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# AtNPF5.5, a nitrate transporter affecting nitrogen accumulation in Arabidopsis embryo

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**Dipeptide (Leu-Leu) and nitrate transport activities of 26 Arabidopsis NPF (NRT1/PTR Family) proteins were screened in *Saccharomyces cerevisiae* and *Xenopus laevis* oocytes, respectively. Dipeptide transport activity has been confirmed for 2 already known dipeptide transporters (AtNPF8.1 and AtNPF8.3) but none of the other tested NPFs displays dipeptide transport. The nitrate transport screen resulted in the identification of two new nitrate transporters, AtNPF5.5 and AtNPF5.10. The localization of the mRNA coding for NPF5.5 demonstrates that it is the first NPF transporter reported to be expressed in Arabidopsis embryo. Two independent homozygous *npf5.5* KO lines display reduced total nitrogen content in the embryo as compared to WT plants, demonstrating an effect of NPF5.5 function on the embryo nitrogen content. Finally, *NPF5.5* gene produces two different transcripts (*AtNPF5.5a* and *AtNPF5.5b*) encoding proteins with different N-terminal ends. Both proteins are able to transport nitrate in xenopus oocytes.**

Nitrate ($NO_3^-$) is the main nitrogen source for most plant species[1]. The uptake of nitrate by plant roots and its translocation to the other organs involve a set of membrane transport proteins with different functional properties: low, high or dual affinity; influx, efflux or bidirectional transport; pH dependencies; channels or transporters[2]. The different nitrate transporters are found within 5 protein families: NRT2[2]; SLAC/SLAH[3]; ALMT[4]; ClC[5] and NPF (NRT1/PTR Family)[6]. Members of this last family, NPF, display sequence and structure homologies with animal and bacterial PTR proteins[7,8]. The structure of NPF proteins comprises twelve transmembrane helices with cytosolic N- and C-terminal ends and with a long cytosolic loop between the 6th and the 7th transmembrane domain. The funding member of this family AtNPF6.3/NRT1.1/CHL1 is a nitrate transporter playing several roles in roots: it is involved in nitrate influx[9] and translocation to the shoot[10], but it is also a nitrate sensor[11–14]. Since the characterization of AtNPF6.3/NRT1.1, 11 other NPFs have been characterized as nitrate transporters in Arabidopsis[6,15]. The NPF family of Arabidopsis comprises 53 members, and other substrates are also transported by NPF proteins such as glucosinolates[16], histidine[17], dipeptides[18,19], ABA[20,21] and auxin[12]. Up to now, protein sequences cannot be used to predict the substrate specificity of the uncharacterized NPFs.

In order to identify substrates of yet uncharacterized Arabidopsis NPFs, we have designed two functional screens in heterologous systems. The first one uses functional complementation of yeast strain defective in peptide uptake (Y06009, ΔPTR2 Δhis Δleu Δmet Δura). The principle is that the yeast strain deleted for its dipeptide transporter PTR2[19] is not able to grow with dipeptides as the sole source of nitrogen. Expression of plant dipeptide transporters will restore growth[19]. The second screen is functional expression in xenopus oocytes that has been used to identify several transporters[22]. It consists in injecting cRNA coding a protein with unknown function and screening for transport activity by supplying potential substrates in the external medium. This approach has been successfully employed to identify AtNPF2.10/GTR1 and AtNPF2.11/GTR2 as glucosinolate transporters[16].

Here, we expressed 26 Arabidopsis NPFs in yeast and xenopus oocytes, and screened for dipeptide and nitrate transport activity. We identified NPF5.5 (At2g38100) and NPF5.10 (At1g22540) as new nitrate transporters. We demonstrate that *NPF5.5* is expressed in the embryo and affects its nitrogen content.

## Results

To optimize the expression of NPFs in heterologous system we used their Coding DNA Sequence (CDS, from ATG to stop without any plant UTRs). Twenty-six CDS were either obtained from ABRC (in pENTR223) or amplified by PCR and cloned in a Gateway® entry vector (pENTR/D/TOPO or pDONR207 or pCR8-GW-TOPO). LR reactions were performed to transfer these CDS in destination vectors designed for inducible yeast expression (pYES-DEST52®) or xenopus oocyte expression, pGEM-GWC: this vector is based on pGEM-HE[23] with a Gateway® cassette inserted between the 5′ and 3′ β-globin UTR from xenopus. NPFx-pGEM-GWC were linearized and used for *in vitro* cRNA synthesis (see methods for details).

The mutant yeast strain (Y06009, obtained from Euroscarf) used in this study is deleted for the only dipeptide yeast PTR transporter (ΔPTR2). This strain is also unable to synthetize His, Leu, Met and Ura (His⁻, Leu⁻, Met⁻, Ura⁻). Efficient NPF transformation with the pYES-DEST52 vector restored the auxotrophy for uracil. Therefore, standard growth of transformed yeast was achieved adding His, Leu and Met to the medium (Figure 1, black bars). As a consequence, the media used for the screen was the minimal media supplemented with His, Met and Leu in the form of Leu-Leu (Figure 1, green bars). Yeast can grow only if they are able to take up Leu-Leu as Leu source. Yeasts were individually transformed with 26 different NPFs. The only growing strains were those expressing the already known Arabidopsis dipeptide transporters: AtNPF8.1/PTR1 or AtNPF8.3/PTR2[24]. No other NPF complementing the mutant phenotype could be identified among the 26 tests (Figure 1).

In the second screen, xenopus oocytes were injected with NPF coding cRNA and ¹⁵NO₃⁻ accumulation studies were performed: oocytes were bathed in 30 mM K¹⁵NO₃ for 2 hours and ¹⁵N was quantified in oocytes (see methods). In this screen, several NPF-expressing oocytes display ¹⁵N accumulation higher than non-injected ones (Figure 2). The positive controls, NPF6.3/NRT1.1[9], NPF1.1/NRT1.12[15], NPF1.2/NRT1.11[15] NPF2.9/NRT1.9[25] and NPF6.2/NRT1.4[26] display such behavior. Interestingly, expression of one other NPF induced a significant ¹⁵N accumulation, similar to the NPF6.3/NRT1.1 level: NPF5.5 (At2g38100). Another member of this subfamily, NPF5.10 (At1g22540), induce higher ¹⁵N accumulation than control oocytes, even if the accumulation level is lower than for NPF5.5 (Figure 2). This screen should be continued with the NPF that have not been tested but also with the one tested here but that do not display accumulation higher than wild-type. Indeed at
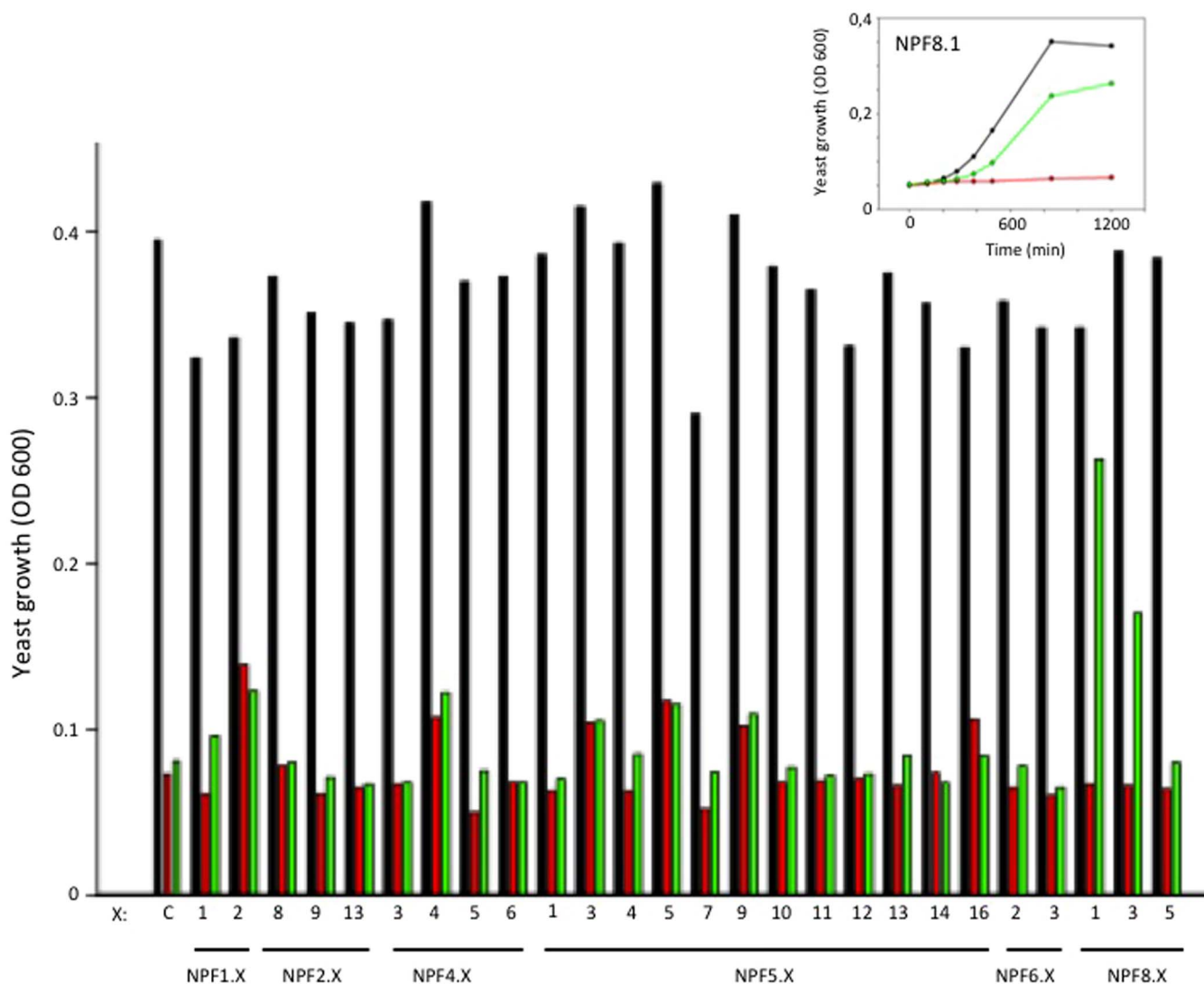


**Figure 1 | Screen for dipeptide transport complementation by NPFs in *S. cerevisiae*.** Control (C, non-transformed) and NPF-transformed ΔPTR2 yeast mutants were grown for 20 hours on SC minimal media (complemented with uracil for control yeast) containing His, Leu and Met (black bars), SC minimal media containing Met (red bars) and SC minimal media containing His, Met and Leu-Leu (green bars). (inset) Growth kinetics in the 3 different media for yeasts expressing NPF8.1. Results from one experiment representative of 2–3 independent repeats.
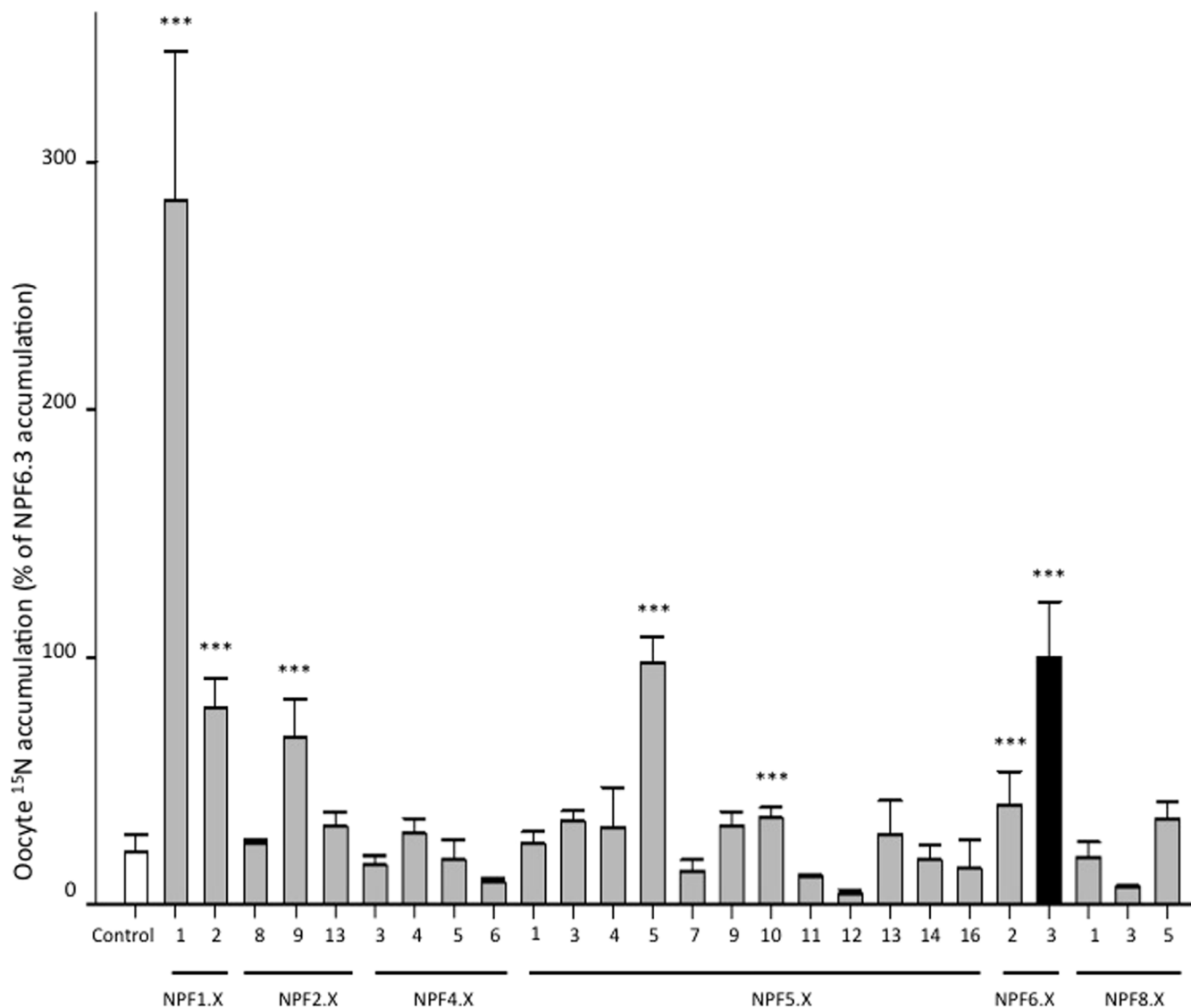
**Figure 2 | Screen for nitrate transport activity of NPFs in xenopus oocytes.** Control (non-injected) and NPF-injected oocytes were bathed in 30 mM K$^{15}$NO$_3$ for 2 hours.$^{15}$N accumulation is expressed as % of accumulation in NPF6.3/NRT1.1-expressing oocytes. Values are mean $+/-$ SEM from 3 experiments. *** indicate significant difference with control oocytes at p < 0.001 (t-test).

least two proteins that have been shown to mediate nitrate influx into the oocytes were not identified in our screen: NPF2.13/NRT1.7[27] and NPF4.6/NRT1.2[28].

RT-PCR has been performed to demonstrate that *NPF5.5* is expressed in roots and shoots[29]. Based on publicly available expression data (eFP browser, http://bbc.botany.utoronto.ca/efp[30]), we found that *NPF5.5* is also expressed in the embryo. This expression is not dependent on developmental stage[31]. Quantitative PCR experiments confirmed the expression of *NPF5.5* in the embryo (Figure 3b). To determine the role of NPF5.5, we isolated embryos from two homozygous KO lines (Salk_039337 and GK756F02, Figure 3a) and found that no full-length *npf5.5* mRNA accumulated in these genotypes (Figure 3b). *npf5.5-1* and *npf5.5-2* mutant plants growth in greenhouse conditions is similar to WT plants. Total nitrogen content of the embryos, analyzed by MS, showed a 7–8% reduction in the two KO lines compared to Col-0 (Figure 3c).

Neither full-length *NPF5.5* cDNA nor 5′-ESTs sequences were present in databases, but data from high-density oligonucleotide arrays spanning the entire genome[32] allowed us to speculate that a *NPF5.5* exon is present before the predicted ATG. We therefore characterized this region, and performed PCR on cDNA. Two different cDNAs with

different in frame ATG were identified (Figure 4a and b). These two cDNAs were named *NPF5.5a* and *NPF5.5b*. They are also both different from the one used in the screen that was cloned from predicted sequences (named NPF5.5 predicted in Figure 4). Nitrate transport activity of the two proteins coded by these two mRNAs was measured in xenopus oocytes. Both NPF5.5a and NPF5.5b expressing oocytes accumulated more$^{15}$N than control oocytes, demonstrating that both versions are nitrate transporters (Figure 4c).

## Discussion

Because nitrogen is an essential nutrient for plants, we have developed functional screens in heterologous systems (yeast and xenopus oocytes) to identify new nitrogen transporters in Arabidopsis. In one family of nitrogen transporters, NPF (NRT1/PTR Family[6]), several proteins have been identified as dipeptides, amino-acids and nitrate transporters, three main forms of nitrogen for nutrition of the plants. We expressed in both yeast and xenopus oocytes 26 of the 53 Arabidopsis NPF CDS. While we were unable to identify new dipeptide (Leu-Leu) transporters in yeast, our screen in xenopus oocytes identified two as yet uncharacterized nitrate transporters: NPF5.5 and NPF5.10. While the NPF5 subfamily is the largest one
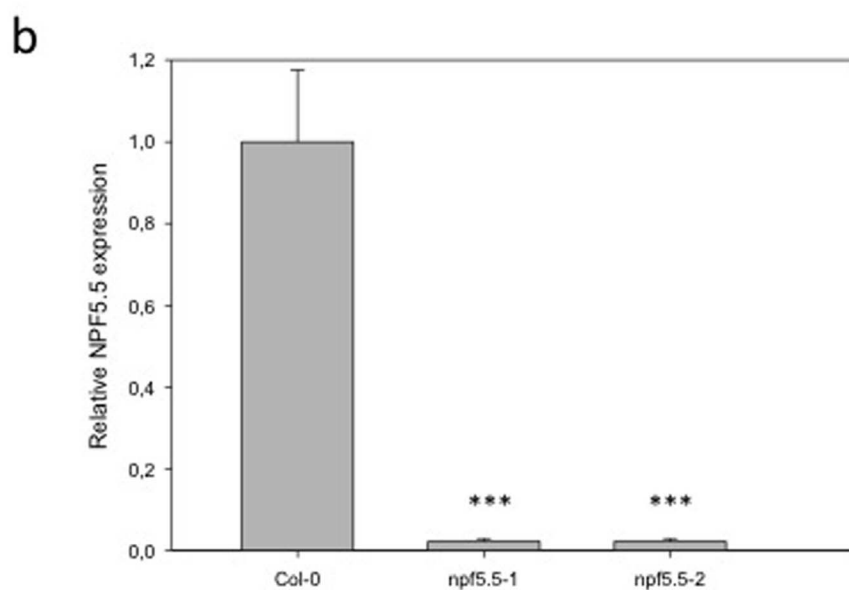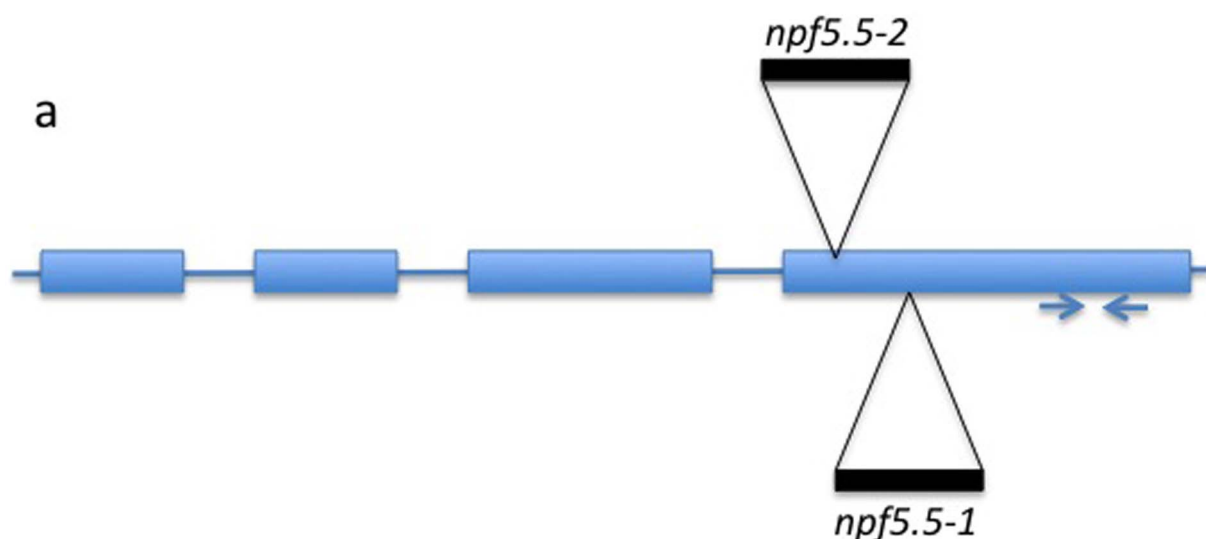
**Figure 3 | Characterization of *npf5.5* KO lines.** (a) Schematic representation of NPF5.5 insertion lines. Blue arrows represent the sens and antisens oligonucleotides used for Q-PCR analysis. (b) Q-RT-PCR analysis were performed on mRNA isolated from embryo at the bent cotyledon stage. Values are mean $+/-$ SEM from 3 experiments. *** indicate significant difference with control oocytes at $p < 0.001$ (t-test). (c) Nitrogen content in embryos of two NPF5.5 KO lines. The total nitrogen content was determined in embryos of two NPF5.5 KO lines (*npf5.5-1* and *npf5.5-2*). Values are expressed in percent of N content decrease compared to total N content in WT embryos. The average was made on 5 batches of 75 embryos, n = 5.
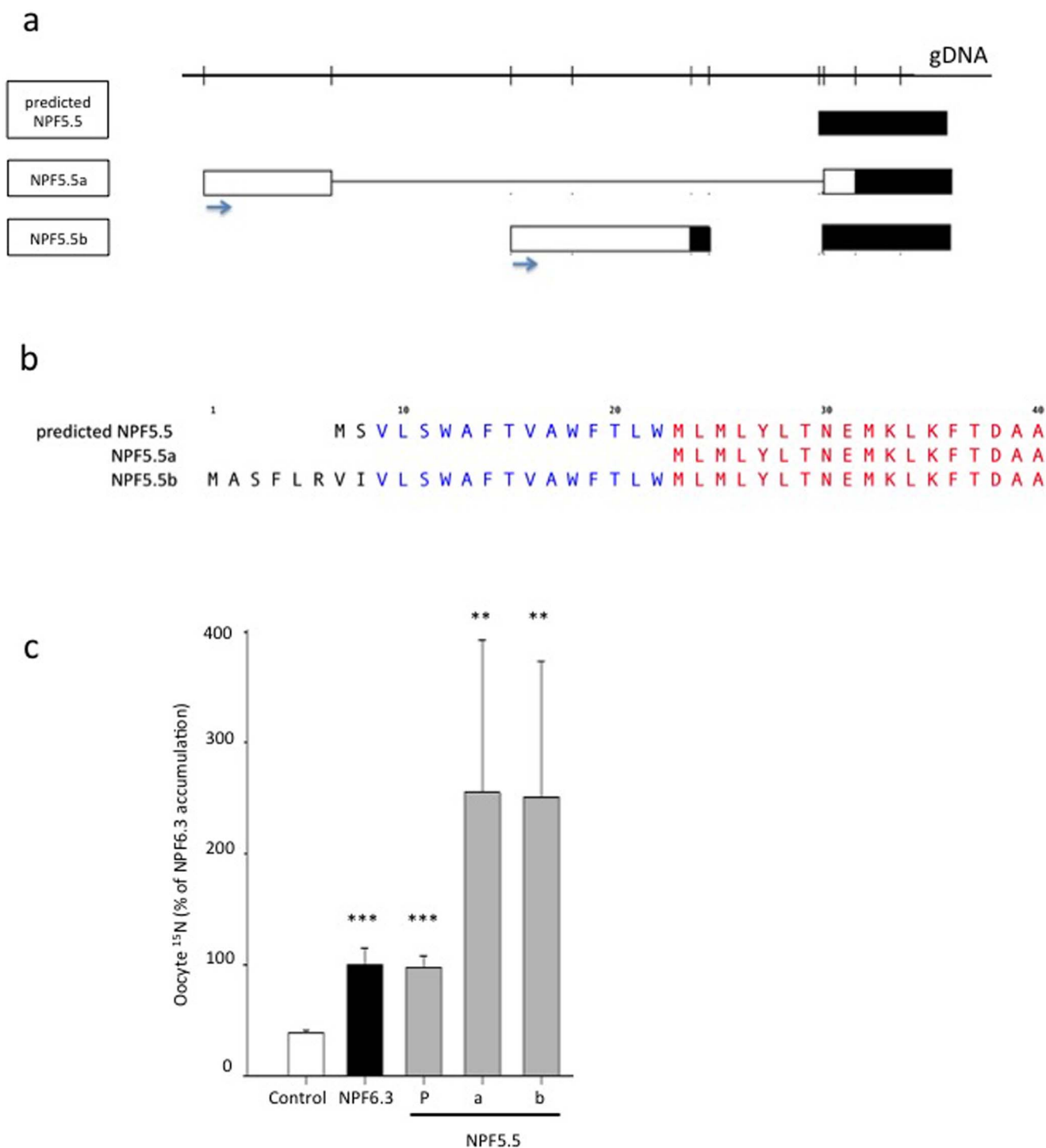
**Figure 4 | Alternative NPF5.5 transcripts.** (a) Schematic representation of NPF5.5 5′ genomic and RNA sequences. Blue arrows represent the sens oligonucleotides used for identification of NPF5.5a and b. (b) Amino-acid alignment of the N-terminal region of the 3 different NPF5.5 proteins. (c) Influx in control (non-injected) oocytes (white bar), in NPF6.3-expresssing oocytes (black bar) and in the 3 forms NPF5.5-expressing oocytes (grey bars) after 2 hours of incubation in 30 mM$^{15}$NO$_3$ solution. P: predicted sequence, a and b: 2 NPF5.5 forms. Influx in control oocytes (white bar), in NPF6.3-expresssing oocytes (black bar) and in NPF5.5-expressing oocytes (grey bars) after 2 hours of incubation in 30 mM$^{15}$NO$_3$ solution. *** and ** indicate significant difference with control oocytes at $p < 0.001$ and $p < 0.005$ respectively (t-test).

in plants (663 members out of 2384 in 31 fully sequenced plant genomes[6]), only one member AtNPF5.2/PTR3 has been functionally characterized in Arabidopsis, and shown to be a dipeptide transporter[33]. None of the NPF5 members (NPF5.1, 3–5, 7, 9–14, 16) tested in this study complemented yeast deficiency in Leu-Leu uptake. Nevertheless, NPF5.5 and NPF5.10 are the first nitrate transporters identified in this subfamily (Figure 2).

Human and animal feeding relies on organic nitrogen produced by plants. Plant seeds provide an important source of this nitrogen. Several nitrate transporters have been demonstrated to be expressed in the seed[2]. For example, NRT2.7 is a high-affinity vacuolar nitrate transporter, highly expressed in seeds and embryos and involved in nitrate accumulation in the seed[34]. NPF2.12/NRT1.6 is expressed in vascular tissues of the silique and in funiculus, and is important for

embryo development[35]. Several organic nitrogen (peptide) transporters are involved in nitrogen accumulation in embryos[18,36].

*NPF5.5* is expressed in different tissues within the plant[29] but is the first NPF transporter gene so far showing an expression in the embryo. Transcriptomic analyses on embryos isolated at different stages showed that *NPF5.5* level is rather stable during embryo maturation[31]. Its expression in the embryo (at bent cotyledon stage) has been confirmed by Q-RT-PCR (Figure 3b). To assess the role of NPF5.5 in the embryo, we have isolated two homozygous KO lines in which no full-length *NPF5.5* mRNA was found (Figure 3b). Nitrogen content (as % of dry weight) was determined for WT and the 2 KO lines on 5 batches of 75 isolated embryos. Embryo nitrogen content is 7–8% lower in both KO lines compared to WT (Figure 3c), demonstrating an effect of NPF5.5 function on the embryo nitrogen content. Since NPF5.5 gene was knock out in all the plant, we cannot exclude that part of this phenotype involves expression of *NPF5.5* in other tissues.

*NPF5.5* is one of the Arabidopsis genes for which there is no cDNA sequence available in databases and we have identified two different mRNAs transcribed from this gene. These different forms most likely arise from different transcription initiation site (instead of alternative splicing). Both proteins encoded by these two different transcripts are nitrate transporters (Figure 4c). Further experiments are needed to identify the role of these different transcripts. Two non-exclusives kind of regulation could exist. The first one is a post-transcriptional regulation due to different 5′-UTR. The second one is a post-translational regulation involving the 22 amino acids extended N-terminus of NPF5.5b that contains phosphorylable residues (2 serines and 2 threonines).

## Methods

**Cloning in Gateway entry vectors.** NPF CDS were either obtained from ABRC (cloned in pENTR223 for NPF2.9, 2.13, 4.3, 4.5, 5.1, 8.1 and 8.3) or cloned in pENTR/ D/TOPO (for clones 2.8, 4.4, 5.3, 5.5, 5.9, 5.10, 5.14 and 6.3), pDONR207 (for clones NPF1.2, 4.6, 5.11, 5.16 and 6.2) or pCR8-GW-TOPO (for clones NPF1.1, 5.4, 5.7, 5.12, 5.13 and 8.5). Each clone was sequenced and compared to Col-0 genomic sequence. LR reaction was performed according to the manufacturer's instructions (Life Technologies), to clone the CDS in yeast expression vector (pYES-DEST52) or xenopus oocyte expression vector (pGEM-GWC).

The primers used for *NPF5.5* variants identification are: NPF5.5a-s (ATGAGAGATATGGCGGCAGAG) and NPF5.5b-s (ATGACCAATATAAC-AGAGTATGATAAAGAA).

**Expression in yeast.** *S. cerevisiae* Y06009 strain (BY4741; MATa; ura3Δ0; leu2Δ0; his3Δ1; met15Δ0; YKR093w::kanMX4), obtained from Euroscarf and defective for dipeptides uptake was transformed with the cDNAs of the 26 NPF members, cloned in the yeast expression plasmid pYES-DEST52. Cells were transformed according to[37] and plated for 3 days on YNB medium (yeast nitrogen base 1.7 g.l$^{-1}$, (NH$_4$)$_2$SO$_4$ 5 g.l$^{-1}$, D-glucose 20 g.l$^{-1}$, agar 20 g.l$^{-1}$) supplemented with the appropriate amino acids for transformant selection (Histidine, Leucine, Methionine). For uptake experiments, 5 ml cultures of cells were grown for two days in SC minimal medium (yeast nitrogen base 1.7 g.l$^{-1}$, galactose 20 g.l$^{-1}$, agar 20 g.l$^{-1}$) supplemented with the appropriate amino acids, washed three times in water and resuspended in the adequate volume of water to have a OD 600 = 1. Ten µl of these cells were grown for 20 h in SC minimal medium supplemented with 20 µg.ml$^{-1}$ of Methionine or 20 µg.ml$^{-1}$ of Histidine, 60 µg.ml$^{-1}$ of Leucine, 20 µg.ml$^{-1}$ of Methionine or with 20 µg.ml$^{-1}$ of Histidine, 20 µg.ml$^{-1}$ of Methionine and Leucine-Leucine 200 µM.

**Expression in xenopus oocytes.** NPFx-pGEM-GWC were linearized and *in vitro* transcribed with mMessage mMachine T7 Ultra Kit following manufacturer protocole (Life Technologies). Oocytes were obtained and injected as previously described[38]. Briefly, 3 days after NPF cRNA injection, injected or control (non-injected) oocytes were incubated 2 hours in 2 ml of ND96 medium (pH 6.5) containing 30 mM of$^{15}$N-nitrate (atom %$^{15}$N abundance: 99,9%). Oocytes were then washed 5 times in 15 ml of ND96 medium (pH 6.5) at 4°C. Batches of 2 oocytes were then analyzed for total N content and atom %$^{15}$N abundance by Continuous-Flow Mass Spectrometry, using an Euro-EA Eurovector elemental analyzer coupled with an IsoPrime mass spectrometer (GV instruments, Crewe, UK).

**Plant material and growth.** The *Arabidopsis thaliana* accession used is Columbia (Col-0). The *npf5.5* mutants are *SALK_039337* (*npf5.5-1*) and GABI_756F02 (*npf5.5-2*) and were obtained from NASC and GABI-kat, respectively.

Arabidopsis seeds were sown in pots (Neuhauss Humin substrate N2, Klasmann-Deilmann, Germany) and grown in greenhouse under long-day conditions (16 H

light at 23°C and 8 H dark at 21°C, humidity is not controlled but is around 50%). When the external light is not sufficient, it is supplemented with sodium vapor lamps (150 µE.m$^{-2}$.s$^{-1}$). Plants are watered once or twice per week. Under these conditions, the seed to seed cycle is 6 to 8 weeks, depending on the season.

**Relative gene expression.** Embryos were isolated (at bent cotyledon stage) from developing siliques of Arabidopsis[39]. Frozen (−80°C) samples were homogenized for 1 min at 30 s$^{-1}$ (Retsch mixer mill MM301; Retsch, Haan, Germany) in 2 mL tubes containing one tungsten bead (2.5 mm diameter). Total RNA was extracted from homogenized tissues by using TRIzol reagent (Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. Three micrograms of RQ DNase (Promega, Madison, WI) digested total RNA was used to prepare cDNA by reverse transcription with M-MLV reverse transcriptase (Promega) and Gene expression was determined by quantitative real-time PCR (Roche Diagnostics, Mannheim, Germany) using LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics). Expression levels of NPF5.5 (F: GTACATTCTACTCGGTTCAATCACA and R: ACTCCCA-CGTTTAATAGAACCA) were normalized to expression levels of the CLATHRIN (At4g24550 F, AGCATACACTGCGTGCAAAG and R: TCGCCTGTGTCA-CATATCTC) and ACTIN (At3g18780.1 F: CTGAGGCTGATGATATTCAACC and R: ACACTGGGAAAAACAGCCC).

**Total nitrogen content.** Embryos were isolated (at bent cotyledon stage) from developing siliques of Arabidopsis[39]. Batches of 75 embryos were analyzed for total N content by Continuous-Flow Mass Spectrometry, using an Euro-EA Eurovector elemental analyzer coupled with an IsoPrime mass spectrometer (GV instruments, Crewe, UK).

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## Author contributions

S.L., B.G., Y.B., C.C.F., C.B., P.T. and B.L. performed the experiments; S.L. and B.L. wrote the manuscript; B.G., Y.B., C.C.F. and A.G. reviewed the manuscript; B.L. designed the experiments.

## Additional information

**Competing financial interests:** The authors declare no competing financial interests.

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