the plant journal
SEB Society for Experimental Biology
The Plant Journal (2015) 81, 377–387 doi: 10.1111/tpj.12740

Urea retranslocation from senescing Arabidopsis leaves is promoted by DUR3-mediated urea retrieval from leaf apoplast

Anne Bohner[1], Soichi Kojima[2], Mohammad Hajirezaei[1], Michael Melzer[1] and Nicolaus von Wirén[1,*]
[1]*Department of Physiology & Cell Biology, Leibniz-Institute of Plant Genetics and Crop Plant Research, Corrensstr 3, D-06466 Gatersleben, Germany, and*
[2]*Department of Applied Plant Science, Tohoku University, Sendai 981-8555, Japan*

Received 14 March 2012; revised 10 November 2014; accepted 20 November 2014; published online 2 December 2014.
*For correspondence (e-mail vonwiren@ipk-gatersleben.de).

SUMMARY

In plants, urea derives either from root uptake or protein degradation. Although large quantities of urea are released during senescence, urea is mainly seen as a short-lived nitrogen (N) catabolite serving urease-mediated hydrolysis to ammonium. Here, we investigated the roles of DUR3 and of urea in N remobilization. During natural leaf senescence urea concentrations and *DUR3* transcript levels showed a parallel increase with senescence markers like *ORE1* in a plant age- and leaf age-dependent manner. Deletion of *DUR3* decreased urea accumulation in leaves, whereas the fraction of urea lost to the leaf apoplast was enhanced. Under natural and N deficiency-induced senescence *DUR3* promoter activity was highest in the vasculature, but was also found in surrounding bundle sheath and mesophyll cells. An analysis of petiole exudates from wild-type leaves revealed that N from urea accounted for >13% of amino acid N. Urea export from senescent leaves further increased in *ureG-2* deletion mutants lacking urease activity. In the *dur3 ureG* double insertion line the absence of DUR3 reduced urea export from leaf petioles. These results indicate that urea can serve as an early metabolic marker for leaf senescence, and that DUR3-mediated urea retrieval contributes to the retranslocation of N from urea during leaf senescence.

Keywords: leaf senescence, urea uptake, urease, phloem transport, nitrogen efficiency, urea metabolism.

INTRODUCTION

In plant tissues, urea is found in micro- to millimolar concentrations that derive essentially from two major sources. On the one hand, urea is taken up via the roots from the soil, where urea represents a degradation product from organic matter and animal excrement, as well as a major form of nitrogen (N) from fertilizer used in agricultural plant production (Kojima *et al*., 2006). On the other hand, urea is generated within the plant tissue by the breakdown of arginine, which itself is a prominent amino acid in storage proteins and accumulates in particular when seed proteins are remobilized for germination (Zonia *et al*., 1995; Witte, 2011). As the arginase reaction releasing urea is localized in mitochondria, urea must be transported into the cytosol, where it is further hydrolyzed to ammonia and CO_2 by the cytosolic, nickel-containing enzyme urease (Faye *et al*., 1986). Support for the essential role of urease in urea recycling came from the analysis of Arabidopsis T-DNA insertion lines defective in the synthesis of the accessory proteins UreD, UreF or UreG, which are required for urease activation and for plant growth on urea as a sole N source (Witte *et al*., 2005). In contrast to the activity of arginase, which increased during germination, urease activity was rather constitutive, suggesting that basal urease activity is not limiting the conversion of nitrogen from urea (urea-N) to ammonia, even under conditions of additional urea release (Cao *et al*., 2010).

Urea should also be generated in senescing leaves, where the accumulation of arginine and a steady increase in arginase activities have been shown to progress with leaf age (Diaz *et al*., 2005). Presuming a constitutively non-limiting activity of urease, as supported by a persistent mRNA expression of urease and its accessory proteins in senescing Arabidopsis or *Oryza sativa* (rice) leaves (Winter *et al*., 2007; Wang *et al*., 2012), ammonia-N can be continuously released from urea for re-assimilation, predominantly by cytosolic glutamine synthetase and glutamate synthase (Masclaux-Daubresse *et al*., 2006). During senescence the total amino acid concentrations decrease,

© 2014 The Authors
The Plant Journal published by Society for Experimental Biology and John Wiley & Sons Ltd.
This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

leaving glutamine, glutamate, asparagine and aspartate as major amino acids in Arabidopsis leaves (Diaz *et al.*, 2005). A comparison of the relative quantities of amino acids present in the phloem sap showed that glutamine was the most abundant amino acid in the phloem (Masclaux-Daubresse *et al.*, 2006; Zhang *et al.*, 2010). Even though amino acids are supposed to constitute the most important fraction for phloem-transported N, their quantitative importance relative to other forms of N involved in the retranslocation of N via the phloem remains unclear. In this context, the role of urea as a quantitatively important metabolite and putative candidate for N retranslocation during leaf senescence has not yet received any attention.

Apart from the enzymatic reactions required for the release and hydrolysis of urea, there is an essential requirement for just one associated urea transport step: the export of urea out of the mitochondria (Goldraij and Polacco, 1999). In general, urea can be transported across plant membranes via high- or low-affinity transport proteins (Kojima *et al.*, 2006). High-affinity transport of urea is mediated at an apparent K_m value of ~4 μM by the rather substrate-specific transporter DUR3 (Liu *et al.*, 2003a). In roots this protein has been localized to the plasma membrane, where is confers urea uptake and contributes to N nutrition (Kojima *et al.*, 2007). Low-affinity urea transport is mediated by aquaporins belonging to the tonoplast (TIPs) or by plasma membrane-intrinsic proteins (PIPs) (Eckert *et al.*, 1999; Liu *et al.*, 2003b). Among those, TIP5;1 has been shown to localize to mitochondria in pollen tubes and to transport urea besides water, when expressed in oocytes. As *tip5;1* mutants were compromised in pollen tube growth under N-limiting conditions, a role for TIP5;1 in N recycling has been proposed that may be the transport of urea from mitochondria to the cytosol (Soto *et al.*, 2010).

The current view on N metabolism in senescent leaves does not consider the contribution of plasma membrane-localized urea importers. Nevertheless, we observed enhanced *DUR3* transcript levels and an accumulation of urea during leaf senescence. We therefore started to investigate the role of this transporter together with the fate of urea in senescing leaves and addressed essentially two questions: (i) is DUR3 involved in urea transport and N remobilization during leaf senescence; and (ii) does urea, which has a favourably narrow C : N ratio, represent a N form for N retranslocation out of senescing leaves.

RESULTS

Nitrogen supply has a weak impact on shoot urea and AtDUR3 gene expression levels during the vegetative growth phase

When Arabidopsis plants were grown in nutrient solution under adequate N provision and kept under short-day conditions, *DUR3* mRNA was hardly found in roots or shoots (Figure 1a). Similar as observed previously (Kojima *et al.*, 2007), *DUR3* transcript levels in roots increased after plants were transferred to N-free nutrient solution for a period of 4 days. In contrast, *DUR3* mRNA levels in N-deficient shoots underwent a rather weak increase (Figure 1a), suggesting a less prominent role of the corresponding transporter in shoots. Under adequate N supply, urea concentrations in roots were threefold higher than in shoots (Figure 1b), but N deficiency decreased the urea concentration in roots by ~85%, whereas that in shoots remained constant. This rapid N-dependent change of urea concentrations in roots suggested that the root urea pool is used to overcome short periods of N deficiency, whereas the more constant pool of urea in shoots may function in another physiological context.

During generative growth, plant and leaf age alter urea accumulation and DUR3 expression

Based on an increase in *DUR3* mRNA levels in senescent and cauline leaves (Winter *et al.*, 2007), we monitored *DUR3* gene expression in hydroponically grown Arabidopsis plants

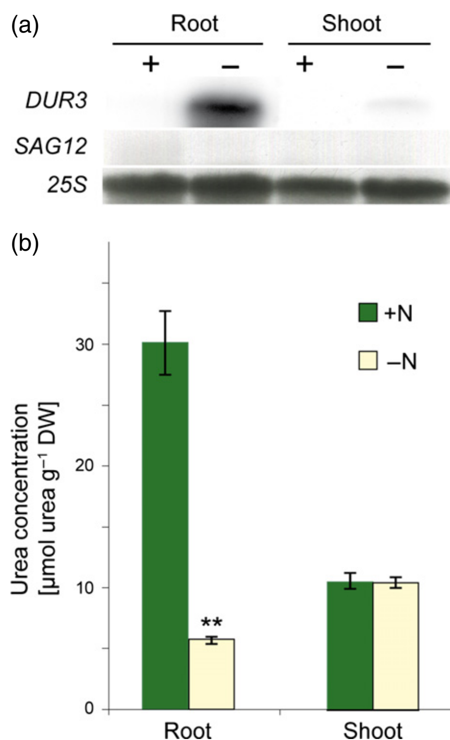

Figure 1. Impact of the nitrogen (N) nutritional status on *DUR3* gene expression and urea concentrations during vegetative growth.
(a) RNA gel blot analysis of *DUR3* and *SAG12* using total root and shoot RNA of 6-week-old wild-type plants cultured under short-day conditions in 2 mM NH_4NO_3 (+N) or in N–deficient nutrient solution for 4 days (–N). A *25S* RNA probe was used to control equal RNA loading.
(b) Urea accumulation in roots and shoots of the same plants. Bars indicate means ± SEs, $n = 6$. The asterisk denotes significant difference according to Tukey's test at $P < 0.001$.

© 2014 The Authors
The Plant Journal published by Society for Experimental Biology and John Wiley & Sons Ltd.,

during a period of 5 weeks after the plants were transferred to long-day conditions. After plants had entered the generative growth phase *DUR3* mRNA levels increased with plant age (Figure 2a). Fractionating leaves into four different classes of increasing tissue age showed that *DUR3* mRNA levels also increased with leaf age. A similar plant and leaf age-dependent increase was also seen for mRNA levels of *ORE1* (Figure 2b), a widely recognized marker for early leaf

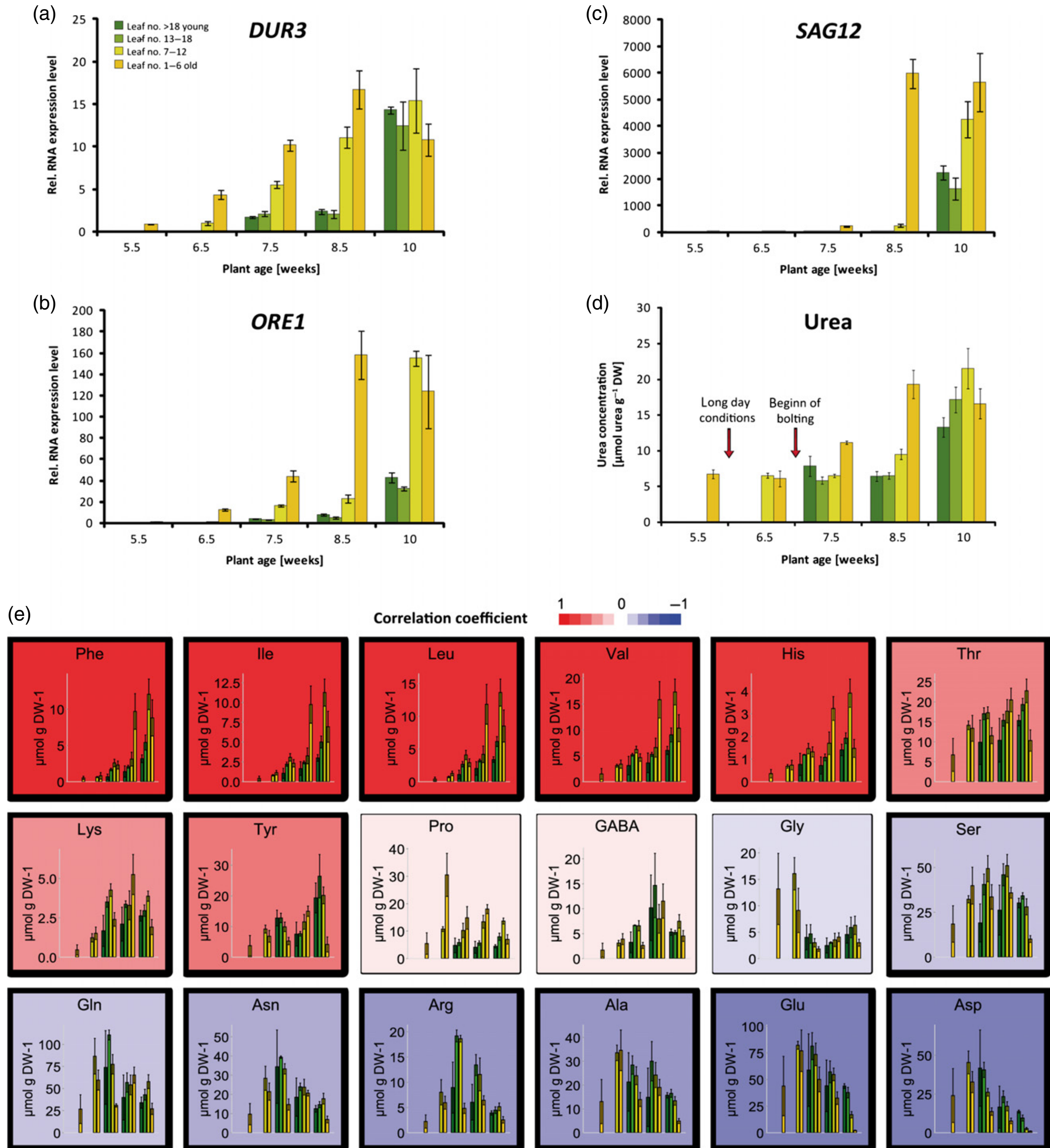

Figure 2. *DUR3* transcript levels in relation to urea and amino acid concentrations in naturally senescing wild-type leaves.
Relative mRNA expression levels of (a) *DUR3*, (b) *ORE1* and (c) *SAG12*. (d–e) Absolute concentrations of (d) urea or (e) amino acids in Arabidopsis leaves. In (e) the correlation coefficient between the concentrations of urea and individual amino acid is represented by a heat map, with red for positive and blue for negative correlations. Plants were pre-cultured hydroponically for 6 weeks in short days before transfer to long days (arrow in d). Leaves were sampled 4 h after the onset of light at five time points (weeks 5.5–10) and fractionated into four pools (leaf nos: 1–6, old; 7–12; 13–18; 18, young). Bars represent means ± SEs, *n* = 5. Relative mRNA levels were determined by qRT-PCR, normalized to *Actin2* so as to show relative expression levels to that for leaf nos 1–6 at 5.5 weeks.

© 2014 The Authors

senescence (Kim *et al.,* 2009). Thus, the senescence-dependent increase in *DUR3* transcript levels occurred even earlier than that of the alternative senescence marker *SAG12* (Figure 2c).

This increase in *DUR3* mRNA levels was closely accompanied by a leaf and plant age-dependent accumulation of urea (Figure 2d; Table S1). Comparison with age-dependent changes in the concentration of amino acids indicated that Phe, Ile, Leu, Val and His followed a highly similar pattern, correlating with *r* values of 0.82–0.87 with urea concentrations in the different leaf samples (Figure 2e; Table S1). Although the majority of the amino acids exhibited weaker or no clear age-dependent changes, Asp and Glu levels in particular showed a pronounced decrease with leaf and plant age, and correlated negatively with urea concentrations. The tight positive correlation between the concentrations of Phe, Ile, Leu, Val, His and urea with transcript levels of *ORE1* and *SAG12* suggested that urea accumulates within a senescence-induced protein degradation programme that completely alters the amino acid composition of Arabidopsis leaves.

Urea accumulation in old leaves depends on DUR3

As *DUR3* gene expression in roots can be induced by its substrate, a possible involvement of the corresponding transporter in urea accumulation was investigated in the leaves of wild-type plants and in two T-DNA insertion lines *dur3-1* and *dur3-3* (Kojima *et al.*, 2007), in which *DUR3* gene expression was completely absent from leaves (Figure 3a). In 5-week-old plants, urea concentrations were still as low as during vegetative growth (Figures 1 and 2), and only older leaves tended to contain more urea (Figure 3b). With the start of bolting in week 6, urea concentrations almost doubled in the middle-aged leaf fraction, whereas they increased by roughly fourfold in old leaves of the wild type. In the two younger leaf fractions, urea concentrations did not significantly differ between wild-type and mutant plants, but in older leaves urea concentrations in wild-type plants were ~40% higher than in *dur3-1* and *dur3-3*. Therefore, DUR3 was involved in the accumulation of urea in old leaves.

Tissue localization of DUR3 promoter activity in senescent leaves

To monitor tissue specificity of the *DUR3* promoter activity in senescing leaves, transgenic plants expressing a *DUR3-pro::GUS* construct were generated, and GUS reporter activity was localized. In leaves of 8-week-old, soil-grown plants, which had entered natural leaf senescence, promoter activity was mainly observed in major and minor veins (Figure 4a); however, GUS activity was difficult to localize precisely as the senescing leaf tissue disintegrated and became fragile. Alternatively, we subjected young plants to N deficiency for 2 weeks and examined lower, senescing leaves that turned severely chlorotic. GUS activity only appeared in the oldest leaves, and was essentially absent from younger leaves (Figure 4b,c). Despite the loss of tissue integrity and the decreased metabolic activity compromising GUS activity in older leaves, GUS activity was especially confined to the leaf tip and along the major veins, but was also apparent outside the vasculature (Figure 4b,d). Leaf cross sections revealed high GUS activity in all cells of the vascular system except the xylem vessels (Figure 4e,f). Moreover, weak GUS activity also appeared in bundle sheath cells around the vasculature and even more weakly in other mesophyll cells. In conclusion, the predominant localization of *DUR3* promoter activity in the vasculature of senescent or severely N-deficient leaves indicated that DUR3 may be involved in urea loading or unloading processes in the vascular system.

The involvement of DUR3 and urease in urea retranslocation

To further characterize the function of DUR3 in leaf urea metabolism, the *ureG-2* mutant line that lacks a functional urease (Witte *et al.*, 2005) was crossed with the *dur3-1* line. As expected, the urea uptake capacity of the *dur3 ureG* double mutant and the *dur3-1* single mutant were severely reduced when plants were subjected to N deficiency. Relative to the wild type, the *ureG-2* mutant showed slightly

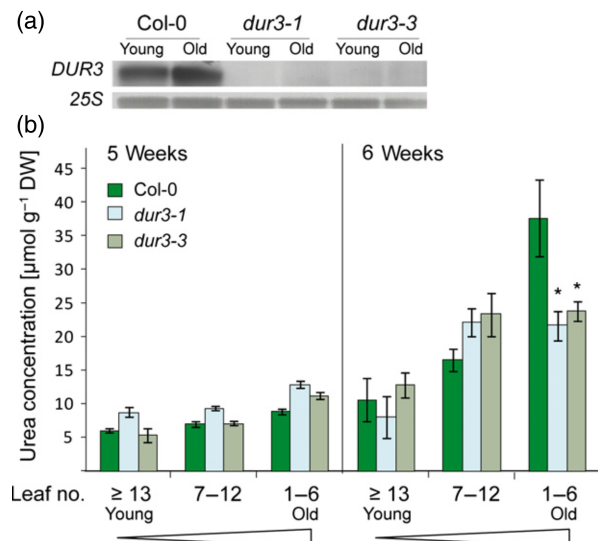

Figure 3. DUR3-dependent urea accumulation in naturally senescing leaves.
(a) RNA gel-blot analysis of *DUR3* on RNA from young or old leaves of wild-type or *dur3–1* and *dur3–3* mutant plants. A *25S* RNA probe was used to control equal loading of RNA.
(b) Urea concentrations in leaves of Col–0, *dur3–1* and *dur3–3* plants. Leaves were separated according to leaf number (leaf nos: 1–6, old; 7–12, middle-aged; ≥13, young). Plants were pre-cultured hydroponically on 2 mM NH_4NO_3 for 4 weeks under short-day conditions before transfer to long days and harvest 1–2 weeks later. Bars indicate means ± SEs, $n = 10$. Asterisks denote significant differences among means at $P < 0.05$ according to Tukey's test.

© 2014 The Authors

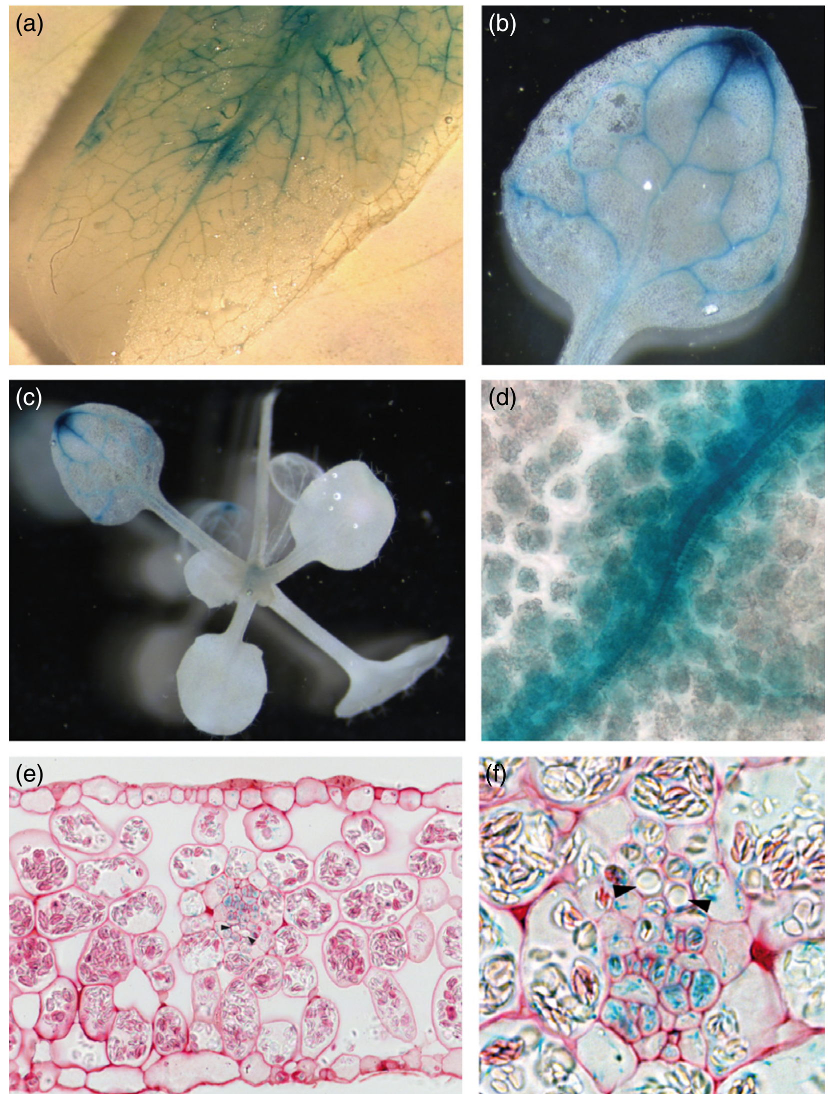

Figure 4. Localization of *DUR3* promoter-GUS activity in senescent or nitrogen (N)-deficient leaves.
(a) *DUR3* promoter-GUS activity in a naturally senescing leaf of Arabidopsis. Transgenic plants expressing a *DUR3pro:GUS* construct were cultivated in the glasshouse on fully fertilized soil for 8 weeks.
(b, c, d) *DUR3* promoter-GUS activity in (b) the oldest leaf, (c) whole shoots and (d) along the vasculature of the oldest leaf.
(e) Cross section of a mature leaf at a vascular bundle.
(f) Magnification of a vascular bundle from another cross section. Arrowheads point to xylem vessels. Transgenic plants in (b–f) were pre-cultured under adequate N supply for 1 week, transferred to long-day conditions and continued to grow on low N for another 2 weeks.

higher *DUR3* mRNA levels and urea influx (Figure 5a,b). In both lines lacking DUR3 urea influx was still higher under N deficiency than under N sufficiency, suggesting that a part of this difference in urea influx was mediated by other, probably low-affinity transport systems that are differently regulated by N (Liu *et al.*, 2003b). These observations further corroborated the positive relationship between urea concentrations and *DUR3* expression levels.

With these lines we first tested the hypothesis whether urea is a form employed for N retranslocation during leaf senescence. To stimulate N remobilization in senescing Arabidopsis leaves, 4-week-old plants were transferred to long days, grown for 2 weeks and then starved of N for 4 days prior to leaf sampling. When DUR3 protein expression was monitored using an anti-DUR3 antibody (Kojima *et al.*, 2007), it showed a gradual increase from young to middle-aged and older leaf fractions in the wild type, and showed an even stronger increase in *ureG-2* mutant plants (Figure 6a). This increase coincided with the degradation of the large subunit of Rubisco, as recorded by the detection of two bands (Kokubun *et al.*, 2002).

In all lines, leaf urea concentrations increased with leaf age (Figure 6b). Relative to the wild type *dur3-1* tended to lower urea concentrations in old leaves, considering that plants were N deficient for 4 days only. In *ureG-2* and *dur3 ureG* plants urea concentrations in the middle-aged and old leaf samples nearly doubled, with respect to concentrations in the wild type. Thus the loss of urease promoted urea accumulation in leaf tissue, whereas the lack of DUR3 did not have a significant impact on urea levels in leaves.

To monitor urea retranslocation via the phloem, petiole exudates from leaves of different ages were analyzed for urea. Urea exudation rates were observed to increase with leaf age (Figure 6c). Unlike in young or middle-aged leaves, there was a massive increase in urea export from

© 2014 The Authors
The Plant Journal published by Society for Experimental Biology and John Wiley & Sons Ltd.,

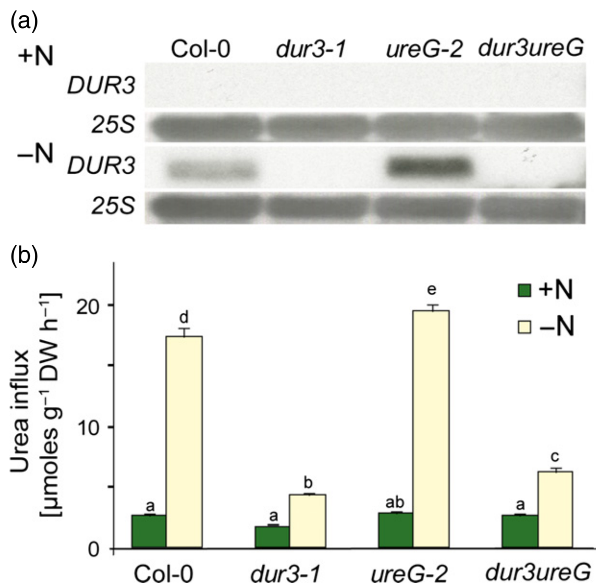

Figure 5. *DUR3* expression and influx of urea in roots of *dur3–1* and *ureGA* single and double mutants.
(a) RNA gel blot analysis of *DUR3* in roots and (b) influx of ^{15}N-labeled urea in roots of Col–0, *dur3–1*, *ureG–2* and *dur3 ureG*. Plants were pre-cultured for 6 weeks in nutrient solution containing 2 mM NH_4NO_3 (+N) or transferred for 4 days to N–free solution (−N) before the influx study. Roots were incubated in 100 µM ^{15}N-labeled urea for 10 min. Bars indicate means ± SEs, $n = 8$. Different letters denote significant differences among means at $P < 0.05$, according to Tukey's test.

the leaf petiole of old leaves, by more than fivefold in *ureG-2* than in the wild type. Thus, urea appeared as an N form being transported in the phloem sap, especially when the degradation of urea in the leaf was inhibited. Relative to the wild type, old *dur3-1* leaves tended to export less urea, but relative to *ureG-2* urea export from the leaf petiole of *dur3 ureG* plants was clearly lower. As there was no significant difference in urea concentrations in old leaves between *dur3-1* and *dur3 ureG* plants (Figure 6b), we concluded that the differences found in net urea export out of old leaves of these lines (Figure 6c) resulted from the urea transport activity of DUR3.

Urea as a form of amino-N in leaf petiole exudates

To estimate the physiological relevance of N being exported from leaves as urea, amino acid concentrations were measured in exudates of leaf petioles, as amino acids are major forms of N transport in the phloem (Masclaux-Daubresse *et al.*, 2010). This analysis was accompanied by large standard deviations that were caused by the analysis of highly diluted amino acids in relatively small sample volumes from senescent leaves close to tissue disintegration (Table 1 and Table S2). In agreement with studies in Arabidopsis on the abundance of amino acids in leaf petiole exudates (Corbesier *et al.*, 2001), glutamine was most abundant, followed by asparagine, serine, glutamate and aspartic acid. When plants were subjected to N deprivation, more amino acids were exported from middle-aged or old leaves than from younger leaves (Table S2). From a quantitative perspective, urea export from old leaves of wild-type plants was 9.5% of the total amino acid concentration, thus urea-N represented 13.6% of the total amino acid-N (Table 1). In both *dur3* backgrounds the lack of a functional urease increased the fraction of urea-N relative to total amino acid-N to >50%.

A loss of DUR3 increases urea concentrations in the apoplast

Based on the observation that the concomitant lack of DUR3 in the *ureG-2* background decreased urea export from the leaf petiole (Figure 6c), we hypothesized that DUR3 retrieves apoplastic urea for phloem loading. Hence, we pre-cultured wild-type and *dur3* lines for 5 weeks before their transfer to long days for an additional week. Four days before harvest N was withdrawn from the nutrient solution to stimulate senescence processes and *DUR3* gene expression in old leaves before the leaf tissue disintegrated (Figure 7a). At this time, urea concentrations in the old leaves of wild-type plants were markedly elevated but not yet significantly different from those in *dur3-1* mutant plants (Figure 7b), so that the concentration gradient driving urea leakage across the plasma membrane of leaf cells was comparable between the two lines. Using a centrifugation-based approach we then collected apoplastic wash fluid from these leaves. Urea levels in the apoplastic wash fluid of both lines increased with leaf age (Figure 7c); however, in old leaves of the *dur3-1* insertion line urea levels were more than twofold higher than in the wild type. Thus, even in the presence of urease DUR3 decreased apoplastic urea pools, clearly supporting a role of DUR3 in the uptake or retrieval of urea from the apoplast of senescing leaves.

DISCUSSION

Leaf senescence is marked by an increase in protein degradation leading to an enhanced generation of urea via the conversion of arginine to ornithine and urea in mitochondria (Witte, 2011). Although urea must thus represent a quantitatively important N metabolite in the front end of N retranslocation, urea has so far not received particular attention in senescence studies, probably because the urea-hydrolyzing enzyme urease is supposed to be non-limiting for ammonia release from urea hydrolysis (Cao *et al.*, 2010; Wang *et al.*, 2012), and thus for re-assimilating ammonia-N into amino acids. In spite of this view, we observed that urea and its high-affinity transporter DUR3 strongly accumulate during leaf senescence, leading us to investigate whether urea is a form of N exported from senescing leaves and whether DUR3 is involved in N retranslocation.

© 2014 The Authors

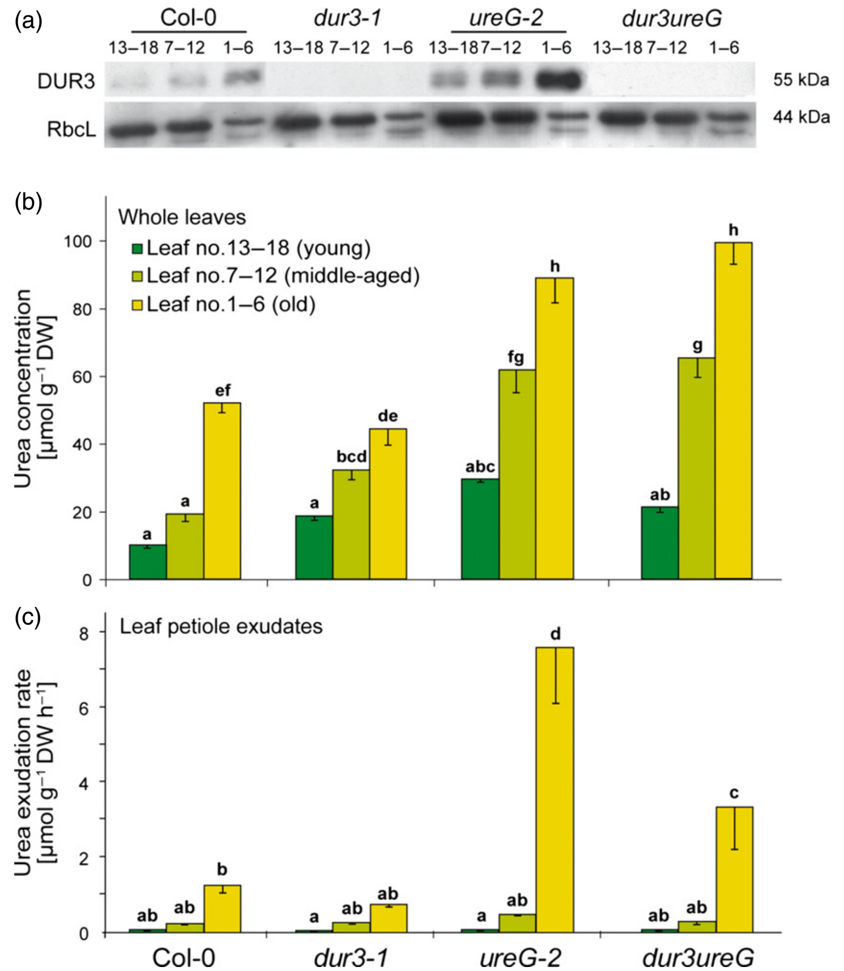

Figure 6. Urea concentrations in leaves and urea exudation rate from leaf petioles of *dur3–1* and *ureGA* single and double mutants.
(a) Protein gel-blot analysis of DUR3 and of the large subunit of RubisCo (RbcL) in young (leaf nos 13–18), middle-aged (leaf nos 7–12) or old leaves (leaf nos 1–6) of Col–0, *dur3–1*, *ureG–2* and ***dur3*** *ureG* plants.
(b) Leaf urea concentrations of the same plants.
(c) Urea exudation rate from leaf petioles over a period of 6 h. Plants were grown hydroponically in short days for 5 weeks with a supply of 2 mM NH_4NO_3, transferred to long days for 2 weeks and continued to grow under nitrogen (N) deficiency for 4 days. Bars indicate means ± SEs, $n = 5$. Different letters denote significant differences among means at $P < 0.05$, according to Tukey's test.

Urea as a metabolic marker for leaf senescence

During vegetative growth urea concentrations in Arabidopsis leaves were lower than those in roots, and were only weakly affected by the form and quantity of N supply (Figure 1; Mérigout *et al.*, 2008). After plants had entered generative growth urea concentrations in Arabidopsis leaves increased with plant age as well as with leaf age (Figure 2d). This increase was paralleled by an increase in the transcript levels of the early senescence marker gene *ORE1* (Figure 2b), emphasizing the tight link of urea accumulation to whole-plant and tissue-dependent senescence (Table S1). Likewise, higher urea concentrations were also measured in an older leaf below the flag leaf in rice plants (Wang *et al.*, 2012). These observations imply that urea concentrations rise whenever leaf N metabolism is altered to take over a source function in N retranslocation (Masclaux-Daubresse *et al.*, 2010), and protein degradation as well as arginase-mediated urea release have set in (Witte, 2011). Indeed, in Arabidopsis leaves Arg was one of the amino acids that decreased with plant age (Figure 2e). An even stronger negative correlation with urea was observed for the concentrations of Asp and Glu, which represent early amino metabolites from the GS-GOGAT cycle. Their decrease might reflect the general switch from anabolic to catabolic N metabolism with increasing plant and tissue age. Likewise, in a metabolic profiling approach with soil-grown Arabidopsis plants, Diaz *et al.* (2005) also observed a relative decrease of Asp and Glu levels in senescing leaves, whereas the proportions of GABA, Leu and Ile, in particular, were found to increase with plant age. Whether the plant- and leaf-age-dependent accumulations of Phe, Ile, Leu, Val and His that closely correlate with *ORE1* and *SAG12* mRNA levels (Figure 2e; Table S1) are primarily a consequence of protein degradation and poor conversion into other amino compounds, or are related to special functions of these amino acids in senescing leaves, is not yet clear. In concert with these five amino acids, urea concentrations rose strictly with plant and leaf age and correlated tightly with *ORE1* and *SAG12* transcript levels ($r > 0.8$; Table S1). In contrast to amino acids, urea is not a precursor or product in anabolic pathways but a pure degradation product of protein catabolism (Witte, 2011), and is thus much less influenced by residual N uptake and renewed synthesis of amino compounds, which may still

© 2014 The Authors
The Plant Journal published by Society for Experimental Biology and John Wiley & Sons Ltd.,

Table 1 Exudation of urea and amino acids from leaf petioles of *dur3* and *ureG–2* single and double mutants

Line	Leaf	Total amino acids (μmol g^{-1} DW h^{-1})	Glutamine (μmol g^{-1} DW h^{-1})	Urea (μmol g^{-1} DW h^{-1})	% urea-N of aa-N
Col-0	Young	5.01 ± 0.57[a,b]	1.63 ± 0.11[b]	0.08 ± 0.19[a,b]	2.2
	Middle-aged	13.35 ± 1.91[a,e]	4.99 ± 0.53[a,b]	0.23 ± 0.07[a,b]	2.2
	Old	13.10 ± 3.25[a,e]	3.82 ± 1.15[a,b]	1.25 ± 1.33[b]	13.6
dur3-1	Young	3.84 ± 0.93[a]	1.02 ± 0.24[b]	0.06 ± 0.98[a]	2.1
	Middle-aged	15.74 ± 1.38[c,e]	5.82 ± 0.33[a,b]	0.26 ± 0.32[a,b]	2.1
	Old	14.69 ± 3.64[b,e]	4.20 ± 1.16[a,b]	0.75 ± 0.03[a,b]	7.2
ureG-2	Young	5.70 ± 0.97[a,c]	2.07 ± 0.34[a,b]	0.09 ± 0.02[a]	2.1
	Middle-aged	17.38 ± 4.96[d,e]	4.53 ± 1.34[a,b]	0.47 ± 0.03[a,b]	3.7
	Old	19.09 ± 5.88[e,f]	5.53 ± 1.70[a,b]	7.62 ± 0.06[d]	56.4
dur3 ureG	Young	4.51 ± 0.48[a,b]	1.37 ± 0.12[b]	0.07 ± 0.04[a,b]	2.2
	Middle-aged	18.81 ± 4.32[e,f]	6.95 ± 1.43[a,c]	0.29 ± 0.00[a,b]	2.0
	Old	8.19 ± 2.43[a,d]	1.90 ± 0.66[a,b]	3.35 ± 0.00[c]	58.9

Concentrations of total amino acids, glutamine and urea were determined in petiole exudates collected from leaves of different age (leaf nos: 1–6, old; 7–12, middle-aged; 13–18, young) from Col-0, *dur3-1* and *ureG* single and double mutants. Plants were grown in nutrient solution with a supply of 2 mM NH_4NO_3 in short days for 5 weeks, transferred to long days for 2 weeks and subjected to N deficiency for 4 days. The relative contribution of urea-derived N to total amino acid-N was expressed as a percentage. Values represent means ± SEs, $n = 5$. Different letters denote significant differences among means over all lines at $P < 0.05$ according to Tukey's test.

occur at a low level in senescing plants (Masclaux-Daubresse *et al.*, 2010). In consideration of this and the large absolute quantities of urea accumulating in senescent leaves, we propose urea as an early metabolic marker for leaf senescence.

DUR3 functions in the retrieval of apoplastic urea

Whenever leaves started to senesce and *ORE1* transcript levels increased, transcript levels of the high-affinity urea transporter *DUR3* showed a parallel increase that went along with urea accumulation (Figure 2; Table S1). In view of elevated *DUR3* transcript levels in urea-resupplied roots (Kojima *et al.*, 2007; Mérigout *et al.*, 2008), it has been suggested that *DUR3* is induced by its own substrate. A similar observation has been made in the roots of *Oryza sativa* (rice), where *OsDUR3* mRNA levels increased after the supply of urea to N-deficient roots (Wang *et al.*, 2012). Substrate induction was also displayed at the protein level, as described in *ureG-2* leaves, where urea accumulated because urease activity was absent, and where DUR3 protein levels increased beyond the levels found in the wild type (Figure 6a). Besides substrate induction, however, senescence-induced regulatory signals are also likely to have contributed to elevated *DUR3* mRNA levels. An *in silico* analysis of the *DUR3* promoter region indicated several binding sites for WRKY- and bZIP-type transcription factors, which are prominent regulators of leaf senescence programs (Lin and Wu, 2004).

The elevated concentration of urea in leaves of the *ureG-2* mutant relative to the wild type went along with a massive increase in urea export from leaf petioles (Figure 6b,c). This relationship also held true in the *dur3* background, where the lack of urease doubled the accumulation of urea in leaves and tripled urea export into the phloem. Thus, urea loading into the phloem profited from a lack of urea hydrolysis, which probably created a steeper urea concentration gradient towards the vascular system. Comparing *dur3-1* and wild-type plants a significant contribution of DUR3 to urea translocation only became evident in tendency, whereas in the *ureG-2* background the absence of DUR3 significantly decreased urea export via the leaf petiole. With respect to the localization of DUR3 to the plasma membrane (Kojima *et al.*, 2007), this finding is consistent with a role of DUR3 directly in or upstream of the phloem-loading process.

Recovering the apoplastic wash fluid from wild-type leaves clearly showed that urea leakage to the apoplast increased with leaf senescence (Figure 7). To a limited extent urea export across the plasma membrane may occur via diffusion, but can be accelerated by urea-permeable members of the PIP family (Gerbeau *et al.*, 1999) acting as importers or exporters of urea, primarily determined by the direction of the urea concentration gradient (Kojima *et al.*, 2006). In the present experiments, apoplastic urea concentrations reached 0.2–1.0 mM, and thus a similar concentration range as apoplastic ammonium concentrations measured in *Brassica napus* (rapeseed) leaves (Nielsen and Schjoerring, 1998). Tracer analysis in rapeseed leaves showed that apoplastic ammonium remained highly buffered, probably because of a rapid exchange of NH_3 leaking via aquaporins and a concomitant NH_4^+ retrieval from the apoplast by *BnAMT1;2* (Pearson *et al.*, 2002). With respect to the significant increase in apoplastic urea concentrations observed in *dur3-1* mutants (Figure 7c) and the abundance of DUR3 when urea is present (Figures 2 and 6), we propose a similar scenario for urea, in which DUR3 takes over the retrieval function for apoplastic urea. As urea concentrations in senescent leaves decreased over

© 2014 The Authors

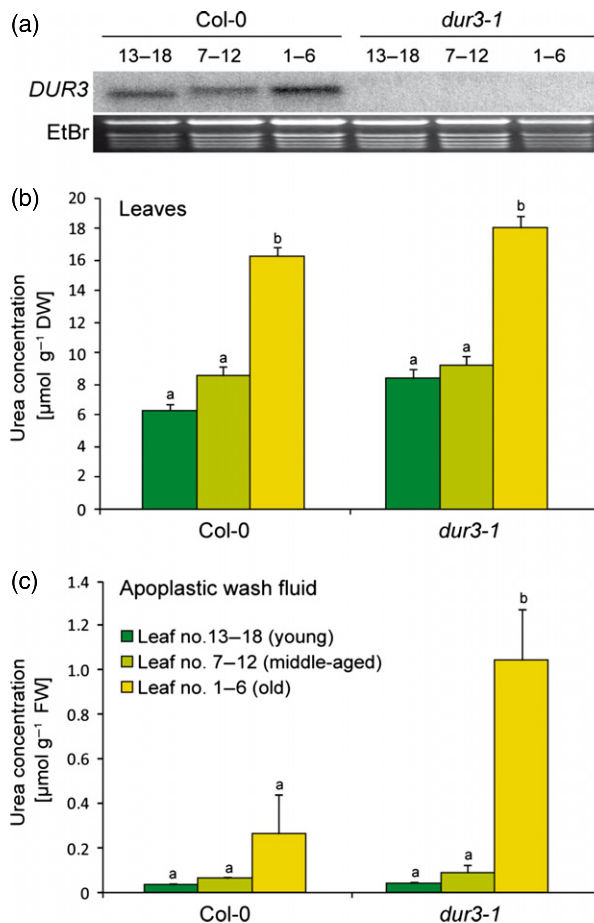

Figure 7. Urea concentrations in leaves and in the apoplastic wash fluid of wild-type and *dur3–1* plants.
(a) RNA gel blot analysis of *DUR3* in young (leaf nos 13–18), middle-aged (leaf nos 7–12) and old leaves (leaf nos 1–6) in Col–0 and *dur3–1*. Urea concentrations in intact leaves (b) and in the apoplastic wash fluid of the corresponding leaf fractions (c). Plants were grown hydroponically under short days for 5 weeks with 2 mm NH_4NO_3, transferred to long days for 1 week and continued to grow under nitrogen (N) deficiency for 4 days. Bars indicate means ± SEs, $n = 5$ (pools of 30–40 plants per replica). Different letters denote significant differences among means at $P < 0.05$, according to Tukey's test.

time in *dur3* mutants, relative to wild-type plants (Figure 3b), failed urea retrieval may favor apoplastic urea degradation by urease, which may have been released from disintegrating cells or may derive from phylloplane bacteria (Holland and Polacco, 1992). Urea levels in old leaves of *dur3* mutants did not drop below wild-type levels when plants were subjected to N deficiency, however (Figures 6 and 7). This may be because of replenishment of the leaf urea pool via the N-deficiency-induced increase in arginase activity, as observed in rice (Cao *et al.*, 2010).

As indicated by the localized activity of the *DUR3* promoter, the function of the corresponding protein in senescing leaves is mainly restricted to the vasculature and the surrounding bundle sheath cells (Figure 4). Interestingly, this restricted localization matches that of the ammonium-reassimilating enzymes cytosolic glutamine synthetase, NADH-dependent glutamate synthase and glutamate dehydrogenase, which are all preferentially expressed in the vasculature or even in phloem companion cells of senescing leaves (AbuQamar *et al.*, 2006; Masclaux-Daubresse *et al.*, 2010). This spatial coincidence further supports the view that DUR3 mediates urea retrieval directly upstream of the ammonium re-assimilation pathway that synthesizes the most suitable amino forms for phloem loading.

The contribution of urea and DUR3 to nitrogen retranslocation

As revealed by ^{15}N tracer studies, urea and ammonium nutrition yielded similarly labeled ^{15}N-amino acid signatures, indicating that both N forms share the same assimilatory pathway (Mérigout *et al.*, 2008), leading from ammonia via the GS/GOGAT pathway to glutamine and glutamate (Masclaux-Daubresse *et al.*, 2010). Indeed, the composition of amino acids in the petiole exudate was not significantly altered if urease activity was lacking, as in *ureG-2* plants (Table S2), and glutamine, glutamate, asparagine and serine remained among the most abundant amino acids, in accordance with previous studies (Corbesier *et al.*, 2001; Zhang *et al.*, 2010). Relative to the most abundant amino acid in the phloem sap, glutamine, urea concentrations accounted for 34% of glutamine-N, corresponding to >13% of urea-N relative to the total amino acid-N in the wild type. Urea export from senescent *ureG-2* leaves showed a sixfold increase so that urea-N accounted for as much as 56% of total amino acid-N, the export of which remained at a similar level (Figure 6c; Table 1). Thus, urea represents a phloem-mobile N form, and from a quantitative perspective, a considerable component in the cocktail of amino-N used for N retranslocation. With respect to its extremely narrow C : N ratio, urea may efficiently contribute to N retranslocation, especially when C availability in source leaves decreases, as is the case at later stages of leaf senescence (Figure 6b; Diaz *et al.*, 2005).

So far, the present view on urea was restricted to its function as a transient intermediate in N catabolism. On top of that, our study identified urea as a quantitatively relevant N intermediate that accumulates during leaf senescence and is used for long-distance transport in the phloem. In this context, DUR3 retrieves urea from the leaf apoplast and contributes to a more efficient export of amino-N from senescing leaves. In the absence of this retrieval function urea levels drop in the long run. As the mobilization and retranslocation of N from senescing leaves is a major factor determining the efficiency of N use in crop plants, the contribution of urea to N retranslocation

© 2014 The Authors
The Plant Journal published by Society for Experimental Biology and John Wiley & Sons Ltd.,

might deserve a closer inspection in future studies dealing with this important agronomic trait in crop plants.

EXPERIMENTAL PROCEDURES

Hydroponic plant culture

Arabidopsis thaliana lines Col-0, *dur3-1, dur3-3* (Kojima *et al.*, 2007) and *ureG-2* (Witte *et al.*, 2005) were used; *ureG-2* plants were crossed with *dur3-1* and *dur3 ureG* double mutant lines were selected by segregation and PCR. Dark-germinated seeds were grown on moistened rockwool for 1 week, before transfer to half-strength nutrient solution containing 1 mM $MgSO_4$, 1 mM KH_2PO_4, 250 μM K_2SO_4, 50 μM KCl, 250 μM $CaCl_2$, 100 μM Na-Fe-EDTA, 30 μM H_3BO_3, 5 μM $MnSO_4$, 1 μM $ZnSO_4$, 1 μM $CuSO_4$ and 1 μM $NaMoO_4$. KOH was used to adjust the pH to 5.8. N supply was in form of 2 mM NH_4NO_3. Nutrient solutions were renewed every week during the first 3 weeks and every 3 days thereafter. To induce senescence, plants were either transferred from short days (10 h light/14 h dark, light intensity 200–280 μmol m^{-2} sec^{-1}; 22°C/18°C, 60–70% humidity) to long days (16 h light/8 h dark) or subjected to N deficiency.

Promoter analysis

To prepare a *DUR3*-promoter:GUS construct, a genomic fragment of 1046 bp located upstream of the *DUR3* translation initiation site was amplified by PCR employing Pfu Turbo DNA polymerase (Stratagene, now Agilent Technologies, http://www.agilent.com). After verification of the DNA sequence, the primers DUR3pro5 (5′-AAAAGCTTAAGGTAAAGAAAGGATACTTGTA-3′) and DUR3pro3 (5′-AAACCATGGTTCCTCTTCTTCTTTACGTTTT-3′) were used to introduce the 5′ end of a *Hin*dIII restriction site and the 3′ end of a *Nco*I restriction site. The resulting *Hin*dIII–*Nco*I fragment of the *DUR3* promoter sequence was cloned into pBI101 (Clontech, http://www.clontech.com) and fused with GUS before the construct was used for *Agrobacterium*-mediated transformation. Shoots were incubated overnight at 37°C in GUS reaction buffer (1 mg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D-glucoronide in 100 mM sodium phosphate, pH 7). Stained shoots were cleared and analysed by light microscopy (Zeiss Axiovert, http://www.zeiss.com) or further embedded in SpurrR resin. Semi-thin sections of 5–8 μm were prepared on a Reichert-Jung Ultracut S (Leica, http://www.leica.com), stained with 2% basic fuchsine (10 sec at 80°C) to improve contrast and examined by light microscopy.

^{15}N influx studies

Urea influx was determined in 6-week-old plants cultured in nutrient solution under short-day conditions, either with continuous supply of N or under N deficiency for 4 days. Roots were washed in 1 mM $CaSO_4$ solution for 1 min, incubated for 10 min in nutrient solution containing 100 μM of ^{15}N-labeled urea (95% ^{15}N). After rinsing in 1 mM $CaSO_4$, roots were separated from shoots, freeze-dried and subjected to ^{15}N determination by isotope ratio mass spectrometry (NU Instruments, http://www.nu-ins.com).

RNA and protein gel blot analysis

After extraction by TRIzol (Invitrogen, http://www.lifetechnologies.com), 10–20 μg of total RNA was run on 3- (*N*-morpholino) propanesulfonic acid (MOPS)-formaldehyde agarose, transferred to Hybond-N+ nylon membranes (Amersham Biosciences, now GE Healthcare, http://www.gelifesciences.com) by blotting and the RNA was cross-linked at 80°C for 2 h. The coding sequence of *DUR3* was used as a ^{32}P-radiolabeled probe for hybridization at 42°C in 50% (v/v) formamide, 1% (w/v) sarcosyl, 5x SSC and 100 μg ml^{-1} yeast tRNA. Membranes were rinsed at 42°C for 40 min first in 2× SSC, 0.1% (w/v) SDS and then in 0.2× SSC, 0.1% (w/v) SDS.

Microsomal membrane fractions were isolated, separated and blotted as described by Loqué *et al.* (2006). In total, 5 μg of protein was run on SDS-PAGE. Antisera of DUR3 (Kojima *et al.*, 2007) and RbcL (Teige *et al.*, 1998) were diluted in blocking solution at 1 : 5000 and 1 : 2500, respectively, whereas the secondary antibody was diluted 1 : 100 000, and detection was carried out using an ECL kit (Amersham). Rainbow marker (Amersham) and Magic marker (Invitrogen) served to verify molecular weight.

Analysis of leaf exudates and apoplastic wash fluids

Leaf petiole exudates were collected in a closed system under controlled light and temperature to prevent transpiration (Corbesier *et al.*, 2001). Leaves were detached from stems close to their base, immersed in EDTA solution and cut a second time. Two or three rinsed leaves were transferred to an Eppendorf tube containing 10 mM EDTA, adjusted to pH 8.5, with petioles remaining immersed, and exudates were collected for 6 h. Amino acids and urea were determined as described in Carvalhais *et al.* (2011) and Kojima *et al.* (2007), respectively.

Apoplastic wash fluids were collected employing the infiltration-centrifugation method (Lohaus *et al.*, 2001). Plants were grown under short days on 2 mM NH_4NO_3 for 5 weeks and then transferred to long days for one additional week. Four days before harvest plants were subjected to N deficiency. Leaves of different ages from 30 to 40 wild-type or *dur3-1* plants were pooled, yielding ~4 g of fresh mass per fraction. Leaves were rinsed before infiltration in ice-cold double-distilled water, before a pressure of 80 hPa was applied five times for 2 min. Leaf tissues were wiped dry and centrifuged at 100 *g* for 20 min. The volume of the collected apoplastic wash fluid was determined by weighing. All experiments were repeated at least twice.

ACKNOWLEDGEMENTS

We thank Bernhard Bauer, Lisa Unrath, Barbara Kettig, Heike Nierig, Susanne Reiner and Twan Rutten (IPK Gatersleben) for excellent technical assistance, Anja Hartmann (IPK Gatersleben) for support in data analysis, and Claus-Peter Witte (Dahlem Center of Plant Sciences, FU Berlin) for providing *ureG-2* seeds. We thank the Deutsche Forschungsgemeinschaft, Bonn, Germany, for financial support in frame of the DFG Research Unit FOR948 (WI1728/14).

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Table S1. Correlation between urea concentrations in leaves and the gene expression levels of *SAG12*, *ORE1* and *DUR3*, or the concentrations of amino acids in leaf samples of different plants and leaf age.

Table S2. Amino acid concentrations in leaf petiole exudates from N-deficient *dur3-1* and *ureGA* single and double mutants.

REFERENCES

AbuQamar, S., Chen, X., Dhawan, R., Bluhm, B., Salmeron, J., Lam, S., Dietrich, R.A. and Mengiste, T. (2006) Expression profiling and mutant analysis reveals complex regulatory networks involved in Arabidopsis response to Botrytis infection. *Plant J.* **48**, 28–44.

© 2014 The Authors

Cao, F.Q., Werner, A.K., Dahncke, K., Romeis, T., Liu, L.H. and Witte, C.P. (2010) Identification and characterization of proteins involved in rice urea and arginine catabolism. *Plant Physiol.* **154**, 98–108.

Carvalhais, L.C., Dennis, P.G., Fedoseyenko, D., Hajirezaei, M.R., Borriss, R., von Wirén, N. (2011) Root exudation of sugars, amino and organic acids by maize as affected by nitrogen, phosphorus, potassium and iron deficiency. *J. Plant Nutr. Soil Sci.* **174**, 3–11.

Corbesier, L., Havelange, A., Lejeune, P., Bernier, G. and Périlleux, C. (2001) N content of phloem and xylem exudates during the transition to flowering in *Sinapis alba* and *Arabidopsis thaliana*. *Plant Cell Environ.* **24**, 367–375.

Diaz, C., Purdy, S., Christ, A., Morot-Gaudry, J.F., Wingler, A. and Masclaux-Daubresse, C. (2005) Characterization of markers to determine the extent and variability of leaf senescence in Arabidopsis. A metabolic profiling approach. *Plant Physiol.* **138**, 898–908.

Eckert, M., Biela, A., Siefritz, F. and Kaldenhoff, R. (1999) New aspects of plant aquaporin regulation and specificity. *J. Exp. Bot.* **50**, 1541–1545.

Faye, L., Greenwood, J.S. and Chrispeels, M.J. (1986) Urease in jackbean (*Canavalia ensiformis* L.) seeds is a cytosolic protein. *Planta*, **168**, 579–585.

Gerbeau, P., Guclu, J., Ripoche, P. and Maurel, C. (1999) Aquaporin Nt-TIPa can account for the high permeability of tobacco cell vacuolar membrane to small neutral solutes. *Plant J.* **18**, 577–587.

Goldraij, A. and Polacco, J.C. (1999) Arginase is inoperative in developing soybean embryos. *Plant Physiol.* **119**, 297–304.

Holland, M.A. and Polacco, J.C. (1992) Urease-null and hydrogenase-null phenotypes of a phylloplane bacterium reveal altered nickel metabolism in two soybean mutants. *Plant Physiol.* **98**, 942–948.

Kim, J.H., Woo, H.R., Lim, P.O., Lee, I.C., Choi, S.H., Hwang, D. and Nam, H.G. (2009) Trifurcate feed-forward regulation of age-dependent cell death involving miR164 in Arabidopsis. *Science*, **323**, 1053–1057.

Kojima, S., Bohner, A. and von Wirén, N. (2006) Molecular mechanisms of urea transport in plants. *J. Membr. Biol.* **212**, 83–91.

Kojima, S., Bohner, A., Gassert, B., Yuan, L. and von Wirén, N. (2007) AtDUR3 represents the major transporter for high-affinity urea transport across the plasma membrane of nitrogen-deficient Arabidopsis roots. *Plant J.* **52**, 30–40.

Kokubun, N., Ishida, H., Makino, A. and Mae, T. (2002) The degradation of the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase into the 44-kDa fragment in the lysates of chloroplasts incubated in darkness. *Plant Cell Physiol.* **43**, 1390–1395.

Lin, J.-F. and Wu, S.-H. (2004) Molecular events in senescing Arabidopsis leaves. *Plant J.* **39**, 612–628.

Liu, L.-H., Ludewig, U., Frommer, W.B. and von Wirén, N. (2003a) AtDUR3 encodes a new type of high-affinity urea/H^+ symporter in Arabidopsis. *Plant Cell*, **15**, 790–800.

Liu, L.H., Ludewig, U., Gassert, B., Frommer, W.B. and von Wirén, N. (2003b) Urea transport by nitrogen-regulated tonoplast intrinsic proteins in Arabidopsis. *Plant Physiol.* **133**, 1220–1228.

Lohaus, G., Pennewiss, K., Sattelmacher, B., Hussmann, M. and Muehling, K.H. (2001) Is the infiltration-centrifugation technique appropriate for the isolation of apoplastic fluid? A critical evaluation with different plant species. *Physiol. Plant.* **111**, 457–465.

Loqué, D., Yuan, L., Kojima, S., Gojon, A., Wirth, J., Gazzarrini, S., Ishiyama, K., Takahashi, H. and von Wirén, N. (2006) Additive contribution of AMT1;1 and AMT1;3 to high-affinity ammonium uptake across the plasma membrane of nitrogen-deficient Arabidopsis roots. *Plant J.* **48**, 522–534.

Masclaux-Daubresse, C., Reisdorf-Cren, M., Pageau, K., Lelandais, M., Grandjean, O., Kronenberger, J., Valadier, M.H., Feraud, M., Jouglet, T. and Suzuki, A. (2006) Glutamine synthetase-glutamate synthase pathway and glutamate dehydrogenase play distinct roles in the sink-source nitrogen cycle in tobacco. *Plant Physiol.* **140**, 444–456.

Masclaux-Daubresse, C., Daniel-Vedèle, F., Dechorgnat, J., Chardon, F., Gaufichon, L. and Suzuki, A. (2010) Nitrogen uptake, assimilation and remobilization in plants: challenges for sustainable and productive agriculture. *Ann. Bot.* **105**, 1141–1157.

Mérigout, P., Lelandais, M., Bitton, F., Renou, J.P., Briand, X., Meyer, C. and Daniel-Vedèle, F. (2008) Physiological and transcriptomic aspects of urea uptake and assimilation in Arabidopsis plants. *Plant Physiol.* **147**, 1225–1238.

Nielsen, K.H. and Schjoerring, J.K. (1998) Regulation of apoplastic NH_4^+ concentration in leaves of oilseed rape. *Plant Physiol.* **118**, 1361–1368.

Pearson, J.N., Finnemann, J. and Schjoerring, J.K. (2002) Regulation of the high-affinity ammonium transporter (BnAMT1;2) in the leaves of *Brassica napus* by nitrogen status. *Plant Mol. Biol.* **49**, 483–490.

Soto, G., Fox, R., Ayub, N., Alleva, K., Guaimas, F., Erijman, E.J., Mazzella, A., Amodeo, G. and Muschietti, J. (2010) TIP5;1 is an aquaporin specifically targeted to pollen mitochondria and is probably involved in nitrogen remobilization in *Arabidopsis thaliana*. *Plant J.* **64**, 1038–1047.

Teige, M., Melzer, M. and Suss, K.H. (1998) Purification, properties and *in situ* localization of the amphibolic enzymes D-ribulose 5-phosphate 3-epimerase and transketolase from spinach chloroplasts. *Eur. J. Biochem.* **252**, 237–244.

Wang, W.-H., Köhler, B., Cao, F.Q. *et al.* (2012) Rice DUR3 mediates high-affinity urea transport and plays an effective role in improvement of urea acquisition and utilization when expressed in Arabidopsis. *New Phytol.* **193**, 432–444.

Winter, D., Vinegar, B., Nahal, H., Ammar, R., Wilson, G.V. and Provart, N.J. (2007) An "electronic Fluorescent Pictograph" browser for exploring and analyzing large-scale biological data sets. *PLoS ONE*, **2**, e718.

Witte, C.P. (2011) Urea metabolism in plants. *Plant Sci.* **180**, 431–438.

Witte, C.P., Rosso, M.G. and Romeis, T. (2005) Identification of three urease accessory proteins that are required for urease activation in Arabidopsis. *Plant Physiol.* **139**, 1155–1162.

Zhang, L., Tan, Q., Lee, R., Trethewy, A., Lee, Y.H. and Tegeder, M. (2010) Altered xylem-phloem transfer of amino acids affects metabolism and leads to increased seed yield and oil content in Arabidopsis. *Plant Cell*, **22**, 3603–3620.

Zonia, L.E., Stebbins, N.E. and Polacco, J.C. (1995) Essential role of urease in germination of nitrogen-limited *Arabidopsis thaliana* seeds. *Plant Physiol.* **107**, 1097–1103.

© 2014 The Authors
The Plant Journal published by Society for Experimental Biology and John Wiley & Sons Ltd.,