Overexpressing NRT2.7 induces nitrate export from the vacuole and increases growth of Arabidopsis

Patrick Armengaud[1*], Alexis De Angeli[2**], Patrick Berquin[1], Virginie Bréhaut[1], Mickaël Durand[1***], Françoise Daniel-Vedele[1], Anne Krapp[1#] and Sophie Filleur[2,3#]

[1] Université Paris-Saclay, INRAE, AgroParisTech, Institut Jean-Pierre Bourgin (IJPB), 78000, Versailles, France; [2] Université Paris-Saclay, CEA, CNRS, Institute for Integrative Biology of the Cell (I2BC), 91198, Gif-sur-Yvette, France ; [3] Université Paris Cité, UFR Sciences du Vivant, 35 rue Hélène Brion, F-75205 Paris Cedex 13, France.

*Present address: INRIA Rocquencourt, Domaine de Voluceau, Rocquencourt - BP105, 78153 Le Chesnay Cedex, France
**Present address: Institute for plant sciences of Montpellier (IPSiM), Université de Montpellier, CNRS, INRAE, Institut Agro, Montpellier, 34060, France.
***Present address: Biomolécules et Biotechnologies Végétales, EA2106, Université de Tours, Tours, France

Email addresses of the authors :
Patrick Armengaud: patrick.armengaud@inria.fr
Alexis De Angeli: alexis.deangeli@cnrs.fr
Patrick Berquin: patrick.berquin@inrae.fr
Virginie Bréhaut : virginie.brehaut@inrae.fr
Mickaël Durand : midurand@univ-tours.fr
Françoise Daniel-Vedele : fravedele@yahoo.fr
Anne Krapp (# co-corresponding authors) : anne.krapp@inrae.fr
Sophie Filleur (# co-corresponding authors) : sophie.filleur@i2bc.paris-saclay.fr

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The overexpression of the nitrate transporter NRT2.7 stimulates growth when nitrogen supply is limiting by increasing the export of nitrate from the vacuole, the main cell compartment for nitrate storage.

Abstract

Nitrogen nutrition is essential for crop yield but applying fertilizers has detrimental effects on the environment. Improved nutrient use efficiency is therefore a highly desired trait for developing a more sustainable agriculture. Compartmenting nitrate into vacuoles is one of the option to develop N-efficient crop adapted to less fertilizers. Only few proteins involved in nitrate transport on the tonoplast have been identified. CLCa is the major transporter involved in nitrate storage in Arabidopsis. Several other nitrate transporters amongst NRT2.7 have been localized in this membrane. The transport mechanism of NRT2.7 has not yet been defined as this protein is present mainly in seed cells that are not easily amenable for electrophysiology analysis. We then investigated its function by ectopically overexpressing it in a *clca* knock-out mutant. Although the growth on nitrogen sufficient medium was complemented, nitrate homeostasis was not restored by NRT2.7 activity like for CLCa overexpression. Moreover, NRT2.7 ectopic overexpression in wild-type background increased growth under limited nitrogen supply, suggesting that NRT2.7 stimulates nitrate efflux from vacuoles. This result was confirmed by electrophysiology performed on isolated vacuoles. Possible means of the growth stimulation by NRT2.7 versus CLCa are discussed based on nitrate fluxes through plasma membrane and nitrate homeostasis.

Keywords: Arabidopsis, Electrophysiology, Nitrate homeostasis, Nitrate transporter, NRT, vacuolar membrane

76 **Abbreviations:**

77 CaMV: Cauliflower Mosaic Virus

78 CLC: ChLoride Channel

79 KO: Knock-out

80 N: Nitrogen

81 NPF: Nitrate transporter 1/Peptide transporter Family

82 NRT2: High affinity Nitrate transporter

83 NUE: Nitrogen Use Efficiency

84 WS: Wassilewskija Arabidopsis accession

85

Introduction

Nitrogen (N) is an essential macronutrient acquired by plants from the soil or the air to synthesize amino acids, nucleotides and proteins. Most cultivated plant species rely on soil soluble ammonium and nitrate, the latter being the most abundant oxidized form of nitrogen found in temperate agricultural soils in amounts ranging from 0.1 to 10 mM (Miller *et al.*, 2007). The effect of nitrogen supply on plant growth has long been evidenced and this nutritional benefit was the basis of the fertilizer-driven yield increases (Epstein and Bloom, 2005). Crops are therefore abundantly provided with nitrogen fertilizers to insure best yield. This practice is at high economic and ecological costs since it has been shown that plants use only 25 to 50 % of nitrogen applied in the field (Cassman *et al.*, 2002). Because of both nitrate mobility and soil structure, nitrate leaks from the field, contaminates water effluents and stimulates growth of algae and microorganisms leading to ecological problems such as river eutrophication (Howarth and Marino, 2006). To avoid these problems and move toward a more sustainable agriculture, one means is to reduce fertilizer inputs without affecting crop yields by improving plant nitrogen use efficiency (NUE). This objective is challenging since NUE is a complex trait that includes uptake, utilization and remobilization efficiencies of the whole plant or of specific organs such as grains (Liu *et al.*, 2022). Therefore, research on NUE improvement has been tackled through approaches engineering known molecular players (transporters, enzymes) or heuristically by QTL, GWAS and transcriptome candidate genes approaches. However, to date, crop varieties with optimized NUE are still rare (see for reviews: (Liu *et al.*, 2022; Melino *et al.*, 2022; Govindasamy *et al.*, 2023).

The large number of nitrate transport mechanisms and their multiple level of regulation form a complex network coordinating nitrate fate and homeostasis. The latter is illustrated by remarkably stable cytosolic nitrate levels ranging between 3 and 5 mM (Cookson *et al.*, 2005). Once absorbed into root cells, nitrate has three possible paths (Dechorgnat *et al.*, 2011). A first one is a direct export to vascular tissue partially through xylem associated nitrate transporters (Wang *et al.*, 2018 for review). A second one is the assimilation path where cytosolic nitrate is reduced first by the nitrate reductase to nitrite and then by the plastidial nitrite reductase to form ammonium. Ammonium is then incorporated into organic acids to produce amino acids. The third path of cytosolic nitrate is vacuolar loading. The vacuolar compartment seems to play

important roles in controlling of the plant nitrate status as vacuolar nitrate accumulation has two major functions (Han *et al.*, 2016; Hodin *et al.*, 2023). First, vacuolar nitrate represents a temporary reserve of nitrogen allowing a near constant cytoplasmic provision independently of external fluctuations (van der Leij *et al.*, 1998; Matt *et al.*, 2001). Second, vacuolar nitrate acts as an osmoticum contributing to the osmotic potential, including during stomata movements (McIntyre, 1997; Wege *et al.*, 2014; Hodin *et al.*, 2023).

The first nitrate/proton exchanger mediating nitrate influx into the vacuole, CLCa, was characterized in Arabidopsis (De Angeli *et al.*, 2006). This protein is part of a family of seven members in this species. CLCa and CLCb were shown to preferentially exchange nitrate over chloride against proton (De Angeli *et al.*, 2006; Bergsdorf *et al.*, 2009; Wege *et al.*, 2010; Barbier-Brygoo *et al.*, 2011). Both of these exchangers are located on the vacuolar membrane. CLCa activity accounts for up to 50% of vacuolar nitrate content whereas it was proposed that CLCb mediates nitrate efflux from the vacuoles when plants are starved (Geelen *et al.*, 2000; Shi *et al.*, 2023). Impaired nitrate flux into vacuoles through CLCa was shown to reduce nitrate uptake, nitrate content and expression level of several nitrate transporter genes but to increase nitrate assimilation and consequently NUE (Monachello *et al.*, 2009; Hodin *et al.*, 2023).

Next to CLC transporters, some members of NRT2 and NPF transporters family are localized in the vacuolar membrane (Hu *et al.*, 2016; He *et al.*, 2017; Wang *et al.*, 2018*b*; Lu *et al.*, 2022). Among them, NRT2.7 belongs to the seven members of the NRT2 gene family in Arabidopsis that encodes major high affinity nitrate transporters. All NRT2 proteins except NRT2.7 are localized at the plasma membrane. NRT2.7 forms indeed a distinct monophyletic group within the Arabidopsis NRT2 family. The expression of the *NRT2.7* gene occurs mainly in seeds, but its expression has been also described for leaves and roots (Orsel *et al.*, 2002; Okamoto *et al.*, 2003; Chopin *et al.*, 2007). NRT2.7 is able to mediate nitrate fluxes in Xenopus oocytes. The main phenotype of *nrt2*.7 knock-out mutants is a greatly decreased nitrate content in seeds which highlights the major roles of NRT2.7 in nitrate accumulation at reproductive stage (Chopin *et al.*, 2007).

Regarding nitrate as a signal, a nutrient or an electro-osmotic charge, further work in characterizing transporters and channels involved in vacuolar fluxes will shed light on the putative sensing and signaling properties of the largest compartment of plant cells.

152 The mechanism of nitrate transport by NRT2.7 was not investigated *in planta* as this
153 protein is essentially present in seeds. So, in this report, we first investigated the impact
154 of a NRT2.7 overexpression in Arabidopsis on nitrate homeostasis, activities of plasma
155 membrane nitrate uptake systems, growth and vacuolar nitrate currents. Functional
156 characterization of nitrate transporters *in planta* often requires the genetic removal of
157 the quantitatively most important components to uncover more subtle or fine-tuning
158 mechanisms. For example, the nitrate transport activity of NRT2 family members are
159 masked by the NRT2.1 activity and has to be assessed in the *nrt*2.1 deficient
160 background (Chopin *et al.*, 2007; Kiba *et al.*, 2012; Lezhneva *et al.*, 2014). Here we
161 applied a similar strategy and used the *clca* mutant background in addition to a wild-
162 type background, to test the physiological impact of NTR2.7 activities *in planta* and to
163 test by electrophysiology the effect the overexpression of NRT2.7 in *clca* on vacuolar
164 nitrate currents. We thus elucidate further the function of NRT2.7 by asking the
165 following questions: (i) whether or not overexpression of NRT2.7 nitrate transporter
166 would give similar effects as overexpression of the CLCa nitrate exchanger in the *clca*
167 knock-out genetic background, (ii) how the NRT2.7 overexpression in wild-type
168 background would affect nitrate homeostasis and growth under nitrate sufficient and
169 limiting conditions and, (iii) what are the consequences of *NRT2.7* overexpression on
170 nitrate fluxes through the vacuolar membrane.

171

172 **Material and methods**

173 *Plant material and growth conditions*

174 All *Arabidopsis thaliana* genotypes used in this study were obtained or generated in
175 the Wassilewskija (WS) background. The *clca-2* T-DNA knock-out plant line originates
176 from the Versailles collection (FST 171A06, line EAE89] whereas *clca-1* was isolated
177 in Geelen *et al.* (2000). The *CLCa-OX* (*clca-2*) line (*clca-2* knock-out mutant
178 overexpressing *CLCa* under the 35S promoter of the Cauliflower Mosaic Virus, CaMV)
179 originates from Wege *et al.* (2010). The *NRT2.7-OX* (*clca*) lines (*clca* knock-out mutant
180 overexpressing *NRT2.7* under the 35S promoter of the CaMV) was obtained by
181 crossing NRT2.7 overexpressors (in the WS background, Chopin *et al.*, 2007) with the
182 *clca* mutants (*clca-1* and *clca-2*).

183 Seeds were sterilized, stored for 3 days at 4°C to synchronize germination and sown
184 in 120 x 120 mm square Petri dishes containing either 35 or 70 mL nutrient medium to
185 monitor root architecture (4-5 plants/plate) or to produce material for analysis (12-15
186 plants/plate). Media differed in their nitrate content (0.2|2|20 mM) and consisted
187 respectively of 0.2|0.7|7 mM KNO_3; 0|0.65|6.5 mM $Ca(NO_3)_2$; 0.5|0.5|1.5 mM $MgSO_4$;
188 0.625|0.625|0 mM KH_2PO_4; 0|0|1.875 mM NaH_2PO_4; 0.75|1.25|1.25 mM NaCl;
189 0.65|0.25|0 mM $CaCl_2$ and 1.05|0.55|0 mM KCl. For all media, micronutrients (in µM)
190 consisted of 42.5 FeNaEDTA, 0.16 $CuSO_4$, 45 H_3BO_3, 0.015 $(NH_4)_6Mo_7O_{24}$, 0.01
191 $CoCl_2$, 0.38 $ZnSO_4$, 1.8 $MnSO_4$. All media were supplemented with 1% sucrose, pH
192 balanced at 5.8 before autoclaving and solidified using 1% agar (Sigma A1296). Petri
193 dishes were sealed using 3M adhesive tape and placed below tubes delivering 65 µE
194 irradiance for 16h/day in a tissue culture room with a thermoperiod day/night of
195 22/18°C. At 12 days after germination (14 days after sowing), plants were harvested
196 (or sampled for ^{15}N uptake) between 10 and 12 am (after 3 to 5h light).

197 *Growth measurements*

198 Root architecture was measured at 3, 6 and 9 days after germination by scanning Petri
199 dishes using a flat-bed scanner (Epson Perfection 4490 Photo) at a 200 dots per inch
200 resolution. Total root length was acquired by analyzing root morphology with the EZ-
201 Rhizo software (Armengaud *et al.*, 2009). Plant biomass was measured directly on a
202 microbalance (Mettler Toledo XS3DU) for fresh weight or after sampling in tin capsules,
203 dried 48h at 80°C and weighted for dry matter measurements.

204 *RNA extraction and RT-qPCR analysis*

205 RNA was extracted from both roots and shoots using the Trizol reagent (Invitrogen)
206 according to the provider recommendations. One microgram was treated with DNase
207 and reverse transcribed using the Quantitect kit (Qiagen) following the manufacturer
208 instructions. For qPCR analysis, specific primers were used for NRT2.7 (5'-
209 CCTTCATCCTCGTCCGTTTC-3' (forward) and 5'- AATTCGGCTATGGTGGAGTA-3'
210 (reverse)) and EF1α (At5g60390) 5'- CTGGAGGTTTTGAGGCTGGTAT-3' (forward)
211 and 5'- CCAAGGGTGAAAGCAAGAAGA-3' (reverse). The reactions were performed
212 in a Realplex mastercycler (Eppendorf) using commercial SYBR green mastermix
213 (Roche). Samples were subjected to 2 min pre-incubation at 95°C followed by 40
214 amplification cycles of 15 s at 95°C, 15 s at the melting temperature of the primers set

215 and 20 s at 68°C. NRT2.7 transcript levels were quantified using linear regression of
216 quantified amplicon range and expressed relatively to EF1α.

217 *Nitrate, C/N analysis*

218 Frozen plant material was ground in liquid nitrogen using a Retsch mill grinder (MM301,
219 Retsch GmbH, Germany). An aliquot of 20 mg of frozen powder was used to prepare
220 an ethanol-water extract as described in Cross *et al.* (2006). All metabolites
221 measurements were performed from this extract. Nitrate content was assayed after its
222 reduction by vanadium(III) combined with detection by the acidic Griess reaction as
223 described in Miranda *et al.* (2001). Total carbon and nitrogen content were determined
224 using an elemental analyzer (EA, Flash 2000, Thermo Scientific) on aliquots of 0.5 to
225 2 mg dry weight in tin capsules. EA settings consisted of 20 sec of oxygen flux at 80
226 mL/min and helium at 140 mL/min for 5 min.

227 *^{15}N uptake measurements*

228 Influx of $^{15}NO_3^-$ was assayed as previously described (Orsel *et al.*, 2004). Two to three
229 12 day-old plants were first transferred to 0.1 mM $CaSO_4$ for 1 min. To measure high
230 affinity ^{15}N nitrate uptake, plants were then transferred to a liquid 0.2 mM nitrate
231 containing medium (the same as described earlier but without sucrose) enriched at
232 99% with $^{15}NO_3^-$ (Sigma). To measure low affinity transport capacity, plants were
233 instead transferred to a 6 mM nitrate medium consisting of 3 mM $K^{15}NO_3$; 1.5 mM
234 $Ca(^{15}NO_3)_2$; 0.5 mM $MgSO_4$; 0.625 mM KH_2PO_4; 1.5 mM NaCl and the same
235 micronutrients as described before. ^{15}N nitrate uptake was performed for 5 min and
236 plants were finally incubated for 1 min in 0.1 mM $CaSO_4$. Roots were then placed into
237 tin capsules, dried at least 48h at 80°C and sent for ^{15}N elemental analysis using an
238 ANCA–MS system (PDZ Europa, Crewe, UK) at INRAE Montpellier. Nitrate uptake
239 was calculated from the ^{15}N content of the roots (0.5 to 1.5 mg DW). Each uptake
240 experiment included at least 5 measurements per condition and genotype.

241 *Patch-clamp analyses*

242 Patch-clamp experiments were made in the whole-vacuole configuration with an
243 access resistance between 1.5-3 MΩ. Patch-clamp recordings were made with an
244 EPC8 amplifier (HEKA, Lambrecht-Pfalz Germany). Data were acquired with an
245 AD/DA converter Instrutech LIH 8+8 under the control of the software PatchMaster
246 (HEKA, Lambrecht-Pfalz Germany) and analysed with FitMaster software (HEKA,

247 Lambrecht-Pfalz Germany). Applied membrane potentials were always corrected by
248 the liquid junction potential (Neher, 1992). Current to voltage characteristics were
249 obtained measuring the amplitude of the stationary current. Measurements were done
250 after waiting that the vacuolar lumen solution was equilibrated with the pipette solution,
251 typically 10 minutes after the establishment of the whole-vacuole configuration.
252 Cytosolic side solutions were changed using a gravity driven perfusion system. Bis-
253 Tris-propane (BTP) was chosen as a large impermeable cation in order to minimize
254 cation currents. The solution used for patch-clamp experiments were (in mM): Nitrate
255 vacuolar solution: 100 BTP, 200 HNO_3, 0.1 $CaCl_2$, 5 $MgCl_2$, 5 MES, pH = 5.5. Chloride
256 vacuole solution : 475 MES, 15 HCl, 60.5 BTP, 0.1 $CaCl_2$, pH = 5.5. Chloride cytosolic
257 solutions: 7.5 BTP, 15 HCl, 0.1 $CaCl_2$, 2 $MgCl_2$, 15 MES, pH = 7. Nitrate cytosolic
258 solution: 0.1 $Ca(NO_3)_2$, 2 $Mg(NO_3)_2$, 15 MES, pH = 7 with BTP. Osmotic pressures of
259 the cytosolic and vacuolar solutions were adjusted with sorbitol to 640 and 650 mOsm,
260 respectively.

261 *Statistical analysis*

262 Statistical analyses were performed using the rcompanion and coin packages in the R
263 environment except for the electrophysiology results. For those, a Mann-Whitney test
264 was applied using GraphPad Prism 8 software. All the results of the statistical analysis
265 can be found in the supplementary table.
266

267 Results

268 *Both CLCa and NRT2.7 overexpression restore growth of the clca-2 mutant*

269 To set a suitable genetic background for NRT2.7 functional studies, we characterized
270 the phenotypes of *clca* mutants on a range of nitrate supplied as a sole nitrogen source
271 in vertical Petri dishes. We used *clca-1* and *clca-2* knock-outs (Geelen *et al.*, 2000;
272 De Angeli *et al.*, 2006) which both showed a significant decrease in shoot fresh weight
273 (-13 to -45%) and root growth rate (-8 to -40%) when compared to the WS wild-type
274 (Supplementary Fig. S1A-B and S2A-B). This decrease was more important under
275 non-limiting nitrate supply (2 and 20 mM). As expected *clca mutants* showed reduced
276 nitrate content compared to WS under all N supplies in both shoot and root
277 (Supplemental Fig. S1C-D and S2C-D).

278 To study the activity of NRT2.7 we overexpressed ectopically NRT2.7 in the *clca-2*
279 background and as control in the WT. These lines have been obtained by crossing the
280 *clca* mutants with the NRT2.7 overexpressing lines *NRT2.7-Ox1 (WS)* and *NRT2.7-*
281 *Ox2 (WS)* (Chopin *et al.*, 2007). Shoot NRT2.7 expression was increased 9 and 10
282 times, respectively compared to WS (Supplementary Fig. S3). Both lines NRT2.7-Ox1
283 (*clca-2*) and NRT2.7-Ox2 (*clca-2*) showed similar phenotypes and representative data
284 for one line are presented. For comparison, we included in our studies a *clca-2* mutant
285 overexpressing the *CLCa* cDNA under the control of the strong 35S promoter of the
286 CaMV (referred thereafter as CLCa-Ox (*clca-2)*) (Wege *et al.*, 2010). In this line, *CLCa*
287 transcript levels were increased 30-fold compared to WS (Wege *et al.*, 2014).
288 Based on the more pronounced differences in growth between *clca-2* and WS under
289 non-limiting external N supply, we cultivated the plants on 2 mM nitrate medium for all
290 further analyses using both *clca-2* lines overexpressing either *CLCa* or *NRT2.7*
291 compared to the controls WS, *NRT2.7* over-expressing lines (NRT2.7-Ox1 (WS)) and
292 *clca-2.* Both *CLCa* and *NRT2.7* overexpression restored the *clca-2* growth phenotypes
293 for shoot biomass and total root length (Fig. 1A and 1C). This growth restoration was
294 also observed in both lines overexpressing *NRT2.7* in the *clca-1* knock-out background
295 and occurred regardless external nitrate availability (Supplementary Fig. S2A-B). Wild-
296 type plants overexpressing NRT2.7 showed no significant difference in shoot fresh
297 weight and an important increase of 160% in total root length compared to the wild
298 (Fig. 1B-C). The growth stimulation observed for NRT2.7 overexpressing lines both in
299 the *clca-2* and the WS background, seems thus to be independent of the *clca-2*
300 mutation. The physiological changes triggered by NRT2.7 overexpression in the *clca*
301 mutants might thus differ from the complementation by CLCa while both led to restored
302 growth.

303 *NRT2.7 and CLCa overexpression have opposite effects on both nitrate content and*
304 *nitrate uptake capacities*
305 To decipher the underlying mechanism that impact growth of these overexpressing
306 lines, we determined nitrate content in roots and shoots. As described earlier, nitrate
307 content of *clca-2* was 53 and 60 % lower than the WT level in shoots and roots,
308 respectively. Expression of *CLCa* in the *clca-2* mutant restored nitrate levels in shoots
309 and roots, whereas *NRT2.7* overexpression in the *clca-2* background led to an about
310 two times decrease of the nitrate content when compared with the *clca-2* mutant (Fig.

311 2A and 2C). Likewise, *NRT2.7* overexpression does not complement *clca-1* phenotype
312 for shoot and root nitrate content whatever is the external nitrate concentration for plant
313 growth (Supplementary Fig. S2C-D). This means that the increased growth observed
314 in the *NRT2.7-Ox (clca-2)* and *NRT2.7-OX (clca-1)* lines occurred without restoring
315 nitrate content. Similar to the effect of *NRT2.7* overexpression observed in the *clca-2*
316 mutant background, overexpression of *NRT2.7* in wild-type background was
317 accompanied by a slight decrease in nitrate content in roots but no significant
318 modification was observed in shoot (Fig. 2B and 2D).
319 In order to explain the impact on nitrate levels, we then investigated how nitrate uptake
320 was affected in these lines. Nitrate uptake was measured using ^{15}N labelled nitrate at
321 concentration of 0.2 and 6 mM to monitor both high and low affinity transport system
322 activities (HATS and LATS, respectively, this latter activity being obtained as the
323 difference between uptake measurements at 6 mM and 0.2 mM). Figure 3 shows that
324 the HATS activity was decreased in *clca-2* whereas the LATS activity was not
325 significantly different from that of wild-type plants. The *CLCa-Ox* (*clca-2*) line exhibited
326 the same uptake capabilities for both transport activities as wild-type plants, illustrating
327 a full complementation of the *clca-2* mutant phenotype. The *NRT2.7-Ox* (*clca-2*) line
328 showed similar HATS activity as the *clca-2* knock-out mutant and an increase LATS
329 activity (Fig. 3B and 3C). The *NRT2.7-Ox* (WS) lines displayed comparable tendencies
330 with a 2-fold increased activity of the LATS compared to WT (Fig. 3C). Thus, whereas
331 *CLCa* overexpression in the *clca* mutants increased mainly HATS activity compared to
332 wild-type, *NRT2.7* overexpression had the opposite effect, supporting the hypothesis
333 that these two nitrate transporters have different functions *in planta* when
334 overexpressed ectopically.

335 *NRT2.7 overexpression leads to increased biomass under limited and non- limited*
336 *conditions*

337 Plants stimulate their root growth when their N nutritional status is low (Jia and von
338 Wirén, 2020). Given the important increase in root growth of the *NRT2.7-Ox* (WS) lines
339 in wild-type background under non-limiting N supply (Fig. 1), we further studied the
340 growth-promoting effect of *NRT2.7* overexpression under limiting N supply to better
341 understand the changes of nitrate fluxes through the vacuolar membrane on N status.
342 Plants were grown on vertical agar medium containing 0.2 mM nitrate for 12 days.
343 Shoot biomass and total root length of *NRT2.7-Ox* (WS) lines were increased

344 compared to WT by 50 to 65% and 18 to 32% respectively (Fig. 4A and 4B).
345 Conversely, root nitrate content of the *NRT2.7-OX* (WS) lines was dramatically
346 reduced by 75 to 83% compared to WT, whereas shoot nitrate content was not
347 modified (Fig. 4C and 4D). The carbon to nitrogen (C/N) ratio is a good indicator for
348 plant growth, development and metabolism (Coruzzi and Zhou, 2001). The C/N ratio
349 of *NRT2.7-Ox* (WS) compared to WT was increased in shoots and roots mainly due to
350 a decrease in total N percentage (Fig. 4E). These data suggest that the ectopic
351 expression of *NRT2.7* drives nitrate use for biomass production by increasing nitrate
352 assimilation. This difference may probably due to a lower vacuolar storage capacity
353 and/or an increased nitrate flux out of the vacuole as it was previously showed for other
354 vacuolar nitrate transporters (He *et al.*, 2017; Lu *et al.*, 2022; Hodin *et al.*, 2023; Shi *et*
355 *al.*, 2023) and no difference in nitrate uptake though the HATS was previously
356 observed (Fig. 3).

357 *NRT2.7 overexpression in clca-2 mutant leads to increased nitrate fluxes out of the*
358 *vacuole*

359 To determine if nitrate fluxes across the vacuolar membrane of the *NRT2.7-Ox* (*clca-*
360 *2*) line were different from those of the knock-out mutant *clca-2*, we extracted vacuoles
361 from mesophyll protoplasts of the *clca-2* and the two *NRT2.7-Ox* lines (*clca-2*) and
362 performed patch clamp experiments in whole-vacuole configuration (Fig. 5). In order
363 to maximize the fluxes of nitrate directed from the vacuole to the cytosol and from the
364 cytosol to the vacuole, we performed two independent series of experimentations
365 designed as follow. In both experiment series, we used the non-permeant cation
366 BisTriPropane in order to limit the vacuolar currents mediated by cations. With the aim
367 to highlight the efflux of nitrate from the vacuole (positive ionic currents), in the first
368 series of experiments (Fig. 5B), we used bi-ionic conditions with 200 mM nitrate in the
369 vacuole and 19.2 mM chloride in the cytosolic-side solution. The vacuoles extracted
370 from *clca-2* knock-outs presented a mean current density of +1.3 ± 0.4 pA/pF at +43
371 mV and a mean reversal potential (E_{rev}=-8 ± 3 mV) that were comparable to those
372 previously observed in similar ionic conditions (De Angeli *et al.*, 2006; Wege *et al.*,
373 2010). Interestingly, in the same ionic conditions vacuoles from *NRT2.7-Ox (clca-2)*
374 plants presented significantly higher current densities compared to *clca-2* currents only
375 at positive vacuolar membrane potentials (from +3 mV to +83 mV, Fig. 5B). These
376 positive currents are compatible with an increased nitrate efflux capacity of the

377 vacuoles from *NRT2.7-Ox (clca-2)* plants. In the second set of experiments, we used
378 an ionic configuration suited to highlight nitrate currents directed from the cytosol to
379 the vacuolar lumen. We performed cytosolic-side solution exchange experiments
380 starting from a chloride based buffer (19.2 mM Cl^- pH 7) to a nitrate based buffer (4
381 mM NO_3^- pH 7) keeping the vacuolar-side buffer constant and with only chloride as a
382 permeant anion (15 mM Cl^- pH 5.5) (Supplementary Fig. S4). Upon the exchange of
383 the cytosolic-side solution a similar increase of the negative current was observed in
384 vacuoles obtained from the tested genotypes. Indeed, the mean current ratios at -60
385 mV were I_{NO3}/I_{Cl}=1.84±0.4 in *NRT2.7-Ox (clca-2)* vacuoles and I_{NO3}/I_{Cl}=1.77 ± 0.44 in
386 *clca-2* vacuole*s*. Conversely, the current densities in presence of nitrate did not show
387 a significant difference between the genotypes, being I_{NO3}=-2.4±1 pA/pF at -60 mV in
388 *NRT2.7-Ox* (*clca-2*) and I_{NO3}=-2.7±0.9 pA/pF at -60 mV in *clca-2.* This second series
389 of experiments indicates that the vacuoles obtained from *NRT2.7-Ox* (*clca-2*) do not
390 display an increased capacity of driving NO_3^- fluxes directed to the vacuolar lumen
391 compared to the vacuoles from *clca-2* under our experimental conditions. Altogether,
392 the results obtained by patch-clamp experiments suggest that, compared to vacuoles
393 from *clca-2*, *NRT2.7-Ox* (*clca-2*) plant vacuoles present a higher capacity to drive
394 nitrate fluxes directed into the cytosol but are not modified concerning nitrate influx into
395 the vacuole.

396

397 ## Discussion

398 Recent studies underline the importance of nitrate fluxes through the vacuolar
399 membrane to enhance NUE by increasing efflux of nitrate from vacuole and/or reducing
400 nitrate storage in this latter compartment (Han *et al.*, 2016; Wang *et al.*, 2018*b*; Hodin
401 *et al.*, 2023; Shi *et al.*, 2023). Therefore, it became essential to characterize the
402 different vacuolar nitrate transporter activities to get an overview of the specificity of
403 each. In this report, we analyzed the function of the vacuolar NRT2.7 transporter by
404 introducing its cDNA under the control of the strong *35S* promoter in *clca* KO mutant
405 or in wild-type. The combined results of on one hand the electrophysiological analyses
406 and on the other hand the phenotypes of the *CLCa* and *NRT2.7* overexpressing lines
407 strikingly demonstrate that both NRT2.7 and CLCa impacts the nitrogen use but
408 through distinct means or signals. The NRT2.7 overexpression in the *clca-2* mutant

409 bypassed the CLCa-dependent reduced growth via a mechanism that uncoupled
410 growth and nitrate accumulation probably related to modified nitrate efflux from the
411 vacuole. This mechanism when acting in a wild type background overexpressing
412 NRT2.7 led to an increase of biomass production mainly through a higher root biomass.

413 *NRT2.7 stimulates nitrate export rather than nitrate import into the vacuole when*
414 *overexpressed ectopically.*

415 We confirmed in this study that *CLCa* mutant physiology was deeply affected by its
416 limited capacity of loading nitrate into vacuoles (Fig. 1 and 2; Supplementary Fig. S1
417 and S2). Besides CLCa, the NRT2.7 nitrate transporter is located at the tonoplast.
418 NRT2.7 translocates nitrate in Xenopus oocytes and its loss-of-function reduced nitrate
419 accumulation in seeds where the gene is chiefly expressed (Chopin *et al.*, 2007).
420 Although *NRT2.7* mRNA were detected in leaves and root, no phenotype could be
421 noticed in the KO mutant for these organs (Orsel *et al.*, 2002; Okamoto *et al.*, 2003;
422 Chopin *et al.*, 2007). To better characterize this transporter, we then overexpressed it
423 in wild-type and *clca* KO mutant backgrounds. The overexpression of *NRT2.7* fully
424 restores the reduced *clca-2* plant growth and even exceeds wild-type biomass but,
425 surprisingly, further diminished the low *clca-2* nitrate content (Fig. 1 and 2). It led to a
426 35% and 23% decreases of shoot and root nitrate pools, respectively, when compared
427 to *clca-2*. Therefore, these results showed that NRT2.7 could indeed rescue the *clca-*
428 *2* growth reduction but through an alternative mechanism rather than a true functional
429 complementation. This hypothesis was confirmed as the NRT2.7 overexpressors
430 (*NRT2.7-Ox* (WS)) showed a 50 to 60% increase in total root length whereas a further
431 depletion of root nitrate content compared to wild-type plants was observed.

432 The decrease of root nitrate content caused by NRT2.7 ectopic overexpression is
433 difficult to conceive with a vacuolar nitrate importer. Chopin *et al.* (2007) reported an
434 influx of ^{15}N labeled nitrate into NRT2.7 expressing oocytes. This result indicates that,
435 in this biological system, NRT2.7 was targeted to the plasma membrane with
436 presumably its cytoplasmic terminus oriented to the oocyte cytoplasm. In this same
437 study, GFP-NRT2.7 translational fusion driven by the 35S promoter in Arabidopsis
438 cells clearly showed a tonoplastic localization. As NRT2.7 cytoplasmic terminus is
439 probably maintained in the plant cell cytoplasm (Jacquot *et al.*, 2020), NRT2.7 would
440 function as a nitrate efflux system from the vacuole to the cytoplasm. This orientation
441 would be more conceivable with the fact that NRT2s are assumed to be H^+/NO_3^-

442 symporters (Forde and Clarkson, 1999) and that roots of NRT2.7 overexpressors
443 become depleted of nitrate at low external nitrate availability (Fig. 4). Supporting this
444 hypothesis, the determination of nitrate fluxes across isolated vacuoles by patch clamp
445 techniques, showed that vacuoles from *NRT2.7-Ox* (*clca-2*) plants present a higher
446 capacity to drive nitrate fluxes directed into the cytosol than vacuoles from *clca-2* plants
447 while the capacity to drive nitrate influx into the vacuole did not vary between these
448 genotypes (Fig. 5 and Supplementary Fig. S4). However, based on these experiments,
449 we cannot exclude that NRT2.7 may regulate positively the activity of nitrate exporters
450 localized in the tonoplast and is not itself the active transporter driving the fluxes.
451 Chopin *et al.* (2007) observed a decrease in seed nitrate content in *nrt2.7* mutants,
452 which was interpreted as a reduced capacity to import nitrate into the vacuole of the
453 embryo. NRT2.7 is highly expressed in seeds and the NRT2.7 promoter drives
454 expression in the embryos where the protein is located at the tonoplast. A decreased
455 nitrate content is thus difficult to explain by the loss-of-function of a vacuolar nitrate
456 exporter protein. However, the transport direction of NRT2.7 in seeds and in shoot/root
457 vacuoles might be different because of distinct types of vacuoles existing in these
458 organs (Shimada *et al.*, 2018). In addition, tonoplast membrane potential might vary
459 and the transport direction may reflect the organ specific ionic and osmotic conditions
460 as it has been discussed previously for NPF6.3 (NRT1.1/CHL1, (Léran *et al.*, 2014) or
461 CLCa (Wege *et al.*, 2014). Besides, nitrate transport properties are regulated via
462 posttranslational protein modifications (e.g. (Wege *et al.*, 2014; Jacquot *et al.*, 2017),
463 a mechanism that should be explored in the future for NRT2.7. However no
464 phosphorylation site in NRT2.7 is found in the PhosPhAt databases (Heazlewood *et*
465 *al.*, 2008). Finally, another hypothesis would be that *nrt2.7* seed nitrate content is
466 decreased because nitrate transport from the roots or shoots towards the seeds is
467 diminished in the *nrt2.7* mutants. Indeed, in Arabidopsis seeds nitrate content seems
468 to rely directly on the nitrate taken up by roots (Schulze *et al.*, 1994). Inflorescence
469 grafting experiments are required to distinguish between seed prone or root/shoot
470 prone determination of the decreased nitrate level in *nrt2.7* seeds. Altogether, these
471 results support that NRT2.7 is essential for nitrate fluxes through the vacuolar
472 membrane. It stimulates nitrate export from the vacuole in seedlings but the contrasting
473 phenotype of modified NRT2.7 expression in reproductive and vegetative organs is a
474 challenging hypothesis and needs further investigation.

475 *Nitrate fluxes through CLCa and NRT2.7 alter root nitrate uptake*

476 As previously reported, we confirmed that the mutation in *CLCa* reduced nitrate uptake
477 activity in the high affinity range (Monachello *et al.*, 2009, Fig. 3) whereas the level of
478 LATS activity was not modified. These results illustrate the existence of a coordinated
479 signaling crosstalk between vacuolar and plasma membrane nitrate fluxes. An obvious
480 signal for this coordinated transmembrane transport would be the nitrate anion itself. It
481 was shown that loss of function of CLCa leads to a faster increase of the cytosolic
482 nitrate concentration in response to an increase of the extracellular NO_3^- concentration
483 in guard cells (Demes *et al.*, 2020). In this scenario, a transitory elevated cytosolic
484 nitrate level would decrease nitrate uptake, increase the nitrate efflux from the cells
485 and/or the assimilation pathways through the nitrate reductase (NR). This was
486 confirmed in a recent study demonstrating that the inhibition of nitrate storage in the
487 vacuole leads to an increase of the NR activity and a better NUE (Hodin *et al.*, 2023).
488 In addition to nitrate, nutrient uptake activity is controlled by a wide range of biophysical
489 (membrane potential, pH) or physiological signals (phosphorylation, metabolites) (see
490 Amtmann and Blatt, 2009 for review). CLCa through its activity as a NO_3^-/H^+ exchanger
491 is known for its function in adjusting the cytosolic pH in guard cells (Demes *et al.*, 2020).
492 More recently, Jain and Schmidt (2024) showed that NRT2.1 phosphorylation level
493 varies according to the environmental pH certainly to adjust cytosolic pH. Then the loss
494 of function of *CLCa* could lead to the inactivation of NRT2.1 to compensate the change
495 in cytosolic pH.

496 The increase of nitrate export from the vacuole due to the NRT2.7 overexpression
497 leads probably to change in nitrate cytosolic content and/or cytosolic pH, as for CLCa.
498 Even if this hypothesis needs to be tested using the nitrate/pH biosensor, ClopHensor
499 (Demes *et al.*, 2020), these modifications could explain the decrease in HATS activity
500 observed in *NRT2.7-Ox (clca-2)* like in *clca-2* plants (Fig. 3). However, the *NRT2.7*
501 overexpression led to an increase of the LATS activity that was not the case when
502 *CLCa* is invalidated. Lejay *et al.* (1999) showed that HATS activity is under the control
503 of both NR activity and N status of the plant whereas that of LATS is controlled solely
504 by NR activity. The discrepancy observed between CLCa and NRT2.7 on the nitrate
505 uptake could suggest that nitrate influx and efflux in and out the vacuole do not have
506 exactly the same effect on N assimilation and N status. The coordination between the
507 direction of vacuolar nitrate fluxes and nitrate uptake at the plasma membrane will
508 need to be further studied in the future.

509 *NRT2.7 overexpression increases growth at low external and internal nitrate*
510 The overexpression of *NRT2.7* led to a higher plant growth when it stimulates nitrate
511 export from the vacuole. This phenotype could be explained not only by the increase
512 of LATS activity but also by a higher nitrate long-distance transport from root to shoot.
513 A study characterizing two *Brassica napus* genotypes differing by their capacity in
514 nitrate storage in root previously showed that the decrease of root nitrate content
515 induces a higher nitrate translocation from root to shoot leading to a better NUE (Han
516 *et al.*, 2016). We can assume that a similar phenomenon occurred in the *NRT2.7*
517 overexpressors. Further studies like, for example, analyzing the expression of *NRT1.5*
518 and *NRT1.8*, two nitrate transporters involved in the long-distance nitrate transport in
519 Arabidopsis (Lin *et al.*, 2008; Li *et al.*, 2010) will be needed to confirm our hypothesis.

520 Interestingly, shoot and root biomass increased in *NRT2.7-Ox* (WS) grown under
521 limiting N nutrition even that a severe decrease of nitrate in root (-75 to -83% compared
522 to wild-type) and a slight decrease of total nitrogen content (-8 to –19% compared to
523 wild-type) occurred in these growth conditions (Fig. 4). These results confirm an
524 apparent disconnection between growth and nitrate content in *NRT2.7* overexpressors
525 plants. C and N metabolism are interconnected and regulate each other (Zheng, 2009).
526 Interestingly, the C/N ratio was higher in the overexpressors compared to wild-type
527 indicating that, even if plants are grown under low external N availability, the
528 photosynthesis outputs higher and could facilitate the increase in biomass. The low
529 internal nitrate levels could be then the consequence of N dilution occurring under
530 accelerated growth. At the opposite, the increase of nitrate export from the vacuole
531 could stimulate photosynthesis as shown in a *Brassica napus* genotype presenting
532 reduced root nitrate storage (Han *et al.*, 2016) or, more recently when Arabidopsis
533 plants overexpressing the CLCb nitrate vacuolar exporter are grown on low nitrate (Shi
534 *et al.*, 2023).

535 In conclusion, we show that the two vacuolar nitrate transporters CLCa and NRT2.7
536 share limited functionality and are not exchangeable. Our results suggest that NRT2.7
537 works as or induces the activity of a vacuolar nitrate exporter when overexpressed
538 ectopically. By exploiting genotypes with modified levels of vacuolar transporters, we
539 show that changes in the nitrate influx or efflux from the vacuolar compartment lead to
540 differences in root nitrate uptake suggesting the existence of a fine regulation between
541 vacuolar and plasma membranes through biophysical and physiological networks of

542 vacuolar nitrate sensing and homeostasis. NRT2.7, whose function *in vivo* seemed to
543 be restricted to metabolism in seeds, provides new physiological features when
544 ectopically overexpressed. The link between NRT2.7 nitrate fluxes and carbon content
545 requires further studies but may be the basis for improving plants with higher C/N ratios
546 accompanied by increased growth when nitrogen is limiting.

547 **Supplementary data**

548 The following supplementary data are available at JXB online.

549 *Fig. S1.* Growth and nitrate content of *clca-2* knock-out mutant grown on different
550 nitrate concentrations.

551 *Fig. S2.* Growth and nitrate content of *NRT2.7* overexpressors in the c*lca-1* knock-out
552 mutant.

553 *Fig. S3.* Level of NRT2.7 expression in NRT2.7-Ox (WS) lines.

554 *Fig. S4.* Nitrate influx in vacuoles from *clca-2 35S:NRT2.7* expressing plants and from
555 *clca-2* knock-out.

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561 **Author contributions**

562 FDV, AK and SF : conceptualization design ; PA, FDV, ADA, AK and SF: experimental
563 design ; PA, ADA, PB and VB performed the experimental work ; PA, MD, FDV, ADA,
564 AK and SF analyzed the data; PA, FDV, AK and SF wrote the manuscript. All authors
565 reviewed the manuscript.

566 **Conflict of interest**

567 The authors declare no conflict of interest.

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572 **Data availability**

All the raw data supporting the findings of this work are available upon request from the corresponding authors, Anne Krapp and Sophie Filleur. Statistical analyses are available in the supplementary table.

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Figures Legends

Fig. 1. Growth effect of *NRT2.7* overexpression in *clca-2* and wild-type backgrounds. Plant were grown on 2 mM nitrate for 12 days. Shoot fresh weight (A, B) and total root length (C, D) were measured on wild-type (WS), *clca-2*, complemented line (*CLCa-Ox* (*clca-2*)) and supplemented line (*NRT2.7-Ox* (*clca-2*)) (A, C) or WS and the two *NRT2.7* over-expressing lines, *NRT2.7-Ox1* (WS) and *NRT2.7-Ox2* (WS) (B, D). The number of samples were 4-5 for shoot fresh weight (one sample = a pool of 3-4 plants) when n=6-17 for total root length on individual plants. Presented data are means ± SEM. Different letters at the top of the bars represent significant difference (p value<0.05).

Fig. 2. Nitrate content of *NRT2.7* overexpression in *clca-2* and wild-type backgrounds. Wild-type (WS), *clca-2*, complemented line (*CLCa-Ox* (*clca-2*)) and *NRT2.7* over-expressing line in *clca-2* (*NRT2.7-Ox* (*clca-2*)) (A, C) or WS and the two *NRT2.7* over-expressing lines, *NRT2.7-Ox1* (WS) and *NRT2.7-Ox2* (WS) (B, D), were grown on 2 mM nitrate. After 12 days on the medium, shoot (A, B) and root (C, D) nitrate contents were measured. Two to three biological replicates were performed on each genotype, one sample corresponding to a pool of at least 50 plants. Presented data are means ± SEM. Different letters at the top of the bars represent significant difference (p value<0.05).

Fig. 3. Effect of *NRT2.7* overexpression in *clca-2* and wild-type backgrounds on nitrate uptake activities. Wild-type (WS), *clca-2*, complemented line (CLCa-Ox (*clca-2*)), *NRT2.7* over-expressing line in *clca-2* (NRT2.7-Ox (*clca-2*)) and the two *NRT2.7* over-expressing lines, NRT2.7-Ox1 (WS) and NRT2.7-Ox2 (WS), were grown on vertical Petri dishes on a medium 2 mM nitrate for 12 days. Uptake experiments consisted of four to five measurements of $^{15}NO_3^-$ for 5 min in a liquid medium containing 6 mM (A=HATS+LATS) or 0.2 mM (B=HATS). The activity of LATS (C) was obtained by subtracting the activity measured at 0.2 mM to the one at 6 mM (see material and methods section for details). Presented data are averages of three independent experiments (n=3-5 by independent experiment). Bars represents means ± SEM. Different letters at the top of the bars represent significant difference (p value<0.05).

Fig. 4. Effect of limiting N supply on the phenotype of *NRT2.7* over-expressers. Wild-type (WS) and over-expressing lines, NRT2.7-Ox1 (WS) and NRT2.7-Ox2 (WS), were

grown in petri dishes for 12 days on 0.2 mM nitrate. Shoot fresh weight (A), total root length (B) shoot and root nitrate contents (C, D) were measured as described in Figure 1. C/N ratios, percentages of N and C contents were measured in shoot (E) and root (F) using an elementary analyser. Data are averages of three to four measurements (bars being the standard errors). Different letters shows significant differences between genotypes ($p<0.05$).

Fig. 5. Nitrate efflux from mesophyll vacuoles is increased in NRT2.7-Ox (*clca-2*) expressing plants compared to clca-2 knock-outs. A. Representative whole-vacuole currents measured in vacuoles extracted from *clca-2* knock-out mutant (left) and *NRT2.7-Ox* (*clca-2*) transgenic line (right) in presence of nitrate only in the vacuolar lumen (200 mM NO_3^- pH 5.5 in the vacuolar buffer, 19.2 mM Cl^- in the cytosolic buffer, see Materials and Methods). Currents were evoked in response to 2 s voltage pulses ranging from -77 mV to +83 mV in +20 mV steps, followed by a tail pulse at +33 mV, with a holding potential at -17 mV. B. Box plot of the mean current density (pA/pF) measured at different positive (left) and negative (right) membrane potentials in vacuoles from *NRT2.7-Ox* (*clca-2*) (vertical diamonds line *NRT2.7-Ox1* (*clca-2*) n=11; horizontal diamonds line *NRT2.7-Ox2* (*clca-2*) n=5) and *clca-2* (n=10) knock-out plants (ionic conditions as in A). Boxes defines the first and the third quartile and the median, the whiskers represent the ninth and the ninety-first percentiles. The two asterisks indicate a statistical significance at 2% level with a Mann-Whitney test.

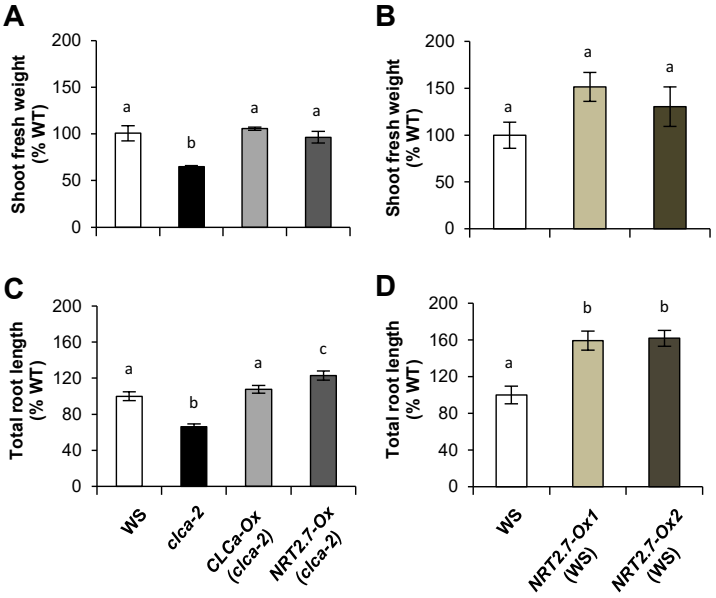

Fig. 1. Growth effect of *NRT2.7* overexpression in *clca-2* and wild-type backgrounds. Plant were grown on 2 mM nitrate for 12 days. Shoot fresh weight (A, B) and total root length (C, D) were measured on wild-type (WS), *clca-2*, complemented line (*CLCa-Ox* (*clca-2*)) and supplemented line (*NRT2.7-Ox* (*clca-2*)) (A, C) or WS and the two *NRT2.7* over-expressing lines, *NRT2.7-Ox1* (WS) and *NRT2.7-Ox2* (WS) (B, D). The number of samples were 4-5 for shoot fresh weight (one sample = a pool of 3-4 plants) when n=6-17 for total root length on individual plants. Presented data are means ± SEM. Different letters at the top of the bars represent significant difference (p value<0.05).

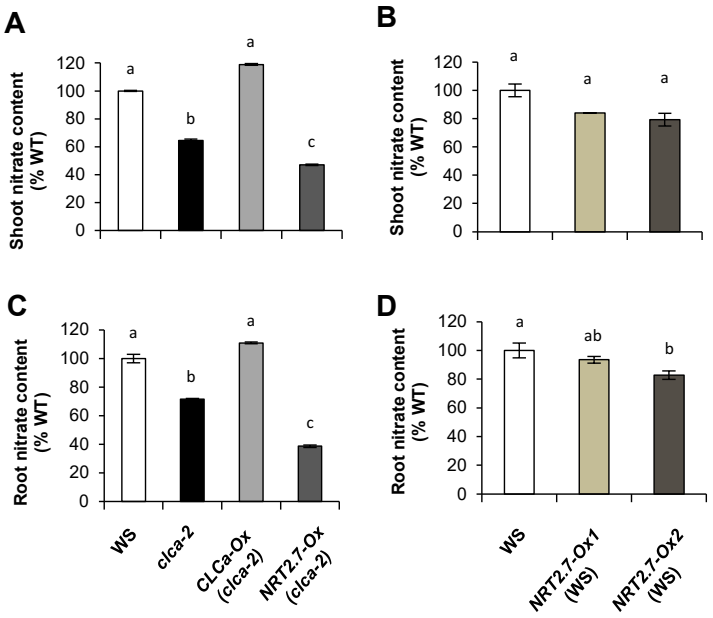

Fig. 2. Nitrate content of *NRT2.7* overexpression in *clca-2* and wild-type backgrounds. Wild-type (WS), *clca-2*, complemented line (*CLCa-Ox* (*clca-2*)) and *NRT2.7* over-expressing line in *clca-2* (*NRT2.7-Ox* (*clca-2*)) (A, C) or WS and the two *NRT2.7* over-expressing lines, *NRT2.7-Ox1* (WS) and *NRT2.7-Ox2* (WS) (B, D), were grown on 2 mM nitrate. After 12 days on the medium, shoot (A, B) and root (C, D) nitrate contents were measured. Two to three biological replicates were performed on each genotype, one sample corresponding to a pool of at least 50 plants. Presented data are means ± SEM. Different letters at the top of the bars represent significant difference (p value<0.05).

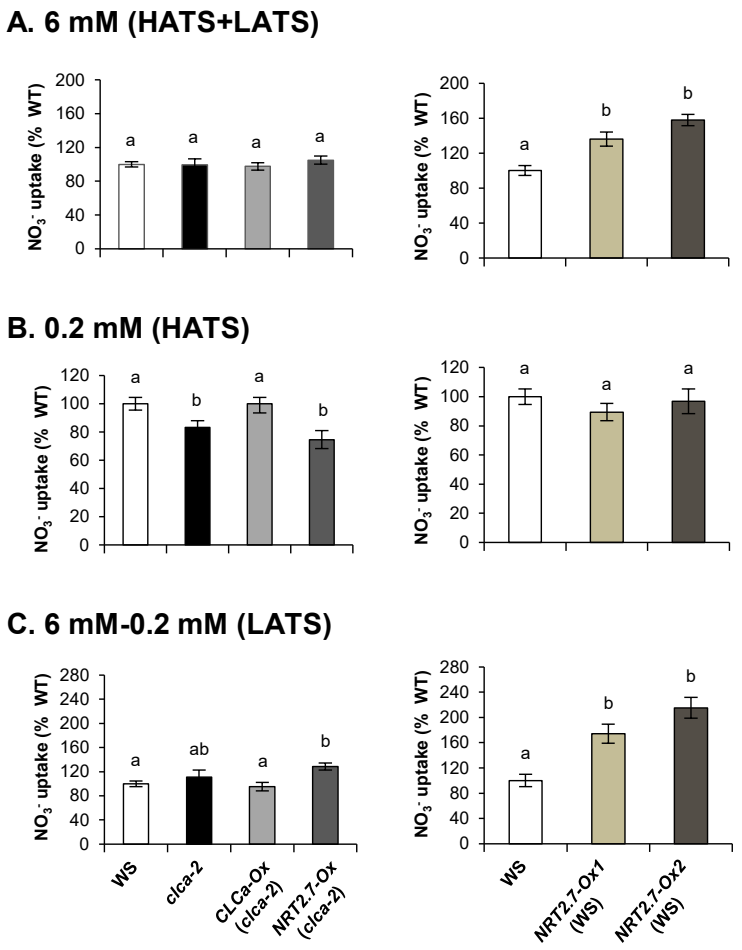

Fig. 3. Effect of *NRT2.7* overexpression in *clca-2* and wild-type backgrounds on nitrate uptake activities. Wild-type (WS), *clca-2*, complemented line (CLCa-Ox (*clca-2*)), *NRT2.7* over-expressing line in *clca-2* (NRT2.7-Ox (*clca-2*)) and the two *NRT2.7* over-expressing lines, NRT2.7-Ox1 (WS) and NRT2.7-Ox2 (WS), were grown on vertical Petri dishes on a medium 2 mM nitrate for 12 days. Uptake experiments consisted of four to five measurements of $^{15}NO_3^-$ for 5 min in a liquid medium containing 6 mM (A=HATS+LATS) or 0.2 mM (B=HATS). The activity of LATS (C) was obtained by subtracting the activity measured at 0.2 mM to the one at 6 mM (see material and methods section for details). Presented data are averages of three independent experiments (n=3-5 by independent experiment). Bars represents means ± SEM. Different letters at the top of the bars represent significant difference (p value<0.05).

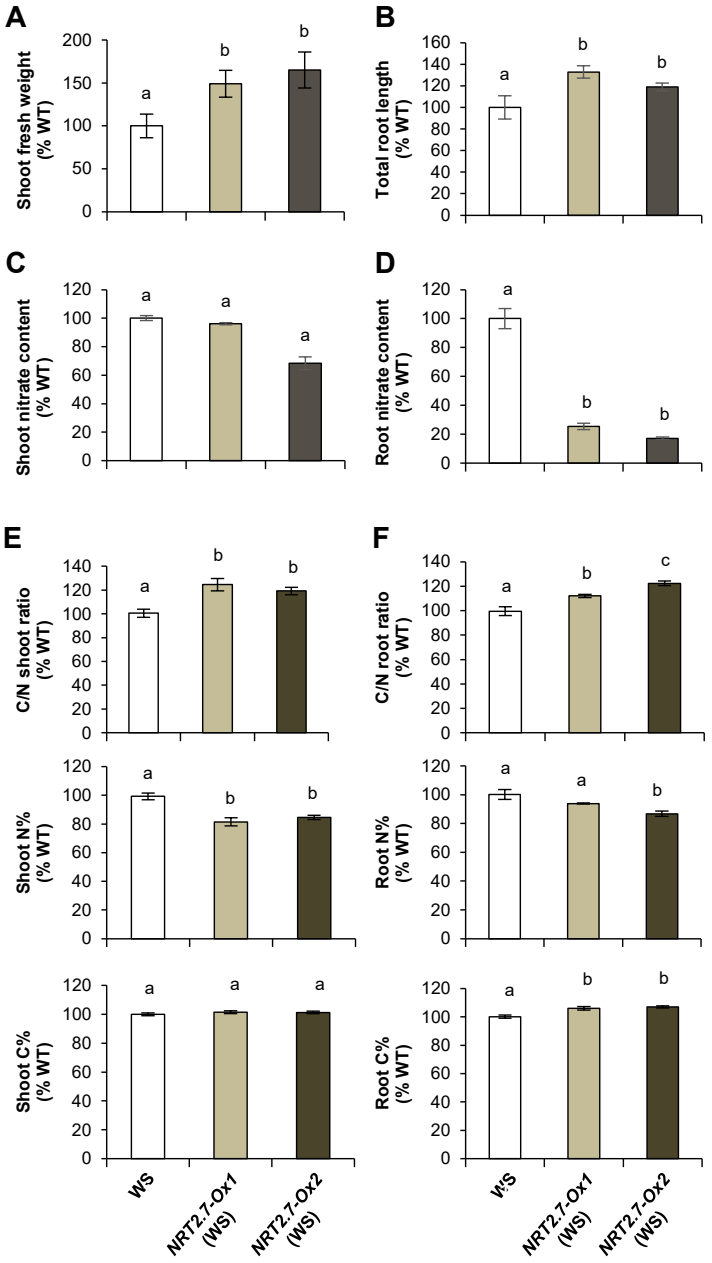

Fig. 4. Effect of limiting N supply on the phenotype of *NRT2.7* over-expressers. Wild-type (WS) and over-expressing lines, NRT2.7-Ox1 (WS) and NRT2.7-Ox2 (WS), were grown in petri dishes for 12 days on 0.2 mM nitrate. Shoot fresh weight (A), total root length (B) shoot and root nitrate contents (C, D) were measured as described in Figure 1. C/N ratios, percentages of N and C contents were measured in shoot (E) and root (F) using an elementary analyser. Data are averages of three to four measurements (bars being the standard errors). Different letters shows significant differences between genotypes ($p<0.05$).

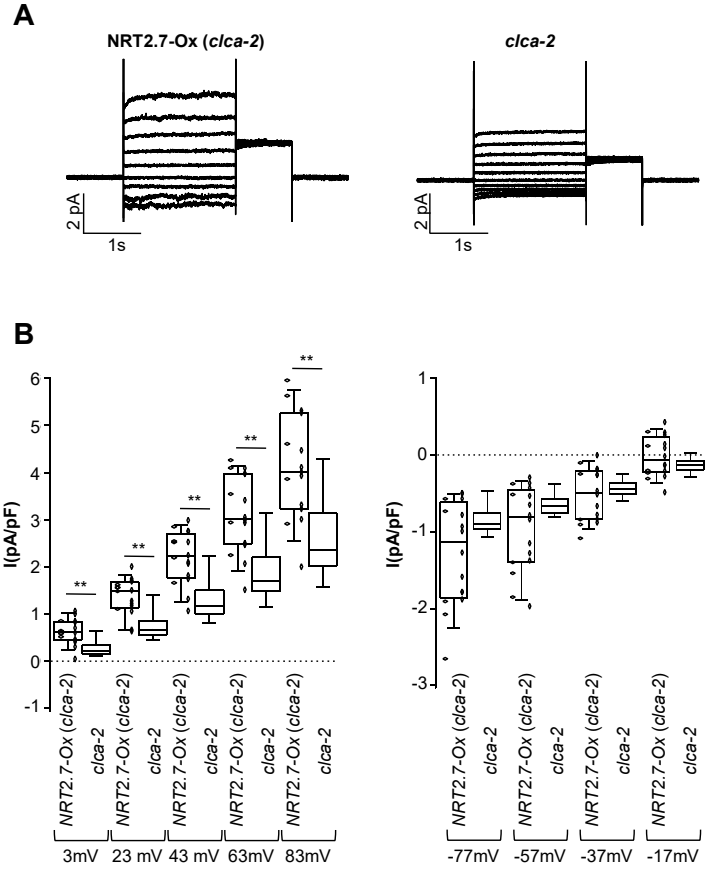

Fig. 5. Nitrate efflux from mesophyll vacuoles is increased in NRT2.7-Ox (*clca-2*) expressing plants compared to clca-2 knock-outs. A. Representative whole-vacuole currents measured in vacuoles extracted from *clca-2* knock-out mutant (left) and *NRT2.7-Ox* (*clca-2*) transgenic line (right) in presence of nitrate only in the vacuolar lumen (200 mM NO_3^- pH 5.5 in the vacuolar buffer, 19.2 mM Cl^- in the cytosolic buffer, see Materials and Methods). Currents were evoked in response to 2 s voltage pulses ranging from -77 mV to +83 mV in +20 mV steps, followed by a tail pulse at +33 mV, with a holding potential at -17 mV. B. Box plot of the mean current density (pA/pF) measured at different positive (left) and negative (right) membrane potentials in vacuoles from *NRT2.7-Ox* (*clca-2*) (vertical diamonds line *NRT2.7-Ox1* (*clca-2*) n=11; horizontal diamonds line *NRT2.7-Ox2* (*clca-2*) n=5) and *clca-2* (n=10) knock-out plants (ionic conditions as in A). Boxes defines the first and the third quartile and the median, the whiskers represent the ninth and the ninety-first percentiles. The two asterisks indicate a statistical significance at 2% level with a Mann-Whitney test.