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

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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 CO₂ by the cytosolic, nickel-containing enzyme urease (Faye et al., 1986). Support for the essential role of urea 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,

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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 it 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 mutant 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...
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 senescence.

**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; 19, 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.

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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::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.](Image)

(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 NH4NO3 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.
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., 2002), 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

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incubated in 100 l
ferred for 4 days to N
for 6 weeks in nutrient solution containing 2 mM NH4NO3 (free solution) 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 NH4NO3 (+N) or transferred for 4 days to N-free solution (−N) before the influx study. Roots were incubated in 100 μM 15N-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.

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 15N-labeled urea in roots of Col-0, dur3-1, ureG-2 and dur3 ureG. Plants were pre-cultured 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.
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 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-OGAT 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...
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 NH4NO3 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.

### 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<sup>ab</sup>           | 1.63 ± 0.11<sup>b</sup>      | 0.08 ± 0.19<sup>ab</sup>| 2.2              |
|       | Middle-aged | 13.35 ± 1.91<sup>a</sup>         | 4.99 ± 0.53<sup>ab</sup>     | 0.23 ± 0.07<sup>b</sup>| 2.2              |
|       | Old      | 13.10 ± 3.25<sup>a</sup>          | 3.62 ± 1.16<sup>ab</sup>     | 1.25 ± 1.33<sup>b</sup>| 13.6             |
| dur3-1| Young    | 3.84 ± 0.93<sup>a</sup>           | 1.02 ± 0.24<sup>b</sup>      | 0.06 ± 0.08<sup>b</sup>| 2.1              |
|       | Middle-aged | 15.74 ± 1.38<sup>ab</sup>        | 5.82 ± 0.32<sup>ab</sup>     | 0.26 ± 0.32<sup>b</sup>| 2.1              |
|       | Old      | 14.69 ± 3.64<sup>ab</sup>         | 4.20 ± 1.16<sup>ab</sup>     | 0.75 ± 0.03<sup>b</sup>| 7.2              |
| ureG-2| Young    | 5.70 ± 0.97<sup>ac</sup>           | 2.07 ± 0.34<sup>ab</sup>     | 0.09 ± 0.02<sup>b</sup>| 2.1              |
|       | Middle-aged | 17.38 ± 4.96<sup>ab</sup>        | 4.53 ± 1.34<sup>ab</sup>     | 0.47 ± 0.03<sup>b</sup>| 3.7              |
|       | Old      | 19.09 ± 5.88<sup>ef</sup>         | 5.53 ± 1.70<sup>ab</sup>     | 7.62 ± 0.06<sup>a</sup>| 66.4             |
| dur3 ureG | Young   | 4.51 ± 0.48<sup>ab</sup>           | 1.37 ± 0.12<sup>b</sup>      | 0.07 ± 0.04<sup>ab</sup>| 2.2              |
|       | Middle-aged | 18.81 ± 4.32<sup>ef</sup>        | 6.95 ± 1.43<sup>ef</sup>     | 0.29 ± 0.00<sup>ab</sup>| 2.0              |
|       | Old      | 8.19 ± 2.43<sup>bd</sup>           | 1.90 ± 0.66<sup>bc</sup>     | 3.35 ± 0.00<sup>b</sup>| 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 NH4NO3 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.
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 dissintegrating 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 15N tracer studies, urea and ammonium nutrition yielded similarly labeled 15N-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; Díaz 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

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**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 [15N]NO3, 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.
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 MgSO4, 1 mM KH2PO4, 250 μM K2SO4, 50 μM KCl, 250 μM CaCl2, 100 μM Na2-Fe-EDTA, 30 μM H3BO3, 5 μM MnSO4, 1 μM ZnSO4, 1 μM CuSO4 and 1 μM NaMoO4. KOH was used to adjust the pH to 5.8. N supply was in form of 2 mM NH4NO3. 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) 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'-AAAAGCTTAGGTAAAGAAAGGATACTTGTA-3') and DUR3pro3 (5'-AAACCATGGTTCCTCTTCTTCTTTACGTTTT-3') were used to introduce the 5' end of a HindIII restriction site and the 3' end of a Ncol restriction site. The resulting HindIII-Ncol fragment of the DUR3 promoter sequence was cloned into p Biał101 (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-glucuronic acid in 100 mM sodium phosphate, pH 7). Stained shoots were cleared and analysed by light microscopy (Zeiss Axiosvert, http://www.zeiss.com) or further embedded in SpurrR resin. Semi-thin sections of 5 μm were prepared on a Reichert-Jung Ultracut S (Leica, http://www.zeiss.com) to long days (16 h light/8 h dark) or subjected to N deficiency.

**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 NH4NO3 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.

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**SUPPLEMENTARY 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 ureG single and double mutants.

**REFERENCES**

AbuQamar, S., Chen, X., Dhawan, R., Bluhm, B., Salmeron, J., Lam, S., Diehrich, R.A. and Mengiste, T. (2006) Expression profiling and mutant analysis reveals complex regulatory networks involved in Arabidopsis response to Botrytis infection. *Plant J.* 40, 28–44.
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., Borris, R., von Wieren, 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 Perilleux, C. (2001) N content of phloem and xylem exudates during the transition to flowering in Sinapis alba and Arabidopsis thaliana. Plant Cell Environ. 24, 367–378.

Diaz, C., Purdy, S., Christ, A., Morot-Gaudry, J.F., Wingler, A. and Masciaux-Daubresse, C. (2005) Characterization of markers to determine the extent and variability of leaf senescence in Arabidopsis. A metabolite 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 Choirsheels, M.J. (1988) 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-TIP1 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. 96, 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 Wieren, 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 Wieren, 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 Wieren, N. (2003a) A dur3 encodes a new type of high-affinity urea/H+ symporter in Arabidopsis. Plant Cell, 15, 789–800.

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

Lohaus, G., Pennewis, 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.

Loque, D., Yuan, L., Kojima, S., Gojon, A., Wirth, J., Gazzarrini, S., Ishiyama, K., Takahashi, H. and von Wieren, 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., Kronenberg, J., Valadier, M.H., Feraud, M., Jougot, T. and Suzuki, A. (2008) 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-Vedele, F., Dechorgnat, J., Chardon, F., Gaufichon, L. and Suzuki, A. (2010) Nitrogen uptake, assimilation and remobilization in plants: challenges for sustainable and productive agriculture. Annu. Bot. 105, 1141–1157.

Mergiout, P., Lelandais, M., Bitton, F., Renou, J.P., Briand, X., Meyer, C. and Daniel-Vedele, 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 NH4+ 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., Erjiman, E.J., Mazzella, A., Amodeo, G. and Muschietti, J. (2010) TIPS-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 amphioblic 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 Provant, 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 urea in germination of nitrogen-limited Arabidopsis thaliana seeds. Plant Physiol. 107, 1097–1103.

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