Introduction

Nitrogen (N) is a crucial plant nutrient; to encourage large yields, farmers tend to apply excess nitrogen fertilizers to their crops. However, crop plants are generally inefficient at nitrogen uptake from the soil, with as much as 50 to 75% of applied N being unused by the plants (Pilbeam, 1996; Hodge *et al*., 2000). Crop plants compete for soil N against soil microbes involved with denitrification and nitrification, volatization to the atmosphere, as well as loss of N by leaching into waterways (Galloway *et al*., 2008). It is important to breed and/or design nitrogen use efficient (NUE) crop plants that can produce the same, or higher yields with less applied N fertilizer. Growth of NUE crop plants, coupled with implementation of best fertilizer management practices, would allow for a reduction of applied N fertilizer per hectare. This would both greatly reduce the N fertilizer expense for the farmer and greatly reduce the environmental pollution from excess N fertilizers.

We have recently developed genetically engineered rice (*Oryza sativa* L.) by introducing a barley alanine aminotransferase *(AlaAT)* cDNA driven by a rice tissue specific promoter, *OsAnt1* (Shrawat *et. al*., 2008). This modification significantly increased biomass and grain yield in the transgenic plants compared to control plants when the plants were grown at a fixed, high amount of ammonium as the N source. As well, we analysed the transcriptomic profile of these transgenic plants grown at the fixed N concentration using Affymetrix Rice GeneChip microarrays to provide further insights into the nature of increased NUE of these transgenics (Beatty *et al*., 2009). In this study, we compared various physiological and genetic data from alanine aminotransferase over-expressing transgenic plants to control plants grown at three different nitrogen levels and demonstrated significant changes between them.

Materials and Methods

T_2 seed from homozygous transgenic rice lines containing the *OsAnt1/AlaAT* construct were grown for 44 days in hydroponic buckets containing a nutrient solution with 0.5 mM, 2 mM or 5 mM ammonium (Shrawat *et. al*., 2008). At 44 days, the rice plants were divided into root and shoot tissues. Samples of roots and shoot were measured and frozen immediately in liquid nitrogen for RNA analysis. The frozen tissues were ground and the RNA extracted using a Qiagen plant RNA extraction kit. Quantitative reverse transcriptase PCR (qRT-PCR) was conducted as described previously (Beatty *et al*., 2009). Root and shoot tissues were dried at 50°C and weighed as described previously (Good *et. al*., 2007). Five grams of each dried shoot and root sample was analysed in a LECO combustion furnace (LECO Co., St. Joseph, MI, USA) for total nitrogen content (Bodycote Testing; Lethbridge, AB, Canada).

Results

Table 1 shows the effect of different nitrogen concentrations on the level of the transgene transcript *HvAlaAT* plus ten other transcripts, in roots and shoots, compared to control plants. 18S rRNA was the endogenous control. Some of these transcripts were selected for analysis based on upregulation of these genes as seen by a microarray analysis of the transgenic lines in a previous study (Beatty *et al*., 2009). The transcripts investigated in this study were; endogenous rice AlaAT (*AlaAT2*), ammonium transporter (*OsAMT1*), glutamine synthetase (*OsGln1;2*), and six other genes that showed 2 fold or more upregulation in the microarray analysis; glycine rich protein (*GRP*) gene, hypothetical protein (*Os8823)*, leucine rich repeat (*LRR*) gene, *OsWAK101*, *OsSAUR39*, *gatA* and an aldehyde dehydrogenase (*OsANT1*).

The transgene *HvAlaAT* was highly upregulated at 0.5mM, 2mM and 5mM NH_4^+ for both lines in roots and shoots. The *GRP* transcript was highly upregulated in the roots of both

transgenic lines at 0.5mM, 2mM and 5mM NH_4^+, but only moderately upregulated in the shoots of AGR-3/8 alone. The hypothetical protein *Os8823* was also highly upregulated in the roots of both lines at 0.5mM, 2mM and 5mM NH_4^+, but only moderately upregulated in the shoots of AGR-1/7. The leucine rich repeat transcript was moderately upregulated in the roots of both lines at both low and high NH_4^+ but not at 2 mM. *OsWAK101* showed moderate upregulation in the roots of AGR-1/7 at both low and medium NH_4^+ but not at high NH_4^+ concentration.

Table 1. The relative quantification (RQ) of 10 selected transcripts in roots and shoots of two transgenic lines, AGR-1/7 and AGR-3/8, at three different ammonium concentrations. Green shading indicates highly expressed transcripts, yellow shading indicates moderately expressed transcripts, no shading indicates no significant increase in expression, all compared to control plants.

Transcript	Line	RQ 0.5mM NH_4^+		RQ 2mM NH_4^+		RQ 5 mM NH_4^+	
		Roots	Shoots	Roots	Shoots	Roots	Shoots
HvAlaAT	AGR-1/7	57.5	19.2	46.5	28.5	55.8	22.4
	AGR-3/8	34.1	23.0	40.7	30.3	29.0	46.7
OsWAK101	AGR-1/7	2.4	0.4	2.9	0.9	1.7	1.2
	AGR-3/8	1.1	0.3	0.6	0.8	1.3	0.9
LRR	AGR-1/7	5.8	1.6	1.7	1.8	6.9	1.2
	AGR-3/8	4.3	1.1	1.8	2.3	3.6	1.8
Os8823	AGR-1/7	60.0	4.5	54.7	5.8	56.0	4.2
	AGR-3/8	23.0	1.1	28.0	1.6	1.9	0.7
OsAMT1	AGR-1/7	1.0	1.1	1.4	3.1	0.7	0.7
	AGR-3/8	1.6	0.4	1.4	0.8	0.7	1.8
OsGln1;2	AGR-1/7	0.5	0.6	1.3	1.8	0.4	1.0
	AGR-3/8	6.4	0.8	2.1	1.1	0.6	2.2
GRP	AGR-1/7	89.7	0.8	132.7	2.2	91.0	0.5
	AGR-3/8	176.2	2.9	106.3	3.0	118.9	3.6
AlaAT2	AGR-1/7	1.8	0.6	0.8	1.5	0.9	0.8
	AGR-3/8	2.2	0.9	1.4	2.2	0.6	2.4
OsANT1	AGR-1/7	0.9	0.6	1.2	0.9	1.1	0.9
	AGR-3/8	1.7	0.5	1.4	1.8	0.4	1.3
GatA	AGR-1/7	1.5	0.7	0.4	0.7	1.5	1.2
	AGR-3/8	0.6	0.7	0.6	1.0	0.8	2.5
OsSAUR39	AGR-1/7	1.7	0.6	0.5	1.0	1.3	0.9
	AGR-3/8	3.3	0.6	0.9	0.7	1.7	0.9

Table 2 shows the level of total biomass and percentage nitrogen in transgenic and control plants. The difference in total nitrogen or biomass was on average between 1.2 and 1.26 times that of the control plants. This indicates that the over-expression of *AlaAT* in the transgenic rice plants was related to the level of available nitrogen.

Table 2. Biomass and percent total nitrogen in shoot and root of control and transgenic plants grown in nutrient solution containing 0.5 mM, 2 mM or 5 mM ammonium. Dried shoot and root samples collected from 44-day-old plants were used to measure biomass and total nitrogen analysis.

Line	$[NH_4^+]$	Measure	Shoot Avg	Root Avg	Total Avg
AGR-1/7	0.5mM	Biomass (g)	6.36 ± 0.21	3.85 ± 0.10	10.21 ± 0.24
		Nitrogen (%)	2.21 ± 0.00	1.25 ± 0.00	1.85 ± 0.00

	2mM	Biomass (g)	14.05 ± 1.54	4.03 ± 0.50	18.80 ± 2.34
		Nitrogen (%)	3.43 ± 0.00	1.55 ± 0.00	2.96 ± 0.00
	5mM	Biomass (g)	16.47 ± 0.57	4.67 ± 0.18	21.14 ± 0.70
		Nitrogen (%)	4.29 ± 0.00	2.62 ± 0.00	3.92 ± 0.00
AGR-3/8	0.5mM	Biomass (g)	6.87 ± 0.23	4.02 ± 0.08	10.89 ± 0.24
		Nitrogen (%)	2.27 ± 0.00	1.36 ± 0.00	1.94 ± 0.00
	2mM	Biomass (g)	16.88 ± 0.58	7.06 ± 0.40	23.94 ± 0.96
		Nitrogen (%)	3.23 ± 0.00	1.63 ± 0.00	2.76 ± 0.00
	5mM	Biomass (g)	17.07 ± 0.59	4.93 ± 0.15	22.00 ± 0.71
		Nitrogen (%)	4.49 ± 0.00	2.12 ± 0.00	3.96 ± 0.00
Nipponbare	0.5mM	Biomass (g)	5.20 ± 0.30	3.10 ± 0.33	8.30 ± 0.63
		Nitrogen (%)	2.32 ± 0.00	1.24 ± 0.00	1.92 ± 0.00
	2mM	Biomass (g)	10.35 ± 0.57	3.65 ± 0.44	14.00 ± 0.99
		Nitrogen (%)	3.47 ± 0.00	1.73 ± 0.00	3.02 ± 0.00
	5mM	Biomass (g)	13.62 ± 0.98	4.25 ± 0.36	17.86 ± 1.30
		Nitrogen (%)	3.57 ± 0.00	2.32 ± 0.00	3.28 ± 0.00

We have previously shown that over-expression of *AlaAT* in canola can have significant effects on seed yield in the field under low nitrogen conditions (Good *et. al.*, 2007) and on biomass and seed yield in rice under controlled environmental conditions (Shrawat *et. al.*, 2008). In this study, we have shown the effect of different nitrogen concentrations on transgenic *AlaAT* transcript expression, biomass and nitrogen accumulation in trangenic and control rice plants.

Conclusions

Nitrogen is both a major agricultural expense and a major environmental pollutant. Improving NUE by increasing the recovery efficiency of the crop plants coupled with using best management practices in N fertilizer application could reduce both the cost of nitrogen fertilizers to the farmer and damage to the environment from N pollution. Our studies demonstrate that the manipulation of *AlaAT* can be affected by the level of available N. In addition, certain transcripts in the transgenic lines can be affected by N levels as well. We are now interested in understanding the functional relationship between the level of over-expressed *AlaAT*, highly upregulated transcripts such as *GRP* and *Os8823* and alanine.We are specifically interested in the role of alanine as an important amino acid for nitrogen uptake and signaling. We are also exploring different engineering approaches to develop NUE plants, including examining novel genes and the selection of tissue specific promoters to drive the expression of genes of interest to produce transgenic plants displaying reliable field-based improvements in NUE. We also plan to test these novel genes and tissue promoters in other crop plants such as barley, wheat and corn.

References

1. Pilbeam, C.J. (1996) Effect of climate on recovery of crop and soil of N-15 labelled fertilizer applied to wheat. *Fert. Res.* **45**: 209-215.
2. Hodge, A., Robinson, D., and Fitter, A. (2000) Are micro-organisms more effective than plants at competing for nitrogen? *Trends Plant Sci.* **5**:304—308.
3. Galloway, J.N., Townsend, A.R., Erisman, J.W., Bekunda, M., Cai, Z., Freney, J.R., Martinelli, L.A., Seitzinger, S.P. and Sutton M.A. (2008) Transformation of the Nitrogen Cycle:Recent Trends, Questions, and Potential Solutions. *Science* **320**, 889-892.
4. Shrawat, A.K., Carroll, R.T., DePauw, M., Taylor, G.J., and Good, A.G. (2008) Genetic

engineering of improved nitrogen use efficiency in rice by the tissue-specific expression of *alanine aminotransferase. Plant Biotechnology Journal*. **6**, 722-32.
5. Beatty, P.B., Shrawat, A., Carroll, R.T., Zhu, T., and Good, A.G. 2009. Transcriptome analysis of nitrogen efficient rice overexpressing alanine aminotransferase. *Plant Biotechnology Journal*, forthcoming.
6. Good, A.G., Johnson, S.J., DePauw, M.D., Carroll, R.T., Savidov, N., Vidamir, J., Lu, Z., Taylor, G. and Stroeher, V. (2007) Engineering nitrogen use efficiency with alanine aminotransferase. *Can. J. Bot*. **85**, 52–262.