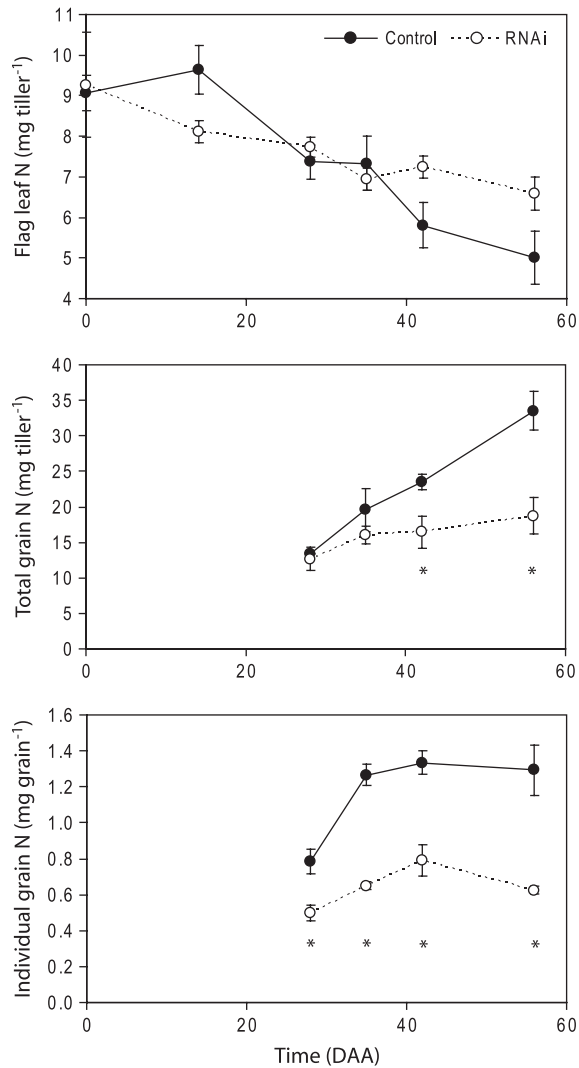


Fig. 2. Nitrogen content of flag leaves and grain in control and RNAi plants over the growth period. (A) Flag leaf N content, (B) total grain N content (per tiller), (C) N content of individual grains. Values \pm SE. Asterisk denotes statistical significance ($P \leq 0.05$).

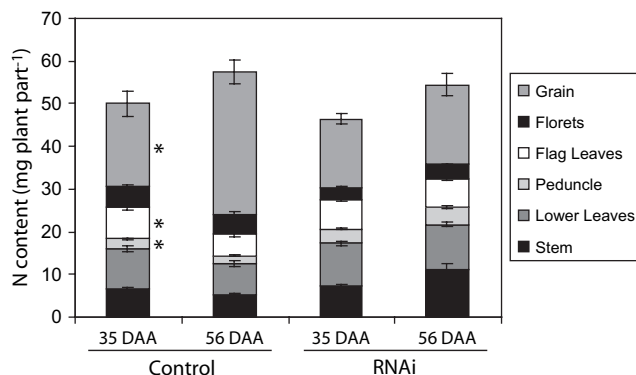


Fig. 3. Stacked bar graph representing nitrogen content in individual vegetative tissues and grain in control and RNAi wheat at 35 DAA and at 56 DAA (maturity). Values \pm SE. Asterisk represents statistical difference ($P < 0.05$) between time points.

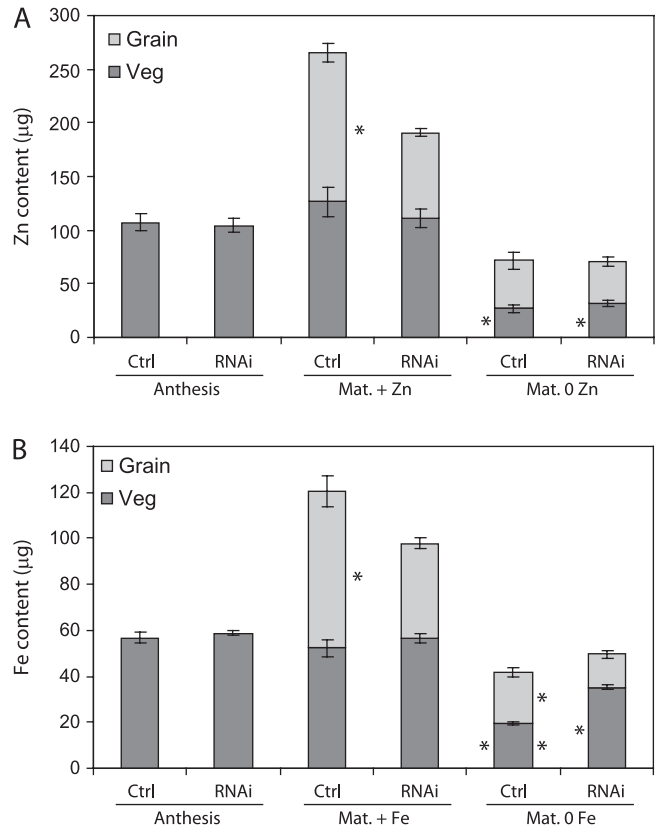


Fig. 4. Stacked bar graph representing (A) Zn content and (B) Fe content in total vegetative tissues and grain in hydroponic grown control and RNAi lines at anthesis and maturity [35 DAA for (A); 42 DAA for (B)]. +Zn, Zn was supplied continuously; +Fe, Fe was supplied continuously; 0 Zn, Zn was omitted from nutrient solution from anthesis onward; 0 Fe, Fe was omitted from nutrient solution from anthesis onward. Values \pm SE. Asterisk to the left of bar represents statistical difference ($P < 0.05$) from anthesis. Asterisk to the right of bar represents statistical difference ($P < 0.05$) between lines at that time point.

complete nutrient solution, the control line exhibited substantial Fe and Zn net remobilization from some vegetative organs (Table 1). The control line exhibited a 54% and 48.3% decrease in flag leaf Fe and Zn content, respectively, between anthesis and maturity (35 DAA for Zn, 42 DAA for Fe; Table 2). For both minerals, the RNAi line remobilized significantly less ($P < 0.05$) Fe (16%) and Zn (14%) from the flag leaves. This indicates that the *NAM* genes influence net remobilization from flag leaves even in the complete nutrient solution. The other minerals tended to accumulate or remain constant in flag leaves between anthesis and 35 DAA, and the effect of the *NAM* genes was not readily apparent (see Supplementary Table S2 at *JXB* online). An exception was Cu, which was also remobilized in both RNAi and control plants (Table 2; Supplementary Table S2 at *JXB* online). These data suggest that under these experimental conditions, the effect of *NAM* genes on mineral remobilization primarily affects remobilizable metal micronutrients, i.e. Fe, Zn, and Cu.

Grain mineral accumulation

Over the time-course of the potting mix experiment, grain Ca and Mg concentrations ($\mu\text{g g}^{-1}$) were similar between lines at most time points, while K was higher in the RNAi line (Fig. 5). Copper, Fe, Mn, P, S, and Zn were at lower concentrations in the RNAi line at most time points. In the hydroponics experiments, the RNAi line produced more, although slightly smaller grains per head than the control line, resulting in significantly higher total grain mass per tiller (see Supplementary Fig. S2 at *JXB* online). Across treatments, the total grain mineral content per tiller was higher in the RNAi line than in the control line for Ca and K, and similar for Mg, Mn, P, and S (with the exception of 35 DAA 0 Zn; see Supplementary Table S2 at *JXB* online). This similar total grain mineral accumulation spread across a higher total grain weight in the RNAi line resulted in lower concentrations of Mg, Mn, P, and S in this line, relative to the control (Fig. 6). Copper, Fe and Zn concentrations were also lower, but by a greater percentage than the other minerals. In terms of total grain accumulation (content), Fe was lower in the RNAi line relative to the control in complete solution at 35 DAA and 42 DAA ($P=0.05$), and in the Fe withheld experiment (36% , $P<0.01$). Grain Zn content in the RNAi line was also significantly lower than the control in complete solution at

35 DAA and 42 DAA ($P < 0.05$), but not in the Zn deficiency treatment (Fig. 6; see Supplementary Table S2 at *JXB* online).

Radiotracer experiment

To determine the effect of *NAM* genes on the short-term translocation of newly absorbed Zn to the grains, a radioactive tracer strategy was used. At mid-grain fill, plants were transferred from a complete nutrient solution to a complete nutrient solution spiked with ^{65}Zn . After 12 h or 24 h, radioactivity was detected in all shoot organs. In three of the four experiments, the proportion of total shoot ^{65}Zn that was partitioned into grain was significantly higher in the control line than in the RNAi line (Fig. 7). This was true regardless of whether the plants were continuously labelled or pulse-labelled.

Discussion

Remobilization: an operational definition

Much of the N imported into wheat grain is derived from protein in vegetative tissues that is degraded to amino acids and recycled by translocation to the grain, i.e. remobilization (Barneix, 2007; Gregersen *et al.*, 2008;

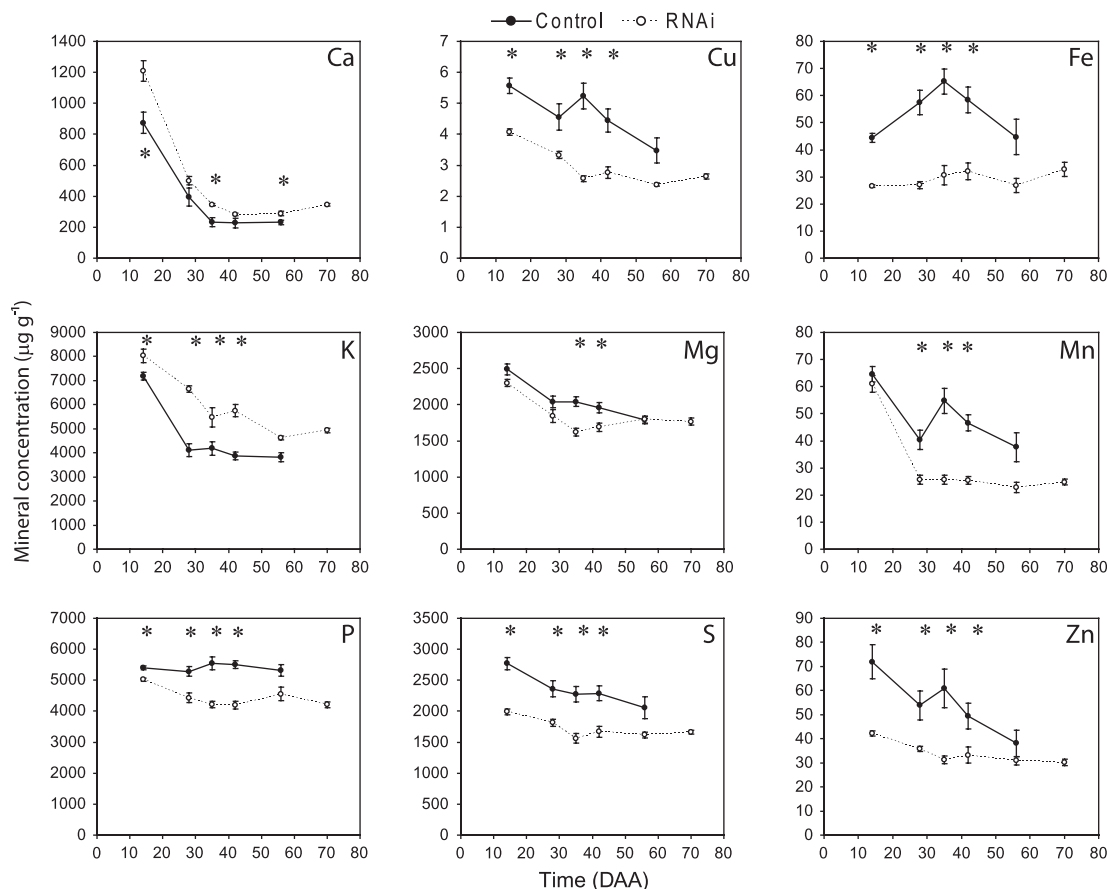


Fig. 5. Grain mineral concentrations over the grain development period in potting mix-grown wheat. DAA, days after anthesis. Values \pm SE. Asterisk denotes statistical significance ($P \leq 0.05$).

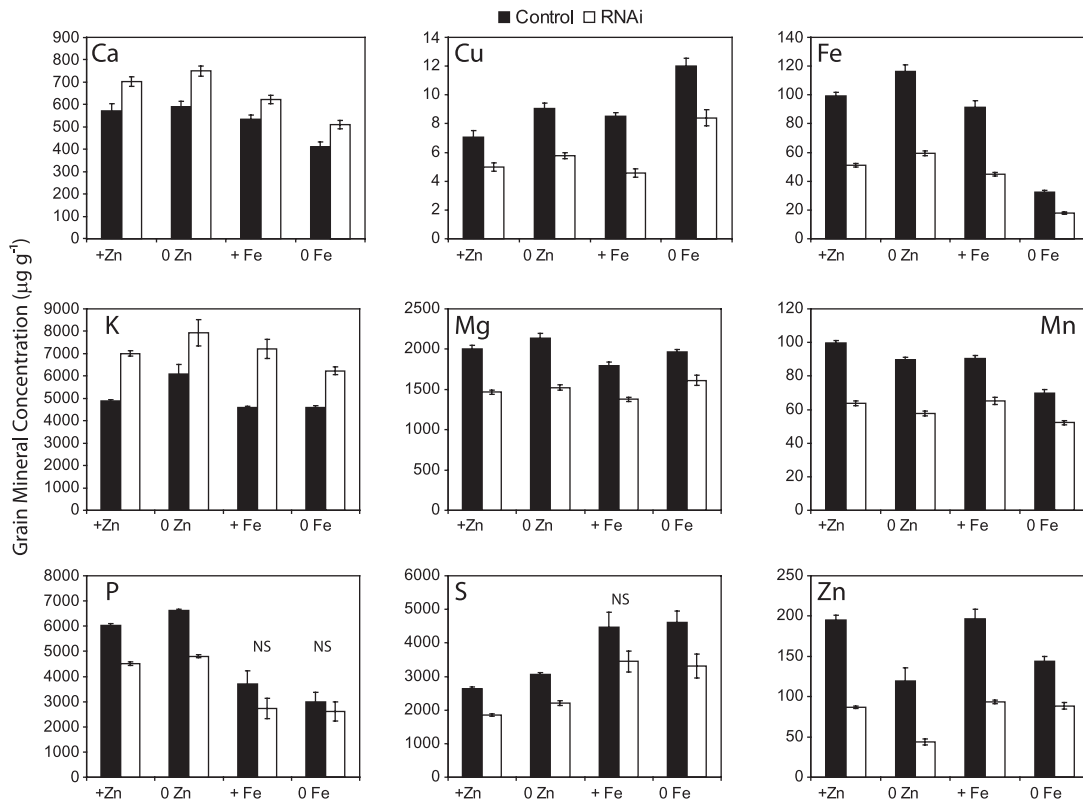


Fig. 6. Grain mineral concentrations of mature control and RNAi lines grown in hydroponics with complete nutrient solution (+Zn, 35 DAA, +Fe, 42 DAA), or nutrient solution lacking Zn or Fe from anthesis onward (0 Zn, 35 DAA; 0 Fe, 42 DAA). Values \pm SE. Unless denoted by NS (not significant), control and RNAi values were statistically different ($P \leq 0.05$).

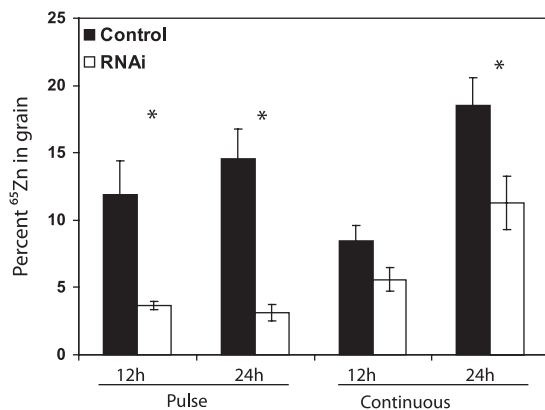


Fig. 7. Short-term partitioning of newly taken up Zn to grain at mid-grain fill. Percentage of total shoot ⁶⁵Zn counts in grain at each sampling for control and RNAi lines grown in complete nutrient solution spiked with ⁶⁵Zn. 12 h and 24 h represent 12 h and 24 h sampling time points. Continuous indicates continuously labelled experiment and pulse the pulse-label experiment. Values \pm SE. Asterisk denotes statistical significance ($P \leq 0.05$).

Masclaux-Daubresse *et al.*, 2008). It is often assumed that certain minerals supplied to seeds also come from remobilized sources, but, unlike N, specific sources of stored or recycled minerals are unknown, and the few studies that have quantified the contributions of minerals remobilized from vegetative tissues to seeds have reached differing

conclusions (Hocking, 1994; Miller *et al.*, 1994; Garnett and Graham, 2005; Peng and Li, 2005; Waters and Grusak, 2008). In this work, net remobilization is defined as the loss of stored mineral content over time from one organ, and subsequent accumulation of that mineral content into another organ. Because net change in mineral content over time is a function of influx and efflux of nutrients, net remobilization will be detected only when efflux exceeds influx. Thus, substantial quantities of a given mineral could pass through an organ without a detectable change in content, resulting in no detectable net remobilization. Likewise, minerals could be remobilized from one subcellular compartment (or organ) while accumulating in another compartment or in the apoplastic spaces (or in another organ) without a change in total content or net remobilization. Since all shoot tissues have been collected and analysed in these experiments, it is possible to assess mineral partitioning to various tissues over time. If the grain mineral pool were to increase while the shoot mineral pool remained constant, then the quantity of mineral translocated to the grain must have passed through the shoot tissues, and would be equal to the quantity entering the shoot during that time period. When comparing lines, if translocation of mineral (from remobilization or pass through) to the grain is inhibited, the decreased flux will be detected as a relative increase in vegetative mineral content and less of an increase in grain mineral content. Although

this discussion will focus on Fe, Zn, and N, other minerals were quantified in order to determine whether the effects of the *NAM* genes were general in nature, or if certain minerals were disproportionately affected. Quantifying tissue DW and multiple minerals also demonstrated that remobilization did not occur for all minerals, and that observed changes in organ content were not secondary effects of changes in growth or organ mass.

Effect of NAM genes on remobilization under different mineral availabilities

The *NAM* genes are members of the NAC transcription factor family and were previously shown to affect grain Fe, Zn, and N content in a dosage-dependent manner. Construction of the RNAi line used here and the resultant alterations in *NAM* transcript levels have been described previously (Uauy *et al.*, 2006b). As transcription factors, *NAM* proteins are predicted to regulate genes that encode for proteins that carry out physiological processes for nutrient remobilization and/or translocation to grain. The RNAi and control lines used in our work only differ in their relative *NAM* gene expression, and are otherwise isogenic. Therefore, differences in Fe, Zn, and N dynamics between the control and transgenic lines can be assigned to direct or downstream effects of these genes.

Our results indicate that the extent of net remobilization is dependent on availability of mineral inputs and thus will probably be highly dependent on environmental conditions in field-grown plants. In complete hydroponic nutrient solution growth conditions, no significant net remobilization of Fe or Zn was observed in either line. Despite this, grain Fe and Zn contents and concentrations were substantially higher than those from plants grown in potting mix, where remobilization of both Fe and Zn was observed in control lines. When hydroponic plants were deprived of Fe or Zn inputs post-anthesis, net remobilization occurred in both the control and RNAi lines, from shoot tissues (Table 2) and also from roots (see Supplementary Table S3 at *JXB* online). Both lines remobilized more than enough of these minerals to account for the entire grain content, although the Fe and Zn quantities accumulated in the grain were substantially lower than in plants on the complete solution treatment. These results suggest that while remobilization and partitioning of Fe and Zn to grain is impaired in the *NAM* knockdown line, this is not due to a complete inhibition of remobilization, as the RNAi line is capable of remobilizing minerals under nutrient-limiting conditions. They also suggest that when Fe and Zn are readily available to the roots, and are adequately absorbed into the plant, this source supersedes the need for remobilization from the leaves. In the absence of sufficient Fe and Zn from the soil, the plant can obtain these minerals from the storage forms present in both shoot and root vegetative tissues. These results are consistent with those obtained when P was withheld from wheat plants during grain development (Peng and Li, 2005).

Remobilization: quantification and putative mechanisms

In potting mix growth conditions, net remobilization of Fe and Zn from the control line was observed, but diminished or no net remobilization in the RNAi line. Total accumulation of plant Fe and Zn was similar, but partitioning of these minerals to grain was substantially lower in the RNAi line. In this same experiment, vegetative N content decreased in the control line over time, indicating net remobilization, while there was an increase in N in the RNAi line. Between 35 DAA and 56 DAA, net remobilized N could account for 46% of the increase in grain N in the control line, but accounted for none of the N in the grain of the RNAi line. These results, in combination with the ⁶⁵Zn experiment that demonstrated decreased short-term translocation of Zn to grain, indicate that the translocation of certain minerals and N to grain is impaired in the *NAM* knockdown line. A combination of decreased efflux and sustained influx of minerals into vegetative tissues could account for the lower net remobilization exhibited in the RNAi lines, and could also account for the lower percentage of total Fe, Zn, and N partitioned to grain.

Target genes of the wheat *NAM* transcription factors have not been identified, and the molecular mechanism by which the *NAM* proteins affect translocation to grain (net remobilization plus pass through) is currently unknown. A microarray study of senescence in *Arabidopsis* leaves revealed a large number of up-regulated transporter proteins, including *OPTs*, *YSLs*, and *ZIPs* (Van der Graaff *et al.*, 2006). It is possible that *NAM* proteins regulate similar transporter genes in wheat and that these genes are needed for effective Fe and Zn remobilization. Other possible explanations include indirect effects on phloem loading for efflux of Fe, Zn and N (as amino acids) from leaves; or an effect on the rate or timing of disassembly of the internal sources of these elements. The latter hypothesis is supported by the observation that lines with a functional copy of the *NAM-B1* gene had higher soluble protein and amino acids concentration in the flag leaves than near isogenic lines with a non-functional *NAM-B1* gene (Kade *et al.*, 2005).

The reduced expression of *NAM* genes and the accompanying delay of normal vegetative development, i.e. senescence, may result in a disruption of the normal source and sink tissue relationship. Delayed senescence and the accompanying degradation of proteins may result in a situation where the substrates for transporters (amino acids or minerals) are decreased or not present, or are only present (or available) later in the grain-filling period and thus are less efficiently translocated out of source tissues. Hundreds to thousands of proteins are estimated to interact with Zn ions as structural or catalytic components or as substrates (Broadley *et al.*, 2007), thus substantial quantities of Zn could be released during protein degradation. Similarly, Fe from the degradation of chloroplast proteins could be released during leaf senescence. Delayed degradation of chloroplasts containing these proteins, as suggested by the delay in leaf yellowing in the RNAi line, may explain why

remobilization of Fe was inhibited proportionally more than the remobilization of Zn. Since the grain of the RNAi plants grew normally (based on appearance and DW gain), the movement of water and photoassimilates did not seem to be impaired. This suggests an inhibition of translocation processes more specific to Fe, Zn, and N rather than a general inhibition of phloem transport.

Practical implications

The average grain Zn concentration of potting mix-grown control plants was similar to that of field-grown wheat (Rengel *et al.*, 1999; White and Broadley, 2005; Morgounov *et al.*, 2007). However, grain of control line wheat grown in complete hydroponic culture had a Zn concentration approximately five times higher ($195 \mu\text{g g}^{-1}$ versus $38 \mu\text{g g}^{-1}$), which parallels the improvements in Zn grain concentrations made via Zn fertilization (Cakmak, 2008). The grain Fe concentration of the hydroponic control line was approximately twice that of potting mix-grown plants ($99 \mu\text{g g}^{-1}$ versus $44.7 \mu\text{g g}^{-1}$). In the RNAi line also, Fe and Zn concentrations in grain from plants grown on complete hydroponic solution were significantly higher than in grain from potting mix-grown plants (Fe, $51 \mu\text{g g}^{-1}$ hydroponic versus $29.9 \mu\text{g g}^{-1}$ potting mix; Zn, $87 \mu\text{g g}^{-1}$ hydroponic versus $29 \mu\text{g g}^{-1}$ potting mix). These results suggest that wheat grain is already capable of accumulating several-fold higher Fe and Zn concentrations than are usually obtained in field situations.

Because the RNAi line had lower partitioning of Fe and Zn to grain under both high and low availability, grain concentrations of these nutrients can possibly be increased by improvements in the efficiency of translocation. Indeed, overexpression of an *Arabidopsis* Zn transporter in barley resulted in increased seed Zn concentration (Ramesh *et al.*, 2004). However, constitutive overexpression of a Zn transporter in rice resulted in the aberrant distribution of Zn within the plant (Ishimaru *et al.*, 2007). Overexpression of transporters may need to be targeted spatially and temporally to result in the desired increases of nutrients in the target tissue.

The transgenic line also showed reduced translocation of N. It is estimated that grain protein in the control line was 19.7%, while protein in the RNAi line was one-third lower, at 13.0%. These values are higher than normally observed in field situations, possibly as a result of the continuous supply of N to the plants. While decreased expression of the *NAM* genes negatively affects the accumulation of Fe, Zn and protein in the grains, increasing the transcript levels of the *NAM* genes above the levels normally found in current commercial varieties can result in increased protein. The B genome copy of the *NAMI* gene (*NAM-B1*) is non-functional or deleted in modern bread wheat (Uauy *et al.*, 2006b), and introgression of a functional copy from wild wheat can significantly increase Fe, Zn, and N grain concentration in certain genotype–environment conditions (Uauy *et al.*, 2006a), and can also result in a significant increase in total N content (grain N concentration \times grain

yield) (Brevis and Dubcovsky, 2009). However, accelerated senescence in several isogenic lines containing a functional *NAM-B1* allele resulted in reduced grain-filling periods and reduced kernel weights. Therefore, the best genotype–environment combinations must be determined in the breeding process to deploy *NAM-B1* cultivars effectively.

Conclusions and future directions

Use of the *NAM* knockdown line for comparative physiology has allowed us to understand further the movement of minerals through the plant and eventually to seeds. The results suggest that *NAM* genes affect remobilization and/or pass through of Fe, Zn, and N from vegetative tissues to grain. The transgenic lines analysed here provide a valuable entry point for deciphering specific genes and processes involved in nutrient movement to seeds. Knowledge of genes operating downstream of the *NAM* genes could provide new targets to engineer a more efficient efflux and/or remobilization of Fe, Zn, and N from source tissues at the proper developmental stages. In addition to the improvements in wheat nutritional value, the improvement of mineral partitioning to grain would be an environmentally and economically beneficial improvement, because less fertilizer might be required to produce grain of similar N and mineral concentration. Projects in this direction are currently in progress in our laboratories.

Supplementary data

The following supplementary data are available at *JXB* website online.

Supplementary Table S1. Mineral contents for shoot organs of control and RNAi wheat grown in potting mix.

Supplementary Table S2. Mineral contents for shoot organs of control and RNAi wheat grown in hydroponics.

Supplementary Table S3. Dry weights and Fe and Zn contents and concentrations in roots of hydroponic plants.

Supplementary Fig. S1. Dry weight and seed number characteristics.

Supplementary Fig. S2. Grain characteristics of hydroponically grown wheat.

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