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Engineering nitrogen use efficiency in *Oryza sativa* by the developmental over-expression of barley alanine aminotransferase using a novel rice promoter

by

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Abstract

Nitrogen is the main limiting component to plant growth and development. With increasing cost of food production the development of nitrogen use efficient (NUE) crop plants has become of interest. In our group, the over-expression of barley alanine aminotransferase (*HvAlaAT*) cDNA by the *btg26* and *OsANT1* promoters has yielded NUE plants. Through a detailed bioinformatic analysis, the *PBpr1* promoter was selected, cloned, and coupled to *HvAlaAT* for over-expression in rice (*Oryza sativa* cv. Nipponbare). Overall, rice lines carrying *PBpr1::HvAlaAT* resulted in higher seed yield, biomass and tillering compared to non-transformed Nipponbare. Molecular analysis of these lines showed high levels of *HvAlaAT* mRNA and alanine aminotransferase (AlaAT) protein. Transgenic shoots exhibits high levels of AlaAT activity at all times while root activity increased only after active tillering, indicating developmental regulation of the gene in roots. Analysis of *PBpr1* pattern expression, however, showed no definitive staining despite the observed over-expression of *HvAlaAT* at the transcript and enzyme activity level.

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List of Abbreviations

ABA……. Absicisic acid

ABRE…….. Absicisic acid responsive element

ADP……. Adenosine Diphosphate

AlaAT…….. Alanine aminotransferase

ALDH…….. Aldehyde dehydrogenase

Ap ……Aldehyde dehydrogenase promoter

ApG……. Aldehyde dehydrogenase promoter GUS

APS…….. Ammonium persulphate

ATP……..Adenosine Triphosphate

ATG……. Translation start site

mA……... Miliampere

BNF……..Biological nitrogen fixation

bp……. Base pair

BLASTn……...Basic local alignment search tool: nucleotide

BSA……. Bovine serum albumin

^{o}C……. Degrees celcius

CoA……..Coenzyme A

CAMV……. Cauliflower mosaic virus

CsVMV……Cassava vein mosaic virus

cDNA……...Complementary DNA

c.v……Cultivar

DAG……Days after germination

ddCT…… Delta delta cycle threshold

DNA…….Deoxyribonucleic acid

DTT……..Dithiothreitol

EDTA……...Ethylenediaminetetraacetic acid

ELISA…….. Enzyme-Linked Immunosorbent Assay

EU…… European Union

µE…….Photon flux density unit

GA……Gibberellic acid

g; mg; µg……..Gram; milligram; microgram

GFP…….. Green fluorescent protein

GS…… Glutamine synthetase

GUS……. β-glucuronidase

Gw……Grain weight

hr, hrs……... hour; hours

HPLC……... High performance liquid chromatography

IME…….. Intron mediated enhancements

kDa…… KiloDaltons

L; mL; µL…….Litre; millilitres microlitre

MeJA……Methyl jasmonic acid

M; mM; µM …… Molar; milimolar; micromolar

m; cm; µm; nm…….Metre; centimetre; micrometer; nanometre

min……... Minutes

MMSDH…….. Methylmalonate-semialdehyde dehydrogenase

MMT……Million metric tonnes

mRNA……..messenger RNA

MS……Murashige and Skoog

N……...Nitrogen

NADH……..Nicotinamide adenine dinucleotide

NB……Nipponbare

NCBI……National centre for biotechnology information

N/ha……..Nitrogen per hectar

NUE……. Nitrogen use efficiency

NUpE……... Nitrogen uptake efficiency

NUtE…… Nitrogen utilization efficiency

Ns……. Nitrogen supplied

Nt……. Total plant nitrogen

OD……Optical density

P5P……... Pyridoxal-5-phosphate

PAGE……... Polyacrylamide gel electrophoresis

PCR……..Polymerase chain reaction

PLACE…….Plant cis-acting regulatory DNA elements

PlantCARE……...Plant cis acting regulatory elements

PMSF……... Phenylmethanesulphonylfluoride

PPDB……... Plant promoter database

PVPP……Polyvinylpolypyrrolidone

QTL……..Quantitative trait loci

RACE……...Rapid amplification of cDNA ends

RNA……. Ribonucleic acid

rpm…….. Revolutions per minute

qRT-PCR……. Quantitative reverse transcriptase-PCR

SD…… Standard deviation

SDS…….. Sodium dodecylsulphate

Sw…… Shoot weight

TCA……. Tricarboxylic acid

TSS……...Transcription start site

Tris……... Tris (hydroxmethyl) aminomethane

UTR……. Untranslated region

v/v; v/w; w/v…… Volume per volume; volume per weight; weight volume

V……...Volts

Chapter 1

1. Introduction:

1.1. General Introduction

Nitrogen (N) is an essential component to plant growth and development. Currently 103.2 million metric tonnes (MMT) of N fertilizer is applied to agricultural systems globally to ensure maximum crop production in order to meet growing food demands (Good and Beatty, 2011a). Cereal crops only take up 25 to 50% of the applied N and the remainder is lost, causing severe environmental impacts. Efficient N use would be beneficial to crop production because it reduces producer operating costs and environmental pollution, resulting from excess fertilizer application. Previous studies have focused on over-expression of genes involved in primary N metabolism, in hopes of increasing the N use efficiency (NUE) of crop plants, but have met with limited success. These studies generally used constitutive promoters to drive transgene over-expression which may be energetically unfavourable to the plants' growth and development (Yaish, 2010; Bhatnagar-Mathur *et al.,* 2008). In order to overcome this obstacle, an inducible or tissue specific promoter could allow for the controlled transgene over-expression at the desired organ and developmental stage and so provide for the most efficient production of the desired enzyme, thus increasing the efficacy of metabolic processes and potentially improving the NUE of the plant. Our lab has previously developed NUE plants by over-expressing a barley (*Hordeum vulgare*) alanine aminotransferase (*HvAlaAT*) cDNA using a drought inducible promoter

btg26 in canola (*Brassica napus*), which resulted in increased biomass and seed yield. This phenotype was not observed when the constitutive *CAMV35S* promoter was used (Good *et al.,* 2007) or when *btg26::HvAlaAT* was transformed into rice. Similar results were observed when the root specific promoter of the rice homologue of *btg26*, *OsANT1*, was used to drive *HvAlaAT* in rice (Shrawat *et al.,* 2008, Beatty *et al.,* 2009). These rice plants had increased N uptake efficiency compared to the wild-type plant, Nipponbare (NB). In the research described in this thesis, I examined the *PBpr1* promoter, which is the promoter from a gene that shared high sequence similarity with the *OsANT1* gene in rice, to determine if the over-expression of *HvAlaAT* using the *PBpr1* promoter will produce rice plants with an NUE phenotype. I also characterized the *PBpr1* promoter to determine its tissue specificity, expression patterns and its inducibility, using GUS staining and bioinformatics, comparing it to the *OsANT1* promoter. The *PBpr1* gene is a methyl melonate semialdehyde dehydrogenase gene that has been discovered in previous studies to be highly expressed in plants in the leaf blade and leaf sheath at the seedling stage and also in young roots and stem (Oguchi *et al.,* 2004).

1.2. Importance of N

Nitrogen (N) is critical for plant growth and development because it is an essential component of a variety of organic compounds involved in basic cellular functions, such as amino acids and nucleotides. As a result, all crop plants have a fundamental reliance on N and both biomass and grain yield are severely affected under the conditions of limiting N. In most cases, the supply of naturally

occurring N does not meet the demands of agricultural systems, therefore, application of synthetic fixed N is required.

The global human population was approximately 2.5 billion in 1950, 6 billion in 2000 and is projected to be an average of 8.9 billion by 2050 (United Nations Department of Economic and Social Affairs, 2004). With the population doubling every three to four decades, there are increasing concerns of whether there will be sufficient food, given that there is little arable land that is undeveloped and the significant input cost of items which use and include fossil fuels. In the 1960s, the green revolution brought about an increase of food production by introducing high-yielding, semi-dwarf varieties of wheat and rice which had improved N responsiveness, a higher harvest index and were early maturing, photoperiod insensitive plants (Khush, 1999; Khush 2001). These semi-dwarf varieties had reduced height and therefore could utilize N fertilizers and partitioned resources to maximize grain production. Their short stature prevented lodging and the associated yield losses (Sakamoto and Matsuoka. 2004). However, with the introduction of these varieties, large amounts of chemical inputs such as N fertilizers were required to maintain crop yields (Socolow., 1999; Tilman *et al.,* 2002). In the 1960s, the use of N fertilizer was at approximately ten million metric tonnes (MMT) (Bumb and Baanante, 1996). Currently N fertilizer consumption has increased to 103.2 MMT and is projected to be 151.6 MMT by 2050 (FAO STAT, 2010).

The production of synthetic N fertilizers is mainly carried out using the Haber-Bosch process where large amounts of hydrogen and energy are required

and are usually supplied by natural gas. This process was economically viable before 1995 due to low petroleum prices. The green revolution coupled with the Haber-Bosch process increased the use of N fertilizers 6.9 fold and doubled the global cereal production over the past 40 years (Tilman, 1999), however, the price of petroleum has escalated, thus increasing fertilizer production costs and consequently food prices. The N consumption projected for 2050 is $227.4 billion, representing a large economic burden to the future food production system (Good and Beatty, 2011a). The green revolution boosted food production in the 1970s, but with the continuous increase in the demand for food, there is a requirement for another revolution that reduces the cost of food production and does not cause the same environmental impact as the green revolution.

1.3. The N cycle and anthropogenic alteration of the N cycle

The atmosphere is made up of 78% diN (N_2), however this molecular form of N is not accessible to plants and needs to be fixed to be biologically reactive. Plants can take up N from the soil only as ammonium (NH_4^+), nitrates (NO_3^-) or in the form of organic compounds such as amino acids. Molecular N is relatively inert due to its triple bond and a large amount of energy (945 KJ/mol) is needed to break the triple bond. In nature, N is capable of being fixed by three methods. The high temperature of lightning generates free hydroxyl, oxygen and hydrogen species that react with N_2 to produce nitric acid (HNO_3), which accounts for approximately 8% of globally fixed N. N_2 also reacts with ozone photochemically to produce nitric acid, generating another 2% of the fixed N. Biological N fixation

(BNF) produces almost 90% of the N fixed in nature (Taiz and Zeiger, 2006). Other minor sources of fixed N include compounds such as volcanic ash.

BNF includes free living N fixing bacteria (diazotrophs) such as *Azotobacter* and cyanobacteria that contain the enzyme nitrogenase which convert N_2 to NH_4^+ by hydrolyzing ~16 adenosine triphosphate (ATP) molecules per molecule of N_2 fixed (Howard and Rees, 2006; Saikia and Jain, 2007). Furthermore, symbiotic nodule forming bacteria such as *Rhizobia* form a symbiotic relationship with leguminous plants providing large amounts of N in exchange for photosynthate (Saikia and Jain, 2007). Therefore, legumes are commonly used in crop rotation systems in order to fix N and decrease the use of synthetic fertilizers in agriculture. Plant and animal decaying matter also contribute to the NH_4^+ pool in the soil by ammonification. Therefore, they are also used in farming practices as a source of N fertilizer. Fixed organic N is taken up and used by various organisms and exists in the environment in organic and inorganic form before returning to the atmosphere as gaseous molecular N_2. At the same time, denitrification is also occurring by denitrifying bacteria which convert NH_4^+ and NO_3^- to molecular N_2 that is released back into the atmosphere completing the N cycle (Figure 1.1)(Taiz and Zeiger, 2006).

1.4. Inefficiencies in N use and excessive application of N fertilizers

At present an average of 50 kg of N/ha from BNF is available to agriculture systems (Roger and Ladha, 1990; Roger and Ladha, 1992). However, this amount is far from meeting the demands of N in the agriculture system for

optimal plant growth. On average, as much as 220 kg of N/ha of synthetic fertilizers (that are largely produced by the Haber-Bosch process) is applied globally to meet food production demands. However, the application of N fertilizer is not balanced across the globe, with China and India applying more than optimal amounts of N fertilizer, while sub-Saharan countries have no access to N fertilizers (Ju *et al.,* 2009; Vitousek *et al.,*2009).

The total amount of N taken up and utilized by cereal crops under normal farming conditions ranges between 25 to 50% of the total N applied (Pilbeam, 1996; Raun and Johnson, 1999; Hodge *et al.,* 2000; Ladha *et al.,* 2005). Under ideal field trial conditions however, NUE of crop plants can be up to 80 to 90% (Parry and Hawkesford, 2010). Since NO_3^- is not adsorbed by the soil due to its negative charges, it can move through the soil and leech into groundwater with excess application. Also, surface application of N fertilizers can lead to N loss by NH_3 volatization, which can potentially result in the loss of up to 60 to 70% of the amount of N applied (Mosier. 2001). Excess N is also lost to lakes and rivers, dinitrified by soil microbes and volatization of ammonia in the atmosphere. The application and loss of large amounts of N have caused severe environmental impacts such as acid rain, water and food contamination, greenhouse gas emission and the endangering of aquatic species by creating dead zones in the coastal marine ecosystems due to the depletion of oxygen by drastic algal growth because of high N levels (Johnson *et al.,* 2007). Excessive N fertilizer application could also lead to cancer because under acidic conditions nitrites are converted to nitrosamine, a carcinogen. In addition, NO_3 aerosols, NO_2, and nitric acid in the

air can also cause respiratory illnesses (Taiz and Zeiger. 2006; Johnson *et al.,* 2007; van Grinsven *et al.,* 2010).

Member countries of the European Union (EU) have implemented successful nutrient reduction programs that reduced their total fertilizer use by 56% between 1987 and 2007, resulting in improved water quality in ground water, rivers and lakes (Olesen *et al.,* 2004; The EU Nitrates Directive, 2010, Frederiksen *et al.,* 2007; Good and Beatty, 2011a). This program was carried out in response to the severe pollution that caused water quality to drop to an unacceptable level in 1987. However, in China, the government encourages the use of N fertilizers, at levels that are often beyond the optimal level, in order to attain higher yields as a food security measure (Ju *et al.,* 2009). Studies have shown that increase in fertilizer use is not directly proportional to grain yield after a saturation point (Figure 1.2). Therefore a reduction in applied fertilizer would not reduce food production (Ju *et al.,* 2009). This would not only prove advantageous to the cost of food production by reducing a need for large capital input for agriculture, it would also potentially reduce pollution of the ground and the water systems. Aside from nutrient management, another method of reducing N fertilizer use while at the same time maintaining grain yield is to increase the NUE of crop plants so that they can take up and utilize N more efficiently.

1.5. N use efficiency calculations

NUE calculations have been developed, evaluated and historically standardized to provide a proper measure to determine the NUE of plants. In order to develop NUE plants, specific parameters need to be screened to determine if

plants have enhanced NUE. There are a few ways to calculate NUE either in the growth chamber or under field conditions and their advantages and disadvantages has been extensively discussed by Good *et al.,* (2004) and Dobermann (2005). The most common method, widely accepted agronomically and in the scientific community, is the grain weight (Gw) produced per unit of N supplied (Ns) to the plant.

NUE = Gw/Ns

NUE is comprised of two components, N uptake efficiency (NUpE) and N utilization efficiency (NUtE). The parameter to measure in order to calculate NUpE and NUtE separately is the total plant N measured at maturity (Nt).

NUpE = Nt/Ns

NUtE = Gw/Nt

Therefore, NUE= (Nt/Ns) (Gw/Nt) = NUpE x NUtE

In a growth chamber soil experiment, roots of a plant cannot be separated from the soil cleanly, therefore calculations for NUtE and NUpE cannot be carried out accurately without risks of soil and microbial contamination. When plants are grown hydroponically, both roots and shoots can be measured for total plant N. Since the hydroponic system does not allow for the plants to be grown until maturity, only NUpE can be calculated and Nt measurements are taken at monumental growth stages. In the hydroponic system, the total amount of N taken

up by the plant can be calculated by measuring the N concentration in the hydroponic solution (Moll *et al.,* 1982; Good *et al.,* 2004).

Another simple way of calculating NUE for plants grown in soil where soil N is not measured is to determine the shoot weight (Sw) per unit N content of the shoots (N(s)). In this case, NUE= Sw/N(s). This measurement only takes into account the amount of N taken up into the plant and therefore does not account for soil N content.

1.6. Strategies to increase N use efficiency of crop plants

In face of the potential food crisis, many efforts have been put into developing crops that are NUE in order to increase food production with the same amount of N input or to maintain food production levels with a reduction of N fertilizer use. Traditional plant breeding and transgenic approaches have been used to increase NUE of plants.

Marker assisted breeding has shown that grain yield, plant nitrate content and total kernel weight has a positive correlation to glutamine synthetase (GS) activity in maize (Hirel *et al.,* 2001). Grain yield associated QTLs on chromosome 1 and 5 of maize were also found to be common with QTLs of GS activity at high and low N input respectively. A significant number of yield related QTLs map to *gln4*, which encodes for cytosolic GS, which is located on chromosome 5 of maize. However, nitrate reductase was discovered to be negatively correlated with yield related QTLs (Hirel *et al.,* 2001). It has been reported that marker assisted breeding could bring about 30 to 40% increase in

NUE in crop plants. However, comparisons can be difficult since different farming regimes will provide different levels of N to the plant (Good and Beatty, 2011a).

The transgenic approach has also been used to attempt to develop plants with NUE traits. First, genes that were believed to be involved in key steps in N metabolism were over-expressed in hopes of developing NUE lines. When the cytosolic glutamine synthetase isoenzyme (*GS1*) gene *gln1-3* was over-expressed constitutively using the cassava vein mosaic virus (*CsVMV*) promoter, there was an increase of 30% of kernel number compared to wild-type. However, when *gln1-4* was knocked out, the kernel size decreased significantly in maize (Martin *et al*., 2006). Yamaya *et al.* (2002) found that intraspecies over-expression of a rice (*Oryza sativa* ssp. indica) glutamate synthase gene (*NADH-GOGAT*), involved in N cycling, in a Japonica rice line (*Oryza sativa* ssp. japonica) increased grain yield up to 80%. When antisense *NADH-GOGAT* was driven by the *CAMV35S* promoter, there was a reduction in spikelet weight in rice plants, suggesting the importance of NADH-GOGAT in N remobilization and grain filling. Whole genome transcript profiling has discovered interesting genes that are induced by varying N regimes. An example is an early nodulin gene, *OsENOD93-1*, which responded significantly (by 7 fold) to both N increments and reduction, when over-expressed using a ubiquitin promoter showed increases in amino acid accumulation and N content in roots. This effect was accentuated when N is limiting. In addition, transgenic plants also exhibit 10 to 20% higher seed yield, shoot biomass, number of spikes and spikelets compared to wild-type

plants (Bi *et al.,* 2009). However, these increases in NUE have not been field tested and it is unknown if they will fare the same way as in growth chamber experiments.

Aside from N metabolism genes, genetic manipulation of N transporter genes have also been evaluated for their impact in yielding NUE plants. An example is the constitutive over-expression of *OsNRT2.3b*, the product of splicing of *OsNRT2.3*, a nitrate transporter. It exhibits an increase in grain yield by 41% and NUE of the rice plants by 43%. Interestingly, the over-expression of *OsNRT2.3b* also enhances buffer capacity of the phloem and consequently the adaptability of these transgenic rice plants to different soil pHs (Fan *et al.,* 2010)

In addition, different plants exhibit varying phenotypes with the over-expression of the same genes. For example, when aspartate aminotranferase (AspAT) was over-expressed constitutively in *Arabidopsis*, there was an increase in glycine, alanine, glutamate and asparagine in seeds. While in transgenic AspAT over-expressing rice, there was an increase in seed amino acid concentration and seed protein content (Zhou *et al.,* 2009). However, constitutive over-expression and tissue specific over-expression using the *btg26* promoter in *Brassica napus* yielded no NUE phenotype (Wolansky, 2005). This suggests that the candidate gene, promoter and organism are all important factors to develop NUE plants.

1.7. Genetic engineering of AlaAT to increase NUE

Considerable research in our lab has focused on the over-expression of alanine aminotransferase (AlaAT) to develop crops with an NUE phenotype.

Recently, several QTLs related to increased biomass in maize seedlings were found to be co-localized with AlaAT (Zhang *et al*., 2010). AlaAT is an enzyme that catalyses a reversible transamination of pyruvate with glutamate as the amino donor to produce alanine and 2-oxoglutarate (Muench and Good. 1994). The substrates and products of AlaAT are all important components of both N and carbon metabolism, because AlaAT is directly linked to GS-GOGAT cycle, which is part of the primary N metabolism, and to the carbon sinks in plants by means of 2-oxogluterate and pyruvate. 2-oxoglutarate is an important substrate of the tricarboxylic acid cycle, while pyruvate is involved in many processes involving carbon metabolism such as but not limited to glycolysis, gluconeogenesis, and anaerobic fermentation. Alanine, one of the products of AlaAT, is known to be a neutral amino acid that is non-toxic and is used for transport and storage in plants, especially under anaerobic stress (Vanlerberge and Turpin, 1990) (Good and Beatty, 2011b) (Miyashita *et al.,* 2007). Considering all the biochemical pathways involving AlaAT, it can act as N and carbon shuttle (Good and Muench, 1993) to maintain the balanced ratio of carbon and N input into various biochemical pathways and thus maintaining a favourable growth and development of the plant.

AlaAT is part of downstream N metabolism, therefore its substrates and products may not be as tightly regulated by sensing mechanisms. Hence, genetically manipulating AlaAT may provide a viable option for altering the biochemical balance of N and carbon metabolism, to produce an NUE plant (Good and Beatty, 2011b).

Hordeum vulgare AlaAT (*HvAlaAT*) cDNA was isolated when its expression was highly induced under hypoxic conditions in roots. The original *HvAlaAT* cDNA, was characterized to further understand its role in hypoxic conditions and possible resistance to hypoxia, resulted in an unexpected NUE phenotype when driven by the stress inducible promoter *btg26*.

1.8. Promoters driving AlaAT produce NUE phenotype

The isolation of the promoter of the *Brassica t*urgor *g*ene (*btg26*), which had been shown to be induced by drought, was originally achieved by using a pea cDNA. This gene shared considerable sequence similarity to a *26g* gene that encodes for a turgor protein in *Pisum sativum*. The *btg26* gene is rapidly induced and highly expressed during plant dehydration and heat shock. The *btg26* gene has a 31 to 33% similarity to an aldehyde dehydrogenase gene and is not homologous to any known stress inducible gene products (Stroeher *et al.,* 1995). The promoter *btg26* was shown to be ABA-responsive and its expression root-specific (Stroeher *et al.,* 1995). Therefore, Good *et al.* (2007) isolated the *btg26* promoter to drive the expression of the *Hordeum vulgare* AlaAT (*HvAlaAT*) gene in canola. This produced transgenic canola plants that had higher seed yield and biomass under low N conditions in laboratory and field environments. Higher yields were maintained with a 40% reduction in N fertilizer applied (Good *et al.,* 2007). However, other promoters have been tested driving the same cDNA, including the *CaMV35S* promoter, and a Brassica promoter *trg31*, which was isolated in our lab at the same time as the *btg26* promoter (Good, pers comm.)

showed no NUE phenotype. Additionally, when the *btg26* promoter was used to drive *HvAlaAT* in rice, no NUE phenotype was observed.

By sequence similarity to the *btg26* gene, the *OsANT1* gene was found in rice. The promoter of *OsANT1* was isolated and used to express *HvAlaAT* over-expression in cereal plants. The *btg26* gene shows a ~30% similarity to the dehydrogenase family (Stroeher *et al.,* 1995) and the rice *OsANT1* gene also belongs to the rice aldehyde dehydrogenase family and encodes for a protein called antiquitin in *Arabidopsis* (Lee *et al.,* 1994) hence the promoter name was abbreviated to ANT1 after the protein. The *OsANT1::HvAlaAT* construct was transformed into *Oryza sativa* c.v. Nipponbare. Transgenic lines showed a 40 to 65% increase in NUtE in shoots and a 14 to 53% increase in NUtE in roots (Shrawat *et al.,* 2008). The success of the *OsANT1*:: *HvAlaAT* construct in producing a strong NUE phenotype makes it worthwile to look for other tissue specific promoters for use in NUE studies.

1.9. Importance of promoters for transgenic expression

For transgenic studies, there are different types of promoters that can be used, depending on the goal of the experiment. Promoters are often classified as constitutive, tissue specific and/or inducible. Many transgenic studies to date have used generic constitutive promoters such as the cauliflower mosaic virus (*CAMV35S*) and maize ubiquitin 1 promoter (*ubi-1*) to drive target gene over-expression in plants. This can be a disadvantage as it could be energetically unfavourable for plants to express the gene at all times and it could produce abnormal development, since expression levels of the transgene is not regulated

(Shelton *et al.,* 2002). Constitutive over-expression of the cellulose synthase like gene *CslF6* by the oat globulin promoter *ProASGL* frequently resulted in reduced germination of seeds or seedlings with necrosis on the leaf tips leading to death in severe cases (Burton *et al.,* 2011). The authors suggested that this could have been caused by high uncontrolled production of (1,3;1,4)-b-D-glucan and its non-uniform deposition around the vascular tissues of young leaves, impeding growth, producing leaf necrosis and eventually leading to seedling death. The uncontrolled deposition of (1,3;1,4)-b-D-glucan causing vascular suffocation where high concentrations of viscous (1,3;1,4)-b-D-glucan interferes with water or nutrient transport (Burton *et al.,* 2011).

Using inducible or tissue specific promoters may be a better option to drive transgene expression. Inducible promoters will only drive gene expression when a specific physical, environmental, biological or chemical stimulus is applied. The heat inducible promoter of the *Hvhsp17* gene from wheat can be used for high target gene expression when plants are exposed to 38 to 40^{o}C for 1 to 2 hours (Freeman *et al.,* 2011). This allows for the short term gene expression and control of developmental expression but is limited to tissues that are not severely affected by temperature changes. Also, the over-expression of the *Triticum aestivum* NAC protein (*TaNAC69*), encoding a transcription factor that is involved in drought stress, by the drought inducible promoter *HvDhn4s*, produced wheat plants with significantly higher shoot biomass at the early vegetative stage under mild salt stress and water limitation, compared to the wild-type. Conversely, the *HvDhn8s* constitutive promoter driving *TaNAC69* showed

no significant difference from untransformed controls (Xue *et al.,* 2011). When the drought and cold stress inducible promoter *rdA29* was used to over-express the *DREB1A* gene, which encodes a transcription factor involved in stress tolerance in *Arabidopsis*, normal plants were generated, while constitutive expression driven by the *CAMV35S* promoter resulted in growth retardation under normal growing conditions (Kasuga *et al.,* 1999).

Tissue specific promoters are essential for organ specific and developmental stage specific expression of transgenes. For example, in potatoes, *StRCAp* is engineered into the leaves to produce a toxin as a defense mechanism against predatory insects, but is not expressed in parts consumed by humans (Weber, 2003; Park and Jones, 2008). The use of the 35S promoter has also raised concerns of food safety where the toxin produced in non-target organs might cause the potatoes to be unsafe for consumption. In addition, it may be metabolically taxing for the plant to be constantly producing a secondary metabolite, regardless of the developmental stage or organ, therefore causing plants to be less healthy and potentially compromising yields.

In the case of NUE plant engineering, tissue specific expression of genes might increase the efficacy of N uptake, utilization or remobilization in the plant. In contrast, the use of a constitutive promoter might prove to be a waste of energy because over-expression of non-rate limiting enzymes in certain organs may not produce any phenotype or may even decrease yield.

Development of an NUE plant may also involve the transformation of multiple genes or gene stacking to achieve a satisfactory NUE phenotype, since N

metabolism and transport are very complex processes. In this case, transgenes cannot be regulated using a single promoter due to the potential for gene silencing when the transgenes that are driven by a homologous promoter (Finnegan and McElroy, 1994; McElroy and Brettell, 1994; De Wilde *et al.,* 2000; Halpin *et al.,* 2001; Sunilkumar *et al.,* 2005). Therefore, it may be important to design target gene expression with different tissue specific promoters to avoid silencing.

In addition, the use of the *CAMV35S* promoter may not produce any phenotype because the gene expression or protein expression was not sufficient in a specific organ or developmental phase. Also, plants could turn off the expression of the transgene when it proves to be energetically unfavourable. When *HvAlaAT* was driven by the *CAMV35S* promoter, it did not exhibit any NUE phenotype. However, when the root specific *btg26* promoter was used, it produced plants that had higher NUE (Good *et al.,* 2007).

Similar to over-expression of target genes, promoters when used in a different species may not mimic the expression patterns of its native species. Seed specific promoters from barley (*B-hor* and *D-hor*) and wheat (*HMW-Glu*) did not direct seed specific expression in rice, instead the promoters drove high expression levels in leaf, shoot and maternal seed tissues of rice plants (Wu *et al.,* 1998; Qu and Takaiwa, 2004; Furtado *et al.,* 2008; Furtado *et al.,* 2009). Hence, the effects of using a promoter to drive target gene expression in different organisms cannot be predicted.

1.10. Promoter designing strategies

Unlike designing transgenes, there are to date still no known set "rules" that can be followed to design promoters. Functionality of promoter elements are still largely unknown, and even if known promoter cis-elements are present and active in expressing one gene, this is not sufficient to predict the expression of another gene because cis-elements under the control of one promoter might not be active under the control of another promoter. Although there are no known "rules" that can be followed, there are certainly guidelines from previous studies that may increase the chance of designing a successful promoter for the target transgene.

There are several approaches that have been taken by previous studies to select for a candidate promoter to express target genes. One is to use a promoter trap system that contains a promoter-less reporter gene such as *GUS* or *GFP* on the right border of the T-DNA. When a rice mutant library is generated using these T-DNA lines, high *GUS* expression indicates a strong promoter that can be further studied (Yu *et al.,* 2007). Another approach is to carry out promoter deletion studies to determine which portion of the promoter contains elements that are essential to gene expression.

A strategy that is commonly used is based on the gene homologue of known and experimentally tested promoters from a related species. In our lab it was found that the tissue specific *btg26* promoter from barley drives high levels of *HvAlaAT* expression in canola (Good *et al.,* 2007). The promoter of the rice

homologue of *btg26* gene, named *OsANT1*, was also found to drive high levels of *HvAlaAT* and was also tissue specific (Shrawat *et al.,* 2008).

Another approach is to analyze the promoter using a bioinformatics approach to study expression levels in different tissues, and identify promoter core elements and enhancer elements. Currently there are a large number of databases available to study plant promoters, particularly rice promoters. Some examples are Plant Promoter DataBase (PPDB), PLACE (Plant cis-acting regulatory DNA elements), PlantCARE (Plant cis-acting regulatory elements) and Softberry TSSP. These databases contain comprehensive lists of known promoter elements in plants and analyze possible enhancers, core promoter and inducible or responsive elements that could drive high gene expression. However, real experiments are required to determine the validity of these elements detected, because as mentioned, elements important in the control of one promoter might not be important in the control of another promoter (Tiwari *et al.,* 2003).

Once studies have been done, natural promoters can be designed with elements added or deleted in order to increase strength, specificity and induciblity of the promoter. It was discovered that the addition of the first intron in the alcohol dehydrogenase 1 (*Adh1-S*) gene dramatically enhanced the accumulation of *Adh1-S* mRNA, i.e. 50 to 100 fold more than the *Adh1-S* gene without the first intron (Callis *et al.,* 1987). Other studies have also discovered that promoter proximal introns contain many signals elevating gene expression that are absent in introns that do not enhance gene expression (Rose *et al.,* 2008). Intron mediated enhancements (IME) are more prevalent in monocots than dicots, allowing for its

use in gene over-expression in cereal crops (Simpson and Filipowicz 1996; Clancy and Hannah 2002; Rose, 2002; Samadder *et al.,* 2008). In addition, it was also discovered that in rice, the 5'UTR intron of the rice polyubiquitin gene *rubi3* enhances transcription and post-transcriptional gene expression by 20 fold (Samadder *et al.,* 2008). The two most commonly used promoters for gene expression studies in monocots, *ubi1* and *Act1*, have 5'UTR introns to enhance constitutive gene expression (Christensen and Quail 1996; McElroy *et al.,* 1990; Samadder *et al.,* 2008). However, IME does not occur in all case; the addition of an intron or a 5'UTR intron may have negligible effects on gene expression.

In the design of a synthetic promoter or the use of a natural promoter, there are a few promoter elements or enhancer elements to note. First is the TATA box which is usually situated ~25bp upstream of the transcription start site. The TATA box is the most commonly recognized motif of core promoters by the RNA polymerase II that is required for transcription initiation (Butler and Kadonaga. 2002). However, only approximately 19% of rice promoters are known to have TATA boxes (Civáň and Švec. 2008), indicating that they may not be essential for initiating transcription in plants. Other core promoter elements like Y patches appear to play a more important role because they are found in higher abundance with more than 50% of rice promoters containing one or more pyrimidine Y-patches (Civáň and Švec. 2008). However, the importance of Y patches in driving or regulating transcription is still unknown and their occurrence does not converge with the presence of a TATA box, but Y-patches seem to show

a weak positive correlation with mRNA expression levels in plants (Yamamoto *et al.,* 2009).

Other elements that can be included are plant hormone response elements, environmental response elements or chemical response elements that could potentially be inducible by an external stimulus. For example, when two AGCT light and hormone responsive elements are placed 5 nucleotides apart 50 bps upstream of the TATA box of the *Pmec* minimal promoter, its expression increased 6 fold and the promoter gained salicylic acid inducibility. However, when the two AGCT elements are placed 25 nt apart, they gave abscisic acid inducibility to the promoter (Mehrotra and Mehrotra, 2010).

1.11. Aldehyde dehydrogenase superfamilies in Oryza sativa

There are 20 aldehyde dehydrogenase (*ALDH*) genes identified in rice and these belong to 10 *ALDH* families. Four of the families contain multiple genes while the remaining 6 families are only represented by single genes. The categorizing of the *ALDH* family were determined by protein and DNA sequence alignment. ALDH is a common component of many biochemical pathways involved in many different cell functions. It is commonly tightly regulated since excess ALDH production might have a deleterious effect on the organism's metabolism (Gao and Han, 2009).

In order to hunt for potential promoters that can drive *HvAlaAT* at a similar level to *OsANT1*, bioinformatics was used. *OsANT1* full length cDNA and amino acid sequence was used to mine for a list of ALDH family members in rice (*Oryza sativa*) using the Beijing Genomics Institute Rice Information by means of

blastn and blastp. A total of 36 predicted and expressed genes were found, 25 of which represented unique genes. Of these 25 unique genes, eight did not have all 10 or more known conserved *ALDH* motifs and therefore were not considered to be part of the *ALDH* family. The 15 remaining genes with known ALDH conserved motifs were part of the ALDH protein family. None of these 15 genes had high level of identity compared to *OsANT1,* where the highest level of identity on the genetic level was 30.8% while only 55% protein identity was observed. In terms of the promoter region, *OsANT1* shared a maximum of 48.9% identity with the upstream regions of the 15 *ALDH* genes. The promoter region of *OsANT1* was compared to all the promoters identified from the 15 *ALDH* genes and three of the most closely related family members to *OsANT1*, based on alignment scores and E values, were chosen. Each of these promoters were analysed with promoter prediction software for possible motifs. It is interesting to note that all three of the promoters do not share more than 49% identity to *OsANT1*, leading us to believe that there may be significant differences in expression patterns between *OsANT1* and these promoter homologues. One of these promoters, *PBpr1*, was studied and characterized in this thesis in comparison with *OsANT1*.

1.12. PBpr1 promoter

The *PBpr1* promoter is upstream of the *OsALDH6* gene, which encodes a gene for methylmalonate semialdehyde dehydrogenase in rice (Accession number: gene: AK 121280.1 and mRNA: AF045770.1). AK121280.1 and AF045770.1 are splice variants of each other where AF045770 is shorter than the AK121280.1

proposed mRNA at the 3' exon. However AK121280 appears to be a hypothetical protein designated by GenBank, while AF045770.1 has already been characterized in detail by Oguchi *et al* (2004). *OsALDH6* is homologous to the A*LDH6B2* gene in *Arabidopsis,* which also encodes for a methlymalonate semialdehyde dehydrogenase.

Methylmalonate semialdehyde dehydrogenase (MMSDH) catalyzes the irreversible oxidative decarboxylation of malonate-semialdehydes to acetyl-CoA and methylmalonate-semialdehyde to propionyl-CoA in the distal portions of the valine and pyrimidine catabolic pathways. Since MMSDH generates acetyl-CoA, it is an important factor in the glyoxylate pathway, TCA cycle and fatty acid production. Previous studies have shown that MMSDH is highly down-regulated during oxidative stress due to the restriction in the TCA cycle and the production of ATP (Sweetlove *et al.,* 2002). In two week old rice plants, MMSDH mRNA was found at high levels in roots and leaf sheaths while protein accumulation was found highest in roots followed by leaf blades (Oguchi *et al..,* 2004). Auxin is known to be involved in root development and stem elongation. Therefore it was suggested by Oguchi *et al.* (2004) that accumulation of MMSDH mRNA in the leaf sheath and roots could be auxin and gibberellic acid (GA) induced. Interestingly, with the addition of auxin, MMSDH levels in roots are drastically increased with an increase in rooting also being observed. Furthermore, studies have suggested that MMSDH is involved in root growth, tissue differentiation and thickening growth due to its expression in crown roots, lateral roots and root hairs (Tanaka *et al.,* 2005).

By RT-PCR analysis, the expression of the *OsALDH6* gene was found to be highly expressed in young roots and stems (Gao and Han, 2009). All the previous literature suggests that *PBpr1* could drive high levels of expression at an early developmental stage in the roots, stem and leaf sheath and its expression might be regulated by auxin and GA levels within the plant. MMSDH was found to be localized in the mitochondrial matrix of Arabidopsis, rice, human, bovine and rats which suggests a similarity of function of MMSDH among all these organisms.

The *PBpr1* promoter was selected as a possible candidate for over-expression of *HvAlaAT,* because it had the highest homology to the *OsANT1* promoter compared to all the promoters of the *ALDH* genes in rice. In addition, the *PBpr1* promoter seems to drive a gene (AK121280.1) and produces a protein (methylmalonate semialdehyde dehydrogenase) with known function. In addition, the *PBpr1* gene (AK121280) was found to be the most highly expressed among all the *ALDH* genes in wild-type rice plants in a microarray study that was done in our lab (Beatty *et al.,* 2009, unpublished data).

1.13. Goal of this thesis

Although previous work relating to the characterization of *PBpr1* gene expression has been done in its nascent form, it is still unknown how the *PBpr1* promoter will regulate gene expression when driving *HvAlaAT* in rice plants compared to the OsANT1 lines, in terms of producing an NUE phenotype. The *PBpr1* promoter shares 34.6% identity with *OsANT1* and therefore it is predicted

that its expression pattern will probably differ, compared to *OsANT1*. Therefore, the extensive characterization of the *PBpr1* promoter in spatial and temporal expression was required to determine the tissue specificity of this promoter in driving transgene expression. In addition, the essential promoter element for the inducibility of high levels of transgene expression is of interest.

This thesis aimed to evaluate additional promoters to determine their effectiveness at driving the *HvAlaAT* gene and characterize the *PBpr1* promoter in detail in *O. sativa* c.v. Nipponbare. The *O. sativa* background was used because it is a good model system to study cereal plants due to its relatively small genome. Nipponbare was chosen because the previous work of *OsANT1* characterization was carried out in Nipponbare, and *OsANT1* and *PBpr1* should ideally be in the same genetic background in order to make a valid comparison between the phenotypes. The goals of this thesis were:

1. To determine if the *PBpr1* promoter drives over-expression of *HvAlaAT* in *O. sativa*.
2. To determine if two promoters of gene homologues regulate transgene expression similarly, by comparing *PBpr1* and *OsANT1* driving *HvAlaAT* expression.
3. To characterize the potential NUE phenotype that is produced by *PBpr1* driving *HvAlaAT*.
4. To determine the spatial and temporal expression of *PBpr1* by using it to drive *GUSplus* reporter gene expression.

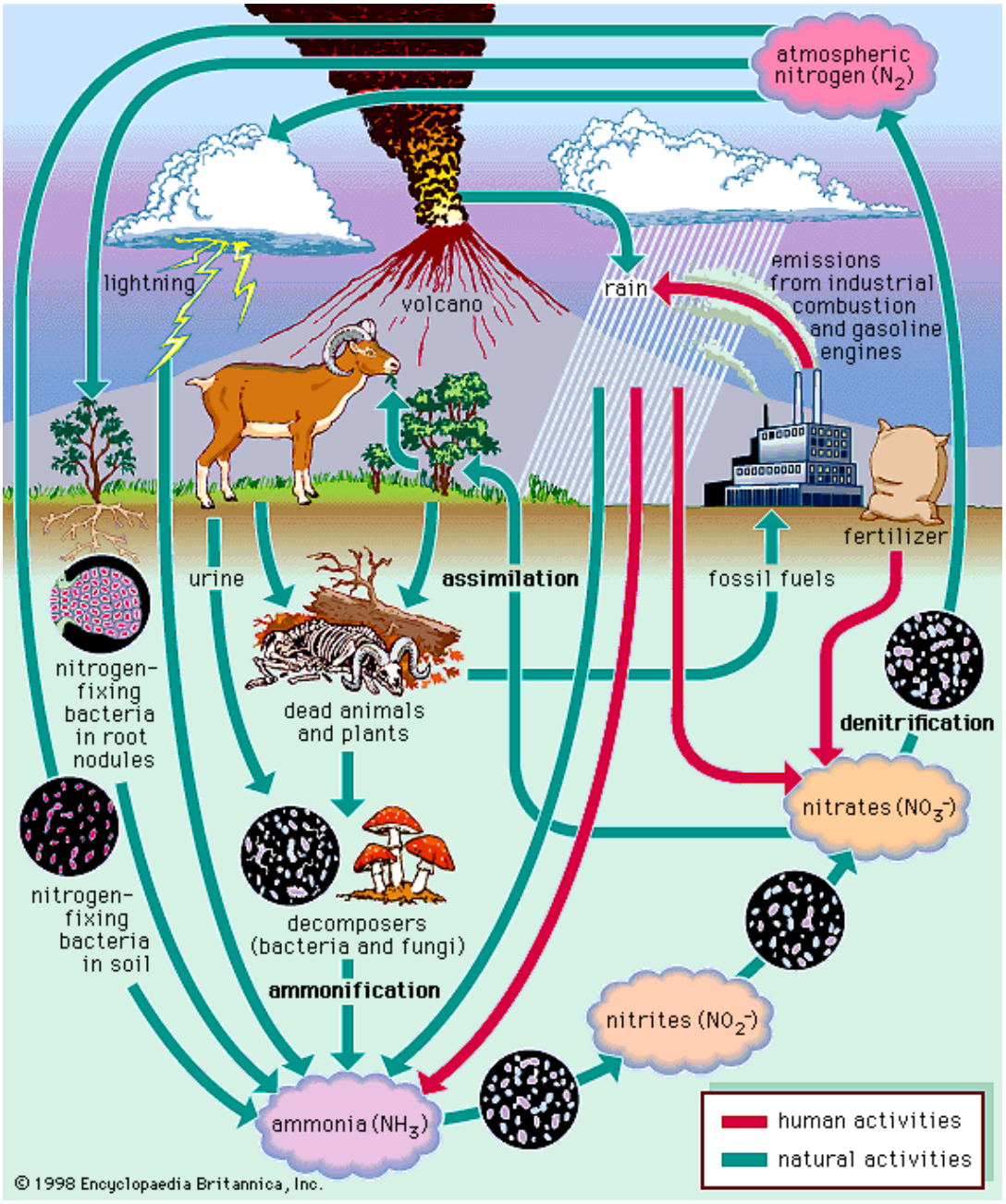


Figure 1.1: Schematic representation of the biogeochemical N cycle (taken from Encyclopedia of Brittanica, (1998))

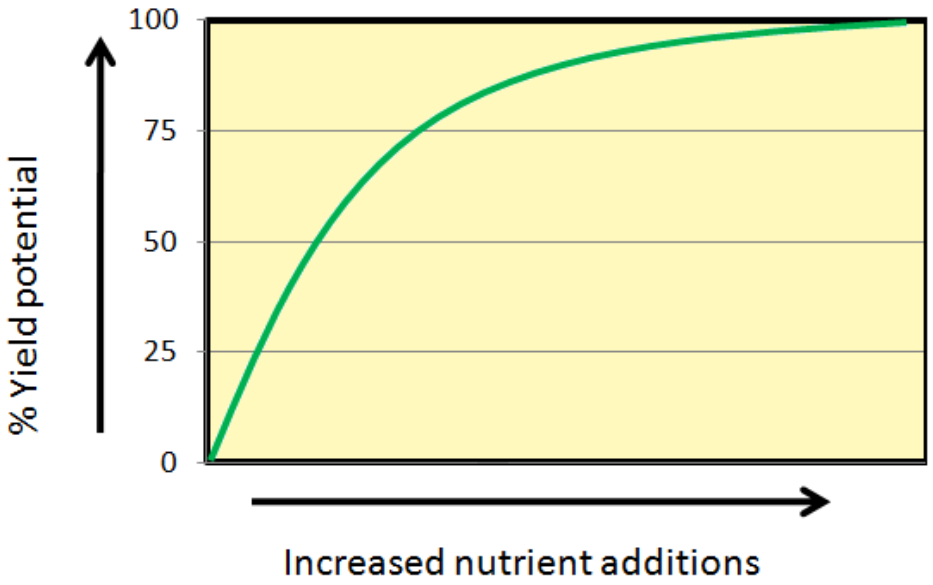


Figure 1.2: A classical crop response curve to added nutrients. The curve represents the yield potential with increasing nutrients levels.

Chapter 2: The phenotypic and genetic characterization of *Oryza sativa PBpr1* promoter over-expression of *Hordeum vulgare AlaAT*

2. Introduction

The importance of understanding how plants acquire and metabolize N and how we might improve the NUE of crop plants has been discussed in detail in Chapter 1. However, recent research by a number of investigators has demonstrated the importance of choosing the correct promoter for expressing a gene. The choice of different promoters to express genes of interest is an area that has not received a lot of attention, as many researchers prefer to use promoters that will over-express the gene at a high constitutive level. However, it would be logical that a promoter should only express the gene of interest, at the appropriate time and within the correct tissue in a plant.

The selection of the *PBpr1* promoter as a candidate promoter was based of a number of factors including homology to *OsANT1* and *btg26*, a microarray study showing expression levels of genes in rice that have increased and decreased expression levels under different nitrogen regimes and promoter analysis using bioinformatic software.

This chapter describes the development and characterization of a number of different independent transgenic lines or events, where the alanine aminotransferase gene (AlaAT) which has been characterized in detail in our lab (Muench and Good, 1994; Good *et al.,* 2007; Shrawat *et al.,* 2008; Beatty *et al.,* 2009), has been expressed using a novel promoter, *PBpr1*. This promoter is from

a gene encoding methyl-malonate semialdehyde dehydrogenase (MMSDH) in rice and has also been characterized in more detail in Chapter 3.

2.1. Materials and Methods

2.1.1. Construction of binary vector and Agrobacterium mediated transformation

For the over-expression of *HvAlaAT*, the *PBpr1* promoter was used. The 733bp *PBpr1* promoter was selected upstream of the MMSDH gene and designed to be cloned using GeneArt (Invitrogen, Life Technologies, Carlsbad, CA, USA). The intragenic region between the MMSDH gene and its upstream gene was ~1 kb and it was selected and designed to be cloned. The construct was designed by introducing the *HvAlaAT* cDNA into pCAMBIA1300 using a PstI/HindIII ligation while the *PBpr1* promoter was introduced upstream of *HvAlaAT* to drive expression using EcoRI/SacI sites (Figure 2.1). However ~300 bp directly adjacent to the ATG of the MMSDH gene was inadvertently lost during the cloning process leaving a 733 bp promoter. The same *PBpr1* promoter was introduced into pCAMBIA1305.1 to drive *GUSplus* for promoter pattern analysis using the EcoRI/NcoI sites. *PBpr1::HvAlaAT* in pCAMBIA1300 and *PBpr1::GUSplus* in pCAMBIA1305.1 were transformed into the *Agrobacterium tumefaciens* strain EHA105 by the freeze thaw method (Weigel and Glazebrook, 2002). Rice callus (*Oryza sativa* c.v. Nipponbare (NB)) was transformed with both constructs, using a *Agrobacterium* transformation system that was developed in our laboratory (Shrawat and Good, 2011). Lines transformed with *PBpr1::HvAlaAT* were named Ap rice lines (Ap designating Aldehyde dehydrogenase promoter) while lines transformed with *PBpr1::GUSplus* were

named ApG lines. Calli that were transformed and regenerated into full plants were designated as T_0 lines and their seeds were designated as T_1 seeds. While this is the nomenclature used in our lab, the terminology is often different in other labs wher T_1 refers to the primary transformants.

2.1.2. Screens for transgenic T_1 plants

Based on seed yield, total above soil biomass and tiller number at maturation, five T_0 lines containing *PBpr1::HvAlaAT* were selected to be continued through the T_1 generation. A total of 20 heterozygous T_1 seeds per line were germinated on 4.5% w/v Phytagar (Invitrogen Life Technologies/Gibco-BRL, Burlington, ON) for three days. Newly imbibed seedlings were then transferred to 2" X 2" potting squares containing wet soilless potting mixture Sunshine Mix #4 (Sun Gro Horticulture, Vancouver, BC) (Canadian Sphagnum peat moss, coarse perlite, starter nutrient charge (with Gypsum), dolomitic limestone and long-lasting wetting agent), with the seeds planted approximate 2 cm below soil level. Leaf tissue was collected from two week old plants to determine if they were transgenic by means of a genomic DNA polymerase chain reaction (PCR) analysis. The primers used were specific to hygromycin resistant gene *hptII* and their sequence was:

(Forward primer hptII-For; ATGAAAAAGCCTGAACTCACCGCG),

(Reverse primer hptII-Rev; GCGCCCAAGCTGCATCATC)

(Integrated DNA technologies, Inc, San Diego, CA, USA). The T_1 population of a single insertion line was expected to have 3:1 ratio of transgenic: null segregant.

Six transgenic plant and two null segregants were selected based on uniform size and height to be continued while the other plants were set aside.

2.1.3. Genomic DNA extraction

Flash frozen tissue samples were ground in DNA extraction buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA pH 8.0 and 0.5% w/v SDS). The reaction mixture was centrifuged at 13000 rpm for 2 min and then the supernatant removed. Three hundred µl of isopropanol was added, mixed and the reaction was again centrifuged at 13000 rpm for 5 min, the supernatant discarded and the pellet left to air dry and later resuspended in 100 µL of 1X TE Buffer (10mM Tris-HCl pH 7.5 and 1 mM EDTA pH 8.0). PCR was carried out on the extracted genomic DNA using Recombinant Taq DNA polymerase (Invitrogen) and a Veriti® Thermal Cycler (Applied Biosystems/Ambion, Streetville, ON).

2.1.4. Soil growth chamber experiments

T_0, T_1, and T_2 generations were planted in soilless-potting mixture Sunshine Mix #4 (Sun Gro Horticulture). Rice plants were grown in growth chambers at 28^{o}C, 70% relative humidity, 14h/10h light/dark photoperiod, photon flux density 750 µE at bench height. T_1 and T_2 generations were germinated in 4.5% w/v Phytagar for 3-4 days and then transferred to 2"x2" square plots and grown for two weeks. Plants were selected for uniform height and size and transferred to 7 inch round pots while others were culled. All plants were fertilized twice a week beginning from when they were transferred into the pots. Each plant obtained 187.5mL of rice fertilizer (0.014% Plant Product 20-20-20,

0.003% Plant Product Micronutrient, 0.009% Plant Product Iron chelate, 15.6 µM $CuSO_4.5H_20$, 30.7 µM $ZnSO_4.7H_2O$ and 674 µM $MgSO_4.7H_2O$) for each application before active tillering stage. Between active tillering and anthesis, 187.5mL of rice fertilizer at a higher concentration (0.023% Plant Product 20-20-20, 0.005% Plant Product Micronutrient, 0.014% Plant Product Iron chelate, 25.0 µM $CuSO_4.5H_20$, 49.1 µM $ZnSO_4.7H_2O$ and 1.079 mM $MgSO_4.7H_2O$) was applied to plants. At anthesis, all plants were covered with clear micro-perforated bags to allow for self-pollination. Once seeds were set, the relative humidity of the chamber was decreased to 50% humidity to prevent fungal growth. At maturity, plants were cut above the soil and weighed for total above ground biomass. Their panicles were also cut to determine total seed weight. Each plant received 0.196 g of nitrogen over its entire life cycle.

In the T_3 generation, three seeds were germinated in a 7 inch round pot and allowed to grow for two weeks. Two of the three seedlings were culled based on uniform height and size of plants remaining in each pot. A fertilization regime similar to that of the T_2 generation was started on the three week old plants. All statistical comparisons carried out for these growth chamber experiments were done using a Student's t-test, with a p-value of more than 0.05 deemed as statistically not significant.

2.1.5. Hydroponic growth chamber experiment

The hydroponic experiment was carried out in a growth chamber at 28^oC, 70% humidity, 14h/10h light/dark photoperiod, photon flux density 750 µE at

bench height. Transgenic T_2 lines, their null segregants, wild-type Nipponbare (NB) and *an OsANT1::HvAlaAT* line (AGR1/7) were germinated in 4.5% phytagar for three days. Newly imbibed seedlings were transferred to germination tanks containing hydroponic Trostle nutrient solution with 4 mM NH_4^+ as a nitrogen source (Trostle *et al.,* 2001). Seedlings were allowed to grow for five days and then transferred to a plastic grid such that the roots of the plants were submerged in the nutrient solution. Fourteen day old plants were transferred to the experimental pots/buckets containing 11 L of the nutrient solution to begin the experiment. Each bucket contained four technical replicates and each line had five biological replicates randomly positioned around the chamber using a Latin square design. The nutrient solution was tested for pH, electro-conductivity and NH_4^+ concentration every 2-3 days and a solution change was carried out at 28, 45 and 52 DAG (days after germination). Plant material was harvested on 28 and 52 day old plants for AlaAT assays and RNA experiments.

2.1.6. Selection of homozygous lines and maintenance of null lines

T_2 seed obtained from T_1 plants were germinated on MS and Hygromycin (4.4 g L^{-1} MS, 30 g L^{-1} Sucrose, 4% w/v phytagar, 50 mg mL^{-1} Hygromycin). Plants whose progeny were all resistant to hygromycin were deemed homozygous while heterozygous plants had a 3:1 ratio of plants that would survive to plants that would die on hygromycin selection. NB and AGR1/7 were used as negative and positive controls and their seeds were germinated on the same media.

Null lines were identified by PCR at the T_1 plant stage using hygromycin specific primers. At the T_2 stage, the absence of the transgene was further confirmed by 0% germination on hygromycin screens. Subsequently, null lines were selected and maintained for a comparison between NB, AGR1/7 and Ap lines.

2.1.7. AlaAT Extraction and enzyme assays

Both shoot and root tissue was harvested for AlaAT enzyme assays in the hydroponic experiments, while only shoot tissue was harvested in potted experiments. In all cases, 150 µg of shoot and 300 µg of root tissue were used for AlaAT extraction. Plant tissue was cut into small pieces and placed into a mortar containing a pinch of sand and polyvinylpolypyrrolidone (PVPP). Plant tissue was ground with a 5:1 ratio (µl:mg) of AlaAT Extraction buffer (100 mM Tris HCl pH 7.8, 5 mM EDTA pH 8.0, 1 mM DTT, 10 mM L-cysteine and 0.1 mM PMSF) to tissue sample weight. The sample was centrifuged at 13,000 rpm for 15 min at room temperature to remove cell debris and subsequently supernatant was transferred to a new 1.5 ml Eppendorf tube and stored in ice. The extraction supernatant containing AlaAT was diluted for spectrophotometric assays.

AlaAT enzyme assays were carried out in 96 well Corning plates. 10 µL of diluted extraction supernatant was added to each well with triplicate technical replicates. Then, 180 µL of AlaAT enzyme assay buffer (0.1 M Tris-HCl pH 8.0, 0.01 M 2-oxogluterate pH 8.0, 0.25 mg mL^{-1} NADH, 0.083% v/v lactate dehydrogenase (LDH), 1 µM pyridoxal-5-phosphate (P5P)) was added to each

well using a multi-channel pipette. The plate containing assay buffer and sample enzyme was analyzed for 4 min at A_{339nm} with 9 second intervals to test for background levels of AlaAT activity. Subsequently, 10 µL of 0.5 M alanine pH 8.0 was added to each well using a multichannel pipette and spectrophotometrically analyzed by a 96 well ELISA plate reader (SpectraMax+) for 5 min at 9 second intervals at A_{339nm}. AlaAT activity was measured in the reverse direction of alanine to pyruvate where alanine is added last to initiate the reaction, and the decrease of A_{339nm} by oxidation of NADH to NAD was monitored.

In order to quantify the amount of protein in each sample, a Bradford protein quantification assay was carried out. Samples were diluted between 1:20and 1:200 depending on the original concentration so that the test concentration can fall within the linear range of 0.05 µg uL^{-1} to 0.5 µg $µL^{-1}$ of the Bradford protein quantification standard curve. A standard curve was constructed using differing concentrations of Bovine serum albumin (BSA). Fifteen µL of protein standard or sample was added into each well and 185 µL of 1/5 diluted Bradford protein assay reagent (Bio-Rad, Mississauga, ON) was also added. The reaction mixture was incubated for 5 min and spectrophotometric analysis (SpectraMax+) at A_{595nm} performed.

2.1.8. Protein SDS-PAGE electrophoresis

In order to validate the results of the AlaAT enzyme assays, an immunoblot analysis was carried out using the protocol of Muench and Good

(1994). AlaAT extraction samples were used in the immunoblot analysis and were diluted to contain equal amounts of protein. Root samples were standardized to a concentration of 0.075 mg mL^{-1}. Shoot samples were diluted 2:15 and standardized to a concentration of 0.124 mg mL^{-1}. Diluted samples were mixed with equal volume of SDS-PAGE loading dye and heated for 10 min at 95^{o}C and then transferred to ice and centrifuged at 13,000 rpm for 10 min.

Samples were loaded in wells in a 4% stacking gel (4% Acrylamide/bis-acrylamide, 0.06 M Tris-HCl pH 6.8, 0.1 % w/v SDS, 0.1% w/v APS, 0.1% v/v TEMED) with a10% v/v separating gel (10% v/v Acrylamide + bisacrylamide, 0.38 M Tris-HCl pH 8.8, 0.1% w/v SDS, 0.1% w/v APS, 0.1% v/v TEMED). The SDS-PAGE gel was ran in protein electrophoresis buffer (3 g L^{-1} Tris, 14.4 g L^{-1} glycine, 1 g L^{-1} SDS at pH 8.3) at 25 mA using a Mini-Protein II electrophoresis cell (Bio-Rad) until the loading dye had touched the interface between the stacking gel and the separating gel, then current was increased to 45 mA.

2.1.9. Immunoblot analysis

The SDS-PAGE gel was equilibrated in transfer buffer (3.03 g L^{-1} Tris, 28.8 g L^{-1} Glycine and 20% v/v methanol, pH 8.3) and transferred to nitrocellulose membrane Hybond-ECL (Amersham, GE Healthcare, Buckinghamshire, England) at 100 V in ice cold conditions for 1.5 to 2.5 hrs using a Mini-Trans blot cell (Bio-Rad). The nitrocellulose membrane was washed twice with TBS (8 g L^{-1} NaCl, 0.02 M Tris-HCl pH 7.6), then blocked with 1% Roche Western Blocking Reagent (Roche Applied Science, Laval, QC) in TBS

overnight at 2 to 8^{o}C. The membrane was then incubated in AlaAT anti-rabbit IgG antibody (Good and Muench, 1992; Muench and Good, 1994) in a 1/3,500 dilution in TBS for 1 hour at room temperature and subsequently washed twice with TBS-T (8 g L^{-1} NaCl, 0.02 M Tris-HCl pH 7.6 and 0.1% Tween 20) and twice with 0.5% Roche Western blocking reagent (Roche). The membrane was then incubated in 1/7,500 horse radish peroxidase (HRP) rabbit anti-goat IgG antibody (GE Healthcare) for 1 hour at room temperature and washed 4 times with TBS-T. Detection of the nitrocellulose membrane was carried out using the Enhanced Chemiluminescence kit (GE Healthcare) and photographic film was used to capture the image.

2.1.10. RNA extraction, cDNA synthesis and quantitative real time PCR

Frozen rice shoot and root samples harvested at 52 DAG were ground into a fine powder in liquid nitrogen and RNA was extracted using the QIAGEN RNeasy plant mini kit (Qiagen Inc, Mississauga, ON) coupled with DNaseI (Qiagen Inc) to ensure the RNA samples were DNA free. RNA concentrations were measured spectrophotometrically using a Nanodrop spectrophotometer (Thermo Scientific, Mississauga, ON). A260/A280 and A260/A230 values of 1.7 or greater were desired for all samples. In order to further confirm the quality of RNA extracted, RNA samples were ran through a 2100 Bioanalyser (Agilent, Cedar Creek, TX) to ensure that the RNA sample is not degraded. Once RNA samples were confirmed to have acceptable quality, they were standardized to a fixed concentration and then 0.5 µg of RNA used for cDNA first strand synthesis. cDNA was synthesized from RNA using the Superscript II reverse transcriptase

enzyme (Invitrogen), Random primers 30µg μL^{-1} (Invitrogen) and oligo dT 12-18 primers (Invitrogen). qRT-PCR reactions were performed on the light cycler ABI Prism 7000 (Applied Biosystems) using the FastStart Universal Probe Master mix (Roche). Gene specific primer and probe pairs that were previously used by Beatty *et al.* (2009) were used to detect differences in gene expression due to over expression of *HvAlaAT*. NB plants were used as a negative control and 18srRNA was used as an endogenous control.

2.2 Results

2.2.1. Screening of primary transgenic plants to be characterized

Twenty seeds were germinated from five independent T_0 plants to generate six independent T_1 lines, designated as; Ap7, Ap17, Ap18, Ap19, and Ap28. The designation AP referred to Aldehyde dehydrogenase promoter representing the fact that Ap is the promoter of a methyl-malonate semialdehyde dehydrogenase.

PCR of genomic DNA from the T_1 lines was carried out to confirm the presence of the transgene (Appendix 1). In the T_1 generation, the seeds from a single plant comprises homozygous, heterozygous and nulls for the transgene and the PCR screens can differentiate homozygous or heterozygous lines from null segregants. When the null segregants were identified, they were kept as negative controls for comparison with the transgenic plants because they have segregated without the transgene but have gone through the same transformation process as the transgenic lines. PCR reactions were carried out using primers that amplify

the hygromycin gene, (*hptII*) using genomic DNA extracted from leaf tissue of 21 day old plants. All NB plants were negative for the *hptII* gene while all AGR1/7 plants were positive for the *hptII* gene. Once the T_1 plants were identified as either transgenic or nulls, 6 transgenics and 2 null plants from each line were selected based on uniform height and size (Appendix 1). AGR1/7 was used as a positive control and NB was used as a negative control in the PCR screens.

2.2.2. Hygromycin screening for homozygous T_2 transgenics

Homozygous T_2 lines were selected based on 100% germination frequency using hygromycin selection. If 35 or 36 seeds germinated on hygromycin containing media, out of a total of 36, the line was considered to be homozygous. Based on this criterion, lines Ap7-12, Ap18-11, Ap28-2 and Ap28-3 were identified as homozygous (Table 2.2) and were chosen to be tested in a hydroponic system. Ap28-2 and Ap28-3 are derived from the same line.

2.2.3. Screening for seed quality in the T_3 transgenics

In order to determine if the seed quality had also been affected in the transgenic lines, seed quality was evaluated by germinating the seeds on media without hygromycin to determine if there was any indication that the transgene affected seed quality (Table 2.2). Additionally, the germination frequency under 'no selection' could then be used to evaluate whether any of the additional lines were homozygous for the insertion. For example, if a line had 70% germination frequency without hygromycin, then with hygromycin only 7 out of 10 seeds were expected to germinate even if the seeds were homozygous. Only Ap17 and Ap19

had been tested under 'no selection' conditions. Ap19-17-2 and Ap17-10-1 had 100% viable seeds and were identified as homozygous (Table 2.2) and selected to be grown on soil together with Ap18-11-4 and Ap28-2-6.

2.2.4. Heterozygous T_0 seed yield and biomass preliminary screens

At the T_0 stage, the independent Ap plants had higher above ground biomass and seed yield compared to NB (Table 2.1). When comparing all Ap lines to NB, Ap has a significantly higher biomass compared to NB ($p<0.001$), but differences in seed weight were not statistically significant (Table 2.1). Five independent Ap lines were selected based on high seed yield and biomass to continue on to the T_1 generation (Table 2.1). When comparing the six lines selected with NB, they had significantly higher seed yield ($p<0.001$) and above ground biomass ($p<0.001$) compared to NB (Table 2.1). There was a strong linear correlation between seed yield and above ground biomass as illustrated in Figure 2.2.

2.2.5. Heterozygous T_1 Ap seed yield and biomass preliminary screens

For further phenotypic analysis and to obtain homozygous seed, T_1 plants of five lines, Ap7, Ap17, Ap18, Ap19 and Ap28, were grown to maturity and biomass and seed yield data collected. Ap7, Ap18 and Ap28 had significantly higher above ground biomass than NB ($p<0.05$), while differences between NB and Ap17 and Ap19 above ground biomass were not significant ($p>0.05$) (Figure 2.3). Ap18 and 28 had significantly higher above ground biomass than AGR1/7 ($p<0.001$), while the other Ap lines did not differ significantly in above ground

biomass as compared to AGR1/7 (Figure 2.3). In terms of seed production, only Ap18 and 28 had significantly higher seed yield than NB ($p<0.05$), all other independent Ap lines had higher seed yield than NB but the differences were not significant ($p>0.05$) (Figure 2.3). In addition, none of the Ap lines showed significant differences in seed yield compared to AGR1/7 ($p<0.05$).

2.2.6. Growth chamber biomass and seed yield analysis of homozygous lines

2.2.6.1. T_2 Biomass of homozygous Ap lines

Homozygous Ap lines at the T_2 generation were grown in both hydroponic and soil conditions. In soil, Ap lines were grown to maturity and total above ground biomass and seed yield data was collected (Figure 2.4). Two of the five Ap lines had higher above ground biomass than NB. Only one of these lines, Ap28-2, was significantly larger than NB and AGR1/7 in terms of biomass ($p <0.001$) (Figure 2.4). Ap28-3, a sibling of Ap28-2, had significantly lower above ground biomass than NB ($p<0.001$) (Figure 2.4). The other Ap lines and AGR1/7 were not significantly different from NB in terms of above ground biomass.

2.2.6.2. T_2 seed yield of homozygous Ap lines

Similar trends were observed in the seed yield of the independent Ap lines, compared to NB and AGR1/7 (Figure 2.4). While Ap28-2 had the highest seed yield of all of the Ap lines, it did not differ from either NB or AGR1/7. Again, Ap28-3, the sibling of Ap28-2, had lower seed yield than AGR1/7. Other lines such as Ap17-10 and Ap18-11 were shown to have higher seed yield than NB and AGR 1/7, but these differences were not significant ($p>0.05$) (Figure 2.4). In

addition, Ap28-3 and Ap19-17 were observed to have lower seed yield than NB and AGR1/7, although these differences were not significant (Figure 2.4). Neither seed yield nor biomass of AGR1/7, when compared to NB, was observed to be significantly different from each other (Figure 2.4). This is not consistent with previous comparative studies or experiments on AGR1/7 vs NB .

2.2.6.3. T_3 above ground biomass of homozygous Ap lines

Homozygous Ap T_3 plants were harvested at 52 DAG in order to assess the changes of biomass at a different developmental stage (Figure 2.5). Plants were harvested and then the dry weight of above ground biomass was measured. Three of the four independent Ap lines, Ap17-10-1, Ap18-11-4 and Ap28-2-6, had higher dry above ground biomass than NB and the nulls at 52 DAG (Figure 2.5). Out of these 3 lines, only Ap28-2-6 had significantly higher above ground biomass than both NB and nulls ($p<0.03$) (Figure 2.5). Ap28-2-6 was the only Ap line that had higher above ground biomass than AGR1/7, however, the difference observed was not statistically significant ($p>0.05$) (Figure 2.5). Ap28-2-6 had above ground biomass similar to NB and the nulls, while it had lower above ground biomass than AGR1/7, although these differences were not significant. AGR1/7 also did not have significantly higher above ground biomass than NB or nulls at 52 DAG (Figure 2.5). Ap18-2N plants had similar above ground biomass compared to NB, which was consistent with previous experiments in the T_1 and T_2 generations (Figure 2.5). Ap18-11-4, on the other hand, had higher biomass than NB and nulls but had lower biomass than AGR1/7. However, the differences

observed between Ap18-11-4 and NB, the nulls ($p>0.05$) or Ap18-11-4 and AGR1/7 ($p>0.05$) were not statistically significant using a Student's t-test.

2.2.6.4. Ap biomass, seed yield and NUE

Ap28, over all 3 generations, has significantly higher above ground biomass than NB (Figure 2.3; 2.4; 2.5). In the T_1 and T_2 generation, Ap28 is higher in above ground biomass than AGR1/7 but not significantly (Figure 2.3; 2.4). Ap18 also exhibits a consistent trend of higher above ground biomass than NB in the T_1 and T_3 generation but not in the T_2 generation (Figure 2.3; 2.4; 2.5). However, differences between Ap18 and AGR1/7 over all the generations are not significant (Figure 2.3; 2.4; 2.5). Other Ap lines were not significantly different from AGR1/7 or NB. Across all generations, Ap 28 had higher seed yield than NB and AGR1/7 while Ap 18 had higher seed yield than NB in the T_1 but not in the T_2 generation (Figure 2.3; 2.4; 2.5).

2.2.7. Ap Plant height and tiller number

2.2.7.1. T_1 Ap lines plant height

Out of the Ap lines analyzed, Ap18 plants were the tallest (Figure 2.6). However, like all the Ap lines, the difference in height was not significant compared to NB. On the other hand, Ap17 plants were significantly shorter compared to both NB and AGR1/7 ($p<0.01$) (Figure 2.6). Although AGR1/7 had slightly taller plants than NB, the differences observed were not statistically significant based on a Student's T-test ($p>0.05$) (Figure 2.6). In later generations

(T_2 onwards), plant height was no longer recorded because it was not informative in determining the difference between independent Ap lines to NB and AGR1/7.

2.2.7.2. T_1 Ap lines tiller productivity

In addition to measuring biomass and seed yield at maturity, the number of productive and non-productive tillers was measured. Tiller productivity and plant height was only recorded in soil grown plants. Tiller productivity of the Ap lines, in comparison with AGR1/7 and NB was determined and all Ap lines had more tillers and more productive tiller than NB (Figure 2.7). All the Ap lines except for Ap17 had more tillers and more productive tillers than AGR1/7. Ap18 had the highest total number of tillers and productive tillers among all Ap lines, AGR1/7 and NB (Figure 2.7). This was consistent with other parameters assessing overall plant size as Ap18 produced the largest plants among all other Ap lines (Figure 2.7). All the Ap lines except Ap17 had significantly higher total number of tillers and productive tillers than NB ($p<0.01$) (Figure 2.7). In comparison to AGR1/7, Ap7, Ap18 and Ap28 had significantly more productive tillers ($p<0.05$), however, the differences between AGR1/7 and all T_1 independent Ap lines in terms of total number of tillers was not significant ($p>0.05$) (Figure 2.7).

2.2.7.3. T_2 Ap homozygous lines tiller productivity

When homozygous T_2 seeds were obtained, they were germinated and raised in soil to assess the stability of the phenotype of different lines containing the promoter/transgene insertion. Ap19-17 and Ap28-2 had significantly more

total tillers and productive tillers than both NB and AGR1/7 ($p<0.02$) (Figure 2.8). Ap28-2 had the highest number of productive tillers among all Ap lines while Ap19-17 had the highest number of total tillers compared to all other lines. Ap17-10, Ap18-11 and Ap28-3 had a higher total number of tillers than NB but the differences observed were not statistically significant (Figure 2.8). In contrast, these lines (Ap17-10, Ap18-11 and Ap28-3) had fewer productive tillers compared to NB, however, this difference was not significant (Figure 2.8). Ap28-2 and Ap28-3 are siblings of the same independently transformed line, but Ap28-2 had significantly higher total tillers ($p<0.05$) and productive tillers ($p\leq0.001$) than Ap28-3 (Figure 2.8). AGR1/7 had fewer productive tillers than NB, although these differences were not significant, while it had the same number of total tillers as NB.

2.2.8. HvAlaAT over-expression measured by AlaAT activity assays

2.2.8.1. Preliminary T_1 screens for AlaAT activity for HvAlaAT over-expression

Shoots of T_1 soil grown Ap plants were harvested between active tillering and maximum tillering stage (40 DAG) to determine their specific AlaAT activity. The different Ap lines had 2.5 to 8 times higher shoot AlaAT activity compared to the null segregants and NB (Figure 2.9). AlaAT specific activity was highest in Ap19 and Ap28 at between 3.3 to 4.3 μmoles NADH min^{-1} mg of protein (Figure 2.9). AGR1/7 containing *OsANT1::HvAlaAT* had higher specific AlaAT activity than nulls and NB in agreement with Shrawat *et al.* (2008). Out of all the Ap lines, only Ap19 had higher average specific AlaAT activity than AGR1/7, but Ap18

and Ap28 had comparable levels of AlaAT activity as AGR1/7. The AGR1/7 used was a T_5 homozygous line while the T_1 Ap lines tested were still segregating. Ap7 had approximately the same levels of AlaAT activity as nulls and wildtype NB (Figure 2.9). Null segregants of the different Ap lines (Ap17N, Ap18N, Ap19N and Ap28N) had similar AlaAT activity as NB (Figure 2.9). Once these lines were confirmed as true nulls, they were selected to be used as negative controls.

2.2.8.2. T_3 homozygous shoot AlaAT activity assay

After a generation of seed increases and homozygous screening of the T_3 plants, lines Ap17-10-1, Ap18-11-4, Ap19-17-2 and Ap28-2-6 were identified as homozygous plants and were raised in the growth chamber until 52 DAG when tissue samples were harvested for AlaAT activity. Trends observed in the T_3 generation were consistent with those of the T_1 plants (Figure 2.10). Ap lines had up to 5.3 times higher shoot AlaAT activity than NB and the nulls. In agreement with T_1 studies in soil, Ap19 had the highest AlaAT activity followed by Ap18 and Ap28 while Ap17 had the lowest AlaAT activity. AGR1/7 was found to have higher AlaAT activity than all Ap lines (Figure 2.10).

2.2.8.3. T_2 homozygous AlaAT activity

Soil experiments with the Ap lines had only allowed testing of shoot AlaAT activity. In order to also assess the AlaAT activity of the roots, homozygous Ap lines were selected to be grown hydroponically. Ap roots and shoot tissue were harvested at the beginning of active tillering (28 DAG) and at maximum tillering (52 DAG).

Shoot AlaAT activity

The Ap lines (with the exception of Ap7-12) exhibit up to 8 times higher shoot AlaAT activity compared to nulls and NB (Figure 2.11). At both 28 DAG and 52 DAG, Ap18 and Ap28 had up to 1.2 and 1.7 times higher AlaAT activity than AGR1/7 respectively. Shoot AlaAT activity of Ap18 and Ap28 was higher at 52 DAG than 28 DAG, whereas native shoot AlaAT activity (the Ap null lines and NB) was observed to drop slightly between 28 and 52 DAG (Figure 2.11).

Between 28 DAG and 52 DAG, AlaAT activities in some of the transgenic, nulls and NB shoots were observed to have decreased with the exception of Ap28-2 (Figure 2.11). In NB, nulls and Ap7, shoot AlaAT activity decreased by 1.6 to 2 times between 28 DAG and 52 DAG. For all Ap lines, with the exception of Ap7, the changes in shoot AlaAT activity was only between 1.1 to 1.2 times between 28 and 52 DAG. The largest change between 28 and 52 DAG in shoot AlaAT activity was observed in AGR1/7 where AlaAT decreased by 1.4 times (Figure 2.11). Ap7 had similar levels of shoot AlaAT activity as NB and its nulls, which was in agreement with the previous T_1 experiments which indicated that Ap7 did not over-express *HvAlaAT* (Figure 2.11).

Root AlaAT actvity

Although all Ap lines except Ap7, had higher root AlaAT activity than NB, similar levels of root AlaAT activity were observed between Ap lines and their corresponding null siblings (Figure 2.12). AGR1/7 was the only transgenic line that had clearly higher level of root AlaAT activity than all other lines (Figure

2.12). However, root AlaAT activity was observed to have dropped in all cases at 52 DAG compared to 28 DAG. The decrease was evident in null segregants and NB where levels of root AlaAT activity were observed to have dropped 2 to 3 times. A smaller decrease of 1.2 to 1.4 times in root AlaAT activity of 28 to 52 DAG was observed in the Ap lines and AGR1/7 (Figure 2.12). With the decrease of root AlaAT activity at 52 DAG, Ap lines and AGR1/7 had up to 3 times higher AlaAT activity than NB and nulls (Figure 2.12). Shoot AlaAT activities in the non-transgenics are always low at both 28 and 52 DAG (Figure 2.11). Root AlaAT activity, on the other hand, is high at 28 DAG and decreases drastically at 52 DAG. Overall root AlaAT activity in the non-transgenics was always higher than shoot AlaAT activity.

2.2.9. HvAlaAT immunoblotting to validate AlaAT assays

2.2.9.1. Soil T_1 Ap lines shoot AlaAT protein levels

Leaf tissue from soil grown T_1 Ap lines at 40 days after germination were harvested and protein extracted to determine the levels of HvAlaAT protein. The presence of high levels of AlaAT is represented by a dark intense 52kDa band. At 40 DAG, Ap shoots showed a thick, dark band at the expected monomer size of *HvAlaAT* of 52 kDa (Figure 2.14). Three siblings of Ap19 and 2 siblings of Ap28 had 52kDa dark bands indicating that they had high levels of HvAlaAT protein. Negative controls, NB and null segregants, did not have any bands at the 52kDa size (Figure 2.14).

2.2.9.2. Soil T_3 Ap lines shoot AlaAT activity levels

After 2 generation of seed increases, 52 day old soil grown T_3 Ap lines were harvested to determine the consistency of the *PBpr1* promoter in over-expressing *HvAlaAT,* by AlaAT activity assays and immunoblotting. T_3 shoots of all the Ap lines tested had high levels of AlaAT protein according to the immunoblot (Figure 2.15). In addition, *HvAlaAT* protein levels in AGR1/7 were found to be comparable but slightly higher than that of the Ap lines. This was consistent with the results obtained in the AlaAT assays (Figure 2.10). The 52kDa *HvAlaAT* band is absent in the nulls and NB indicating the absence of *HvAlaAT* protein (Figure 2.14). Faint non-specific bands are observed across all samples regardless of the absence or presence of the *HvAlaAT* transgene insertion presumably due to the non-specific binding of the *HvAlaAT* antibody (Figure 2.15).

2.2.9.3. T_2 hydroponically grown Ap roots AlaAT protein levels

Roots of hydroponically grown T_2 roots were harvested at 28 and 52 days to assess the changes in the levels of root *HvAlaAT* protein between active and maximum tillering. In lines that did not carry the *HvAlaAT* transgene insertion, the *HvAlaAT* antibody bound to native AlaAT. This was evident where nulls and NB produced two distinct and thin bands approximately at the 52kDa region.The Ap and AGR1/7 root samples, on the other hand, produced a thicker and more intense band that masked the two nonspecific thinner bands at the 52kDa region (Figure 2.16A; 2.17A). Therefore roots of 28 day old Ap plants had higher levels

of *HvAlaAT* protein than the nulls and NB (Figure 2.16A). This did not agree with AlaAT assay results where there were almost no differences between Ap lines and the nulls and NB at this stage (Figure 2.12). In addition, line Ap7-12 does not have the same banding pattern as the other Ap lines but had similar banding patterns to the nulls and NB in agreement with the AlaAT assays (Figure 2.16A).

At 52 days, the nulls and NB were observed to have 2 distinct thin bands which indicate non-specific binding (Figure 2.17A). Again, Ap lines had a dark intense band that masks the presence of the thin bands which indicates the presence of high levels of *HvAlaAT* proteins in the root sample at this time point (Figure 2.17A). The differences in *HvAlaAT* protein levels between transgenics and non-transgenics were in agreement with the AlaAT activity assays (Figure 2.12), with *HvAlaAT* produced in large amounts in the Ap lines. The same band was observed in AGR1/7 indicating high levels of *HvAlaAT* protein. On the other hand, Ap 7-12, similar to 28 DAG and previous generation's AlaAT assays and immunoblots, had the same banding pattern as a non-transgenic (Figure 2.17A).

2.2.9.4. T_2 hydroponically grown Ap shoots AlaAT protein levels

Shoot *HvAlaAT* protein levels of hydroponically grown T_2 Ap plants were also assessed at 28 and 52 days (Figure 2.16B and Figure 2.17B). *HvAlaAT* protein was observed to be high in the shoots of all Ap lines except Ap7-12. Again, AGR1/7 had the same level of banding intensity as all the Ap lines indicating high levels of *HvAlaAT* protein compared to nulls and NB. At 28 and 52 days, protein levels of *HvAlaAT* appeared to be present and high in Ap lines

and absent in the non-transgenics.*HvAlaAT* levels of the two time points (28 and 52 DAG) cannot be compared because they were tested on separate blots (Figure 2.16B and Figure 2.17B).

2.2.10. qRT-PCR analysis to determine gene expression changes from over-expression of HvAlaAT

The mRNA expression levels of seven target genes (barley AlaAT (*HvAlaAT*), native rice AlaAT (*AlaAT2*), a putative glycine rich protein (*GRP*), GS (*OsGln1-2*), ammonium transporter (*OsAMT1*), leucine rich repeats (*LRR*) and a rice wall associated kinase (*OsWAK101*)) were selected from a list of genes that had been found to be differentially expressed in a microarray experiment comparing AGR1/7 to NB (Beatty *et al.,* 2009). Beatty *et al.* (2009) demonstrated that when *HvAlaAT* was highly expressed in AGR1/7, *GRP* was 15 to 400 fold up-regulated and the *LRR* protein and *OsWAK101* were at least 2 fold up-regulated, compared to NB. In this study, I analyzed the differences in gene expression of these genes, between *OsANT1* and *PBpr1* driving *HvAlaAT*, compared to the nulls and NB.

2.2.10.1. Transcript profile of T_3 Ap lines in soil

HvAlaAT was approximately 400 to 1400 fold higher in mRNA expression in the Ap lines and AGR1/7 shoots compared to NB, except for the nulls which did not differentially express *HvAlaAT* compared to NB (Figure 2.18). The qRT-PCR analysis, AlaAT assays and immunoblotting confirmed that the Ap lines were over-expressing *HvAlaAT* compared to the null segregant. Among the Ap

lines tested, Ap28-2-6 had the highest *HvAlaAT* expression at 1400 fold higher followed by Ap19-17-2 with a 1000 fold higher expression relative to NB (Figure 2.18). *GRP* was up-regulated approximately 10 to 30 fold in all Ap lines and AGR1/7 (Figure 2.18). The null lines exhibited 33 fold higher *GRP* mRNA expression compared to NB. *LRR* was 9 to 20 fold up-regulated in the Ap lines and AGR1/7 compared to NB and the nulls, which were 3.4 fold up-regulated compared to NB (Figure 2.18). *OsGln1-2* was not differentially expressed in the Ap lines other than Ap28-2-6, where it was 2.7 fold higher than NB. The null line had the same up-regulation as Ap28-2-6. The native AlaAT was not differentially expressed in any of the Ap lines, compared to AGR1/7 or NB except for the null line at 2.2 fold change (Figure 2.18). *OsWAK101*101 was also not differentially expressed in any line other than Ap28-2-6 at 2.4 fold change compared to NB (Figure 2.18). The mRNA profiles of all target genes tested in Ap lines were similar to the profile of those genes in AGR1/7.

2.2.10.2. Transcript profile of T_2 Ap shoots grown hydroponically

Hydronically grown T_2 Ap shoots were analyzed using qRT-PCR to determine mRNA expression levels compared to NB. In agreement with previous AlaAT assays and western blots, *HvAlaAT* mRNA was highly expressed in all Ap lines with 400 to 800 fold higher expression relative to NB with Ap28-2 shoots having the highest up-regulation, while the null line did not differentially express *HvAlaAT* relative to NB (Figure 2.19). All the Ap lines and AGR1/7 exhibited 20 to 40 fold up-regulation of *GRP* relative to NB. The null line had the highest up-regulation of *GRP* at 40 fold higher expression relative to NB. *LRR* was most

highly up-regulated in AGR1/7 at a 29 fold change relative to NB, while the transgenic Ap lines had a 7 to 11 fold up-regulation (Figure 2.19). The null line had 16 fold up-regulation of *LRR* mRNA relative to NB (Figure 2.19). *OsAMT1* and *OsGln1-2* were not differentially expressed in all Ap lines, AGR1/7 or the null lines compared to NB (Figure 2.19). All lines except the null line did not show any differential expression of native AlaAT compared to NB. The null line had 2.7 fold up-regulation of *AlaAT2* relative to NB. All Ap lines, AGR1/7 and the null line did not show differential expression of *OsWAK101* except for Ap18-11 which showed a 2.5 fold up-regulation relative to NB (Figure 2.19).

2.2.10.3. Transcript profile of T_2 Ap roots grown hydroponically

Hydroponically grown T_2 Ap roots RNA was analyzed for changes in transcript levels in comparison to AGR1/7 and NB. *HvAlaAT* mRNA was highly expressed in Ap lines at an approximately 700 to 2000 fold increase compared to NB. AGR1/7 roots had 2500 fold higher expression of *HvAlaAT* relative to NB (Figure 2.20). As expected, the null line did not show differential expression of *HvAlaAT* relative to NB. *GRP* was highly up-regulated at 200 to 500 fold in both the Ap lines and AGR1/7 relative to NB. The null line also exhibited high up-regulation of *GRP* at a 279 fold increase in expression relative to NB (Figure 2.20). Interestingly, the null line showed the same level of up-regulation in *GRP* as its transgenic sibling at 279 fold increased expression relative to NB (Figure 2.20). *OsGln1-2*, *OsAMT1* and *AlaAT2* were not differentially expressed in the Ap lines, AGR1/7 or the null line relative to NB. In the case of *OsWAK101* and *LRR*, all Ap lines did not show any differential expression in both genes relative to NB

(Figure 2.20). However, *OsWAK101* and *LRR* protein was 3 and 2.3 fold up-regulated in AGR1/7 relative to NB while the null line showed 3 fold down-regulation of *OsWAK101* (Figure 2.20).

GRP was more highly upregulated in roots than in shoots relative to NB in both the Ap lines and AGR1/7. Similarly *HvAlaAT* mRNA was also more highly expressed in roots than in shoots of all the transgenic lines relative to NB (Figure 2.19 and 2.20). Overall there seemed to be no differential expression of *AlaAT2*, *OsGln1-2* and *OsAMT1* in the Ap lines and AGR1/7 relative to NB in either roots or shoots. The null line seemed to have a 2 fold or more up-regulation of *AlaAT2* in shoots in both soil and hydroponic conditions.

2.3. Discussion

Given the importance of developing plants that are nutrient efficient, there is a need to evaluate different genes and regulatory elements and their impact on a plant's ability to efficiently use the limiting nutrient. In this chapter I analyze the over-expression of *HvAlaAT* in rice, using a novel promoter. The promoter had been selected previously within our group based on the factors described in the introduction.

2.3.1. Increased AlaAT activity resulted in the increase of Ap biomass and seed yield

2.3.1.1. T_0 Ap analysis

A total of 39 independent Ap lines were developed of which five were characterized in more detail including the development of homozygous lines,

AlaAT activity assays, immunodetection of the protein, biomass and seed yield. Four of these lines where characterized through to the T_3 generation. In the initial generation (T_0 Ap plants), all five Ap lines had significantly higher seed yield and biomass compared to wild-type (Table 2.2). In transgenic studies, our laboratory has found that for a number of the AlaAT constructs that have been tested, the primary transformants (T_0) frequently exhibit strong phenotypes. This is critical because it allows us to identify candidate lines with strong NUE phenotype for further characterization early on and therefore reduce the number of lines to work with. While some companies have high throughput system to screen large number of lines, this is not the case in most academic labs. Other studies dealing with transgene over-expression in tomato have shown a stronger phenotype in the primary transformants compared to their progeny (Fraser *et al.,* 2002). Many studies do not include T_0 as valid results and therefore the prevalence of this observation is difficult to determine.

2.3.1.2. T_1 Ap analysis

The five Ap lines were propagated and further characterized. As a first step lines with the transgene insertion were selected and nulls and homozygous transgenic lines were selected. Of the four Ap lines, all lines except Ap7 were found to have significantly higher levels of AlaAT activity compared to NB and the null segregants (Figure 2.9). Immunodetection (Figure 2.14) confirmed the high levels of AlaAT protein in the homozygous transgenic lines, clearly demonstrating that the transgenic lines are producing higher amounts of active HvAlaAT protein. At the T_1 stage, AlaAT activity and HvAlaAT protein levels

were variable because of the heterozygosity of the parental T_0 plants (Figure 2.9; 2.14). Only the Ap19 line had higher AlaAT activity than AGR1/7, although these differences were not significant (Figure 2.9).

Ap7 did not exhibit high AlaAT activity levels like the other Ap lines (Figure 2.9). Instead, it had similar levels of AlaAT activity as NB and the null segregants, which suggests that the inserted transgene is not functional. This could mean that there has been a deletion or mutation in *PBpr1* or *HvAlaAT* resulting in a lack of *HvAlaAT*. Alternatively, the transgene may be inserted in a region of the genome that is transcriptionally silent. The low AlaAT activity of Ap7 was validated using immunodetection which also indicated the absence of HvAlaAT band (Figure 2.16; 2.17).

In the T_1 generation, all Ap lines had higher above ground biomass and seed weight than NB, however the difference were significant only in three of the lines (Figure 2.3). Of the three lines that had higher biomass, one line (Ap7) did not over-express *HvAlaAT*. The two Ap lines that were over-expressing *HvAlaAT* (Figure 2.9), had significantly higher above ground biomass than AGR1/7. Seed yield among the other Ap lines and AGR1/7 were similar. However, AlaAT activity did not correlate with high above ground biomass and seed yield (data not shown).

The demonstration that lines Ap18 and Ap28 displayed increased biomass (Figure 2.2) is significant because it means that the *PBpr1* promoter may be a superior promoter when driving *HvAlaAT*. The observation that the line Ap7 also

shows a significantly higher biomass was unexpected, since this line does not appear to over-express this gene. Further analysis of this line was not conducted, however it may be that the tissue culture required to generate the transgenic plants resulted in these changes. Tissue culture effects are common among transformed lines in many plant species. No differences were observed in height of the T_1 lines, while the total number of tillers did show significant differences between two of the four over-expressing lines and NB. In general, the transgenic lines (including AGR1/7) had more tillers, however, a lower percentage of the tillers contained seeds. The observation that the transgenic lines had increased tiller number and number of productive tillers is consistent with what Shrawat *et al*. (2008) observed in the *OsANT1::HvAlaAT* transgenic lines they studied. It is common in many cereal crops that when plants produce more tillers, a greater percentage of the tillers will be non-seed bearing. In addition, other studies have shown that the plant transformation process can cause mutations which may affect fertility (Landsmann and Uhrig, 1985; Miki *et al.,* 2009), and somaclonal variation induced by the tissue culture process can also affect the fertility of tillers and reproductive characteristics (Brauer *et al.,* 2011; Larkin and Scowcroft, 1981).

2.3.1.3. T_2 Ap analysis

Consistent with the T_1 data, T_2 Ap shoots and AGR1/7 shoots were demonstrated to have high AlaAT activity at active tillering (28 DAG) and maximum tillering (52 DAG) (Figure 2.11). This demonstrates that *PBpr1* can drive *HvAlaAT* to produce similar levels of AlaAT activity as *OsANT1* in shoots during vegetative growth, which is the period when plants are actively acquiring

nutrients for increased growth. The increase in AlaAT activity may increase nitrogen uptake and increase plant biomass (Good *et al.,* 2007; Shrawat *et al.,* 2008). Although the nulls and NB exhibit lower overall AlaAT activity compared to the transgenics, there is a clear decrease in the levels of native AlaAT activity from active to maximum tillering (Figure 2.11; 2.12).

In roots, AlaAT activity was also observed to be high at 28 DAG with NB and nulls having almost the same levels of AlaAT activity as the Ap lines. There was no additional increase in AlaAT activity in over-expressing lines (Figure 2.12). Only AGR1/7 exhibited a higher root AlaAT activity indicating that the *OsANT1* drives higher *HvAlaAT* activity in the roots than the Ap lines. Ap lines at the active tillering stage did not have increased AlaAT activity, which might suggest that the *HvAlaAT* gene is not being expressed at this developmental stage (Figure 2.12), however, immunodetection of AlaAT activity indicated higher levels of the protein (Figure 2.16A). This indicates that *PBpr1* is developmentally regulated and drives increased levels of root AlaAT activity later in development. In contrast, AGR1/7 exhibits increased AlaAT activityat active tillering and maximum tillering stages compared to NB (Figure 2.12). Whether these differences are important in affecting any aspect of plant phenotype remains unknown. One explanation for the differences between activity and protein levels in the Ap lines may be that the HvAlaAT protein may have undergone some form of post-translational modification to regulate its function. Since the *HvAlaAT* antibody is a polyclonal, it would detect both functional and nonfunctional forms of the protein.

At the T_2 generation, differences between Ap lines and controls in terms of seed yield and above ground biomass were not significant, with the exception of Ap28-2 (Figure 2.5). However, AGR1/7 also did not exhibit significant differences from NB. Also, the only line that showed significantly higher above ground biomass and seed yield compared to wild-type and AGR1/7, Ap28-2, had a sibling line, Ap28-3, which had similar levels of AlaAT activity (Figure 2.11;2.12) but was not significantly larger than NB (Figure 2.5). Such results raise the question of whether there is a correlation between increased AlaAT activity and increased above ground biomass and seed yield, however, the data was not available at that generation to test this correlation. In other studies, the over expression of *HvAlaAT* by *OsANT1* and *btg26* promoters had resulted in plants with larger biomass and seed yield (Good *et al.,* 2007; Shrawat *et al.,* 2008), however, these studies did not look at the correlation between activity and biomass. The comparison between biomass, seed yield and AlaAT activity may not be valid because AlaAT activity measurements were carried out on plants grown in hydroponics while biomass and seed yield were obtained from soil growth. However, AlaAT activity data has been consistent over all the T_1, T_2 and T_3 generations regardless of the growth condition (Figure 2.9; 2.10, 2.11), indicating that for the assessment of shoot AlaAT, either growth condition should be adequate.

The key question originally raised was whether plants can be developed that have increased NUE. Three of the Ap lines display increased NUE while line Ap28-2 had increased biomass and seed yield (Table 2.3). Similarly, AGR1/7 also

had higher NUE and biomass compared to NB. Two of the three Ap lines with increased NUE had lower biomass indicating a possibility of more nutrients partitioned to seed production.

Increased tiller production in the Ap lines was consistent from the parental T_1 generation on. This increase in tillering was also observed in previous *HvAlaAT* over-expression studies in our lab (Shrawat *et al.,* 2008). Interestingly, AGR1/7 did not produce significantly higher number of tillers and productive tillers compared to NB, which disagrees with the studies done by Shrawat *et al.* (2008). Such variation in tiller number between generations could be due to fluctuations in growth chamber conditions, nutrient stress or the effect of transformation or tissue culture.

Ap28-2 and Ap28-3 were siblings of the same independent line but they had differences in phenotype, with Ap28-2 having significantly higher seed yield and biomass than Ap28-3, and higher levels of tiller productivity than Ap 28-3. The reason for this difference observed is unknown however it could be due to trait instability or poor parental seed quality. In addition, although the Ap lines have been tested to be homozygous plants, there is a possibility of these plants containing multiple transgene insertions. In this case lines could be homozygous at one loci but not another and could produce a hemizygous plant which contains a transgene insertion that is still segregating. Multiple insertions can be detected if an extremely large number of seeds is screened for hygromycin resistance, however, we only used a limited number of seeds per line. Southern blots of

restriction digested DNA probed for the transgene should also be performed in the future on any material that is going to be studied in more detail.

2.3.1.4. T_3 Ap analysis

The T_3 generation allowed us to look at the stability of the trait, and the phenotypic expression of the trait in subsequent generations. AlaAT activity of Ap lines was observed to be in agreement with all previous experiments where Ap lines exhibit high levels of AlaAT activity compared to NB and the null controls (Figure 2.10). AGR1/7 also had high levels of AlaAT activity. Immunoblotting (Figure 2.15) validated the results of the activity assays where all Ap lines and AGR1/7 had high levels of HvAlaAT protein while the HvAlaAT protein was not detected in NB or the null segregants. Overall, the use of activity measurements seems to be both more accurate and convenient to detect over-expression, and immunoblots are likely only required to confirm the presence or absence of the protein.

At maximum tillering the above ground biomass of AGR1/7 was higher than NB (Figure 2.4), in agreement with the hydroponic studies carried out by Shrawat *et al.,* (2008) (Figure 2.5). Ap28-2-6 was the only Ap line in this generation to exhibit significantly higher above ground biomass than either NB or the nulls, although Ap18-11-4 had higher above ground biomass than NB. Ap28-2-6 and Ap18-11-4 were not significantly different from AGR1/7. Other Ap lines showed no difference with NB for above ground biomass, suggesting that there may be no correlation between high shoot AlaAT activity and increased above

ground biomass (Figure 2.13). However, the plants in this experiment appeared to be deficient in micronutrients which caused them to be stunted and severely yellow at a young age. This nutrient deficiency could strongly affect the growth and biomass of the plants.

2.3.1.5. Trait stability over all generations

The assessment of Ap lines through the three generations demonstrated that all Ap lines had higher AlaAT activity than NB (Figure 2.9; 2.10; 2.11; 2.12). Additionally this was confirmed by immunoblotting to find similar abundance of protein levels (Figure 2.14; 2.15; 2.16; 2.17). Also, increased *HvAlaAT* mRNA expression was observed in all of the homozygous generations tested (Figure 2.18; 2.19; 2.20). Of all the over-expressing lines assessed, Ap 18 and 28 had higher biomass, seed yield and number of tillers, therefore producing larger plants than NB consistently across all the generations (Table 2.1; Figure 2.3; 2.4; 2.5; 2.7; 2.8). Such consistency and enhancement in growth suggests that the *PBpr1* promoter, like *OsANT1*, is a good candidate to drive *HvAlaAT* to increase NUE and the trait seemed to be stable across all generations. Overall, Ap lines appeared to stably over-express *HvAlaAT* and also generated larger plants that produced more yield.

2.3.2. Effect of HvAlaAT over-expression on transcript profiles in transgenic Ap lines

Based on activity measurements and transcript abundance (Figure 2.18; 2.19), the *PBpr1* promoter over-expressed *HvAlaAT* in shoots of Ap lines at a similar level as the *OsANT1* promoter, while the nulls were observed to have no

over-expression of *HvAlaAT* in both soil and hydroponic experiments. A previous study within our group had shown that the *OsANT1::HvAlaAT* over-expressers displayed significant changes in certain transcripts, although the functional significance of this observation is unknown (Beatty *et al.,* 2009). The over-expression of *HvAlaAT* by *PBpr1* or *OsANT1* did not affect transcriptional changes in the native AlaAT2 in either soil or hydroponic conditions (Figure 2.18; 2.19). Therefore, transgenic over-expression of an *HvAlaAT* gene did not affect the expression of a homologue (*AlaAT2*) in rice. Additionally, over-expression of *HvAlaAT* by either promoter did not result in differences in gene regulation of the cytosolic nitrogen metabolism gene *OsGln1-2*, an ammonium transporter *OsAMT1*. These results obtained were consistent with the previous study by Beatty *et al.* (2009), where none of the nitrogen metabolism genes were up-regulated in the event of *HvAlaAT* over-expression. Over-expression of *HvAlaAT* did not affect the expression of a gene associated with nitrogen metabolism (*OsGln1-2*), which we choose as a proxy for other nitrogen metabolism genes.

There were a few genes that had changes in transcript profile in response to transgene over-expression itself, tissue culture effects, or the metabolism changes due to higher levels of AlaAT. The *GRP* transcript was highly up-regulated in Ap plants grown in both soil and hydroponic conditions. Glycine rich proteins (*GRP*s) represent a family of cell wall proteins commonly found to be involved in wounding and stress response (Keller *et al.,* 1989; Showalter *et al.,* 1992). Beatty *et al.* (2009), observed *GRP* mRNA to be highly up-regulated in *OsANT1*::*HvAlaAT* lines compared to NB plants and they suggested that *GRP*

could be needed in transgenic AGR1/7 lines to confer plasticity or structural support to cell walls, due to the increase in root bundle and biomass of the transgenic plants (Keller *et al.,* 1989; Farrokhi *et al.,* 2006). It was also speculated that *GRP* could be associated with a receptor kinase and be involved in nitrogen signaling and transduction. *GRP* has not yet been characterized and is still classified as a hypothetical protein in NCBI and Genbank. In this study using both soil and hydroponics, up-regulation of *GRP* mRNA was observed in the Ap lines and AGR1/7. However, *GRP* was also observed to be up-regulated in the null segregants of the Ap lines. This was a surprising observation which suggests that *GRP* over-expression was not induced by *HvAlaAT* over-expression, but rather that *GRP* up-regulation could be attributed to tissue culture effects. Characterization of other *GRP*s has shown that they accumulate in response to hormone induction, differentiation of vascular tissue and excision wounding (Keller *et al.,* 1989; Richard *et al.,* 1999).

The *LRR* protein was more highly up-regulated in Ap shoots compared to controls. However, about a 3 fold up-regulation is found in null segregants indicating that *LRR* may have been up-regulated partly due to the transformation process or tissue culture effect. *LRR* was a nucleotide binding site leucine rich repeat according to motif analysis but its specific function is still unknown. Since *LRR* can be part of a basal or secondary plant related gene (Beatty *et al.,* 2009), a 3 fold up-regulation found in the nulls indicates that the transformation or tissue culture process may have affected *LRR*'s expression. But transgenic Ap plants and AGR1/7 exhibit up-regulation of *LRR* up to almost 20 fold, which was a much

higher up-regulation compared to AGR1/7 (Beatty *et al.,* 2009). This discrepancy could have been due to various environmental stimuli, nutrient deficiency or a stress response since these T_3 plants were yellowing.

OsWAK101 was also not differentially regulated in the Ap shoots as compared to the NB, which was similar to results obtained by Beatty *et al*. (2010). *OsWAK101* belongs to a 125 member wall associated kinase family known to link the cytoplasm to the extracellular matrix, and they are known to be involved in pathogen resistance intercellular communication, heavy metal tolerance and plant development (Anderson *et al.,* 2001; Zhang *et al.,* 2005). *OsWAK101* is speculated to be a wall associated kinase that spans the cell wall and is involved with cellular communication but its specific function is yet to be characterized.

Roots of Ap lines were observed to highly over-express the *HvAlaAT* transgene at similar levels to AGR1/7 while *HvAlaAT* gene expression in null roots was absent and similar to NB plants (Figure 2.20). This over-expression again confirms the AlaAT activity assays and immunoblots that the *PBpr1* promoter increases the expression of the *HvAlaAT* transgene therefore increasing the AlaAT activity in Ap lines.

Similar to the results obtained in the shoots, the over-expression of *HvAlaAT* did not directly impact the gene expression of nitrogen metabolism and ammonium transporter genes *OsGln1-2* and *OsAMT1*, which were not differentially expressed. Also, *OsWAK101*101 did not exhibit differential regulation in roots of Ap lines, AGR1/7 or nulls compared to wild-type plants.

However, unlike shoot transcript profiles, roots of Ap lines and AGR1/7 exhibit no differential regulation in *LRR* compared to NB plants which suggests that *LRR* was only expressed in the shoots at maximum tillering stage.

GRP, same as in shoots, was highly up-regulated in roots of all the Ap lines, AGR1/7 and null segregants. *GRP* was found to be highly up-regulated in AGR1/7 roots compared to wild-type plants in previous studies (Beatty *et al.,* 2009) and was deduced to be involved in nitrogen metabolism. However, with the up-regulation of *GRP* in null segregants, this suggests that *GRP* up-regulation is most likely unrelated to the over-expression of *HvAlaAT*.

According to previous literature on the transcriptome of transgenic plants, most studies cite a small transcript profile difference between transgenics and wild-type plants where less than 0.2% of the genes have differences in regulation. Over-expression of *HvAlaAT* by *OsANT1* produces a difference of 0.11% and 0.07% in root and shoot respectively compared to controls (Beatty *et al.,* 2009), while other over-expression studies have shown less than 0.06% transcript profile changes (Seo *et al.,* 2011) . Although only a small percentage of genes are being differentially regulated in transgenic plants, the assumption that the regulation changes are caused by the transgene itself cannot be made, since transgenic plants have undergone a transformation and tissue culture process, which may have resulted in somaclonal variations within the transgenic plants that could interfere with the phenotype produced by the transgene expression itself.

While experiments for transcript profiling were carried out in both soil and hydroponics, little difference was found between soil and hydroponic shoots using the eight different probe sets. The only difference observed was that the null *LRR* in hydroponics exhibit the same levels of up-regulation as all the Ap shoots in hydroponics while in soil *LRR* was observed to be distinctively less of an up-regulation compared in all the Ap lines. This again indicates that either experimental condition is sufficient to measure changes in transcript profiles and the more intensive hydroponic experiments may not be necessary for this type of analysis.

2.3.3. Post transcriptional regulation of HvAlaAT over-expression

Each of the Ap lines expressed the *HvAlaAT* gene at a 400 to 1400 foldincrease in shoots and 700 to 2000 foldincrease in roots relative to NB in the T_2 and T_3 generations (Figure 2.18; 2.19; 2.20). Each of these over-expressing lines, although exhibiting higher levels of AlaAT activity, only had increased AlaAT activity of up to 8 times in shoots and up to 3 times in roots in the same set of experiments. Therefore, there seemed to be no direct correlation between the extent of gene expression and the AlaAT activity or protein expression levels in the trangenics. Other studies dealing with transgenic plants have also noted that, in transgenic studies, there is frequently no clear correlation between transcriptome, proteome and metabolome driving variations in the plants (Barros *et al.,* 2010). This suggests that there may be a form of post-transcriptional control of the over-expressed *HvAlaAT* in both shoots and roots of rice plants regardless of the growth conditions, although the nature of its specific regulation is still

unknown. Over-expression of *HvAlaAT* in canola and rice also showed similar results indicating a transcriptional control of AlaAT activity (Good *et al.,* 2007; Beatty *et al.,* 2009). It is possible that there is a limit to the increase of AlaAT activity or protein production that is allowed in rice regardless of the transcript levels. In addition, differences between root AlaAT activity and protein levels at active tillering also suggest possible post-translational modifications that may regulate HvAlaAT function.

2.3.4. Conclusion

In interpreting this data, a number of concerns have arisen. Tissue culture effects can heavily influence the changes in transcriptomic profile. This is also observed in other studies where 35% of the transcript changes were not attributed to the transgene over-expression itself (Montero *et al.,* 2011). One method to discern between genes that were differentially regulated by the transgene expression itself and genes that were differentially regulated due to tissue culture or the transformation process is the use of a null segregant, and so address the concern that the transformation and tissue culture can cause large amounts of somaclonal variations and mutations that could mask the true phenotype caused by the gene of interest itself (Brauer *et al.,* 2010; Brauer *et al.,* 2011). A plant line transformed with an empty vector could also act as a negative control for the effects of the presence of hygromycin resistance to the plants but it does not represent a line that has undergone the same transformation, tissue culture and genetic segregation process as a null segregant does. Both the null segregant and the empty vector transformed line would not act as a negative control for a

mutation caused by a transgene insertion since *Agrobacterium* mediated transformations inserts the transgene randomly. The data in this chapter suggests that the substantial differences in transcript profile may not be caused by the transgene, although the specific cause remains unknown. This indicates the need for any comparisons done on transgenic plants to include both wild-type and null segregants, otherwise interesting differences in expression profiles could be mistaken to be caused by the transgene or missed.

The results in this thesis have their limitations due to a number of factors, including the effects of transformation, optimal nutrient application, the variability, quality and cleanliness of the growth chamber, however, these are limitations that all researchers face. Transformation and tissue culturing can affect the overall health of plants and their ability to handle environmental stress (Brauer *et al.,* 2011). Somaclonal variations are thought to be caused by changes in cell cycle or genomic control as a result of the transition of differentiated to undifferentiated cells during tissue culture. These variations could hinder our ability to select for the best possible lines in the early generations. Other studies have shown that the transformation process could affect the health of transgenic plants and produce a negative response of spikelet yield, panicle emergence, fertility and tiller number (Larkin and Scowcroft, 1981; Brauer *et al.,* 2011). In all the soil and hydroponics experiments, plants have undergone some form of nutrient or growth chamber fluctuations that may have induced stress to the plants. AGR1/7, known to have increased biomass, tillering and seed yield compared to NB in previous studies, exhibited no significant differences in these studies.

However, the two Ap lines (Ap18 and 28) have consistently outperformed NB over all the generations regardless of any environmental or nutrient stress present. Taking all of this into consideration, it is still important for us to carry out further work to confirm the NUE phenotype produced by the over-expression of *HvAlaAT* by the *PBpr1* promoter.

Table 2.1: Plant height, biomass and seed yield of T_0 Ap plants and untransformed Nipponbare plants. Plant lines selected for continued experiments are shaded.

Lines	T_0 plant	Plant height (cm)	Total above ground biomass (g)	Seed yield (g)	Biomass without seeds (g)
PBpr1 -1-1	Ap1	81.0	14.0	3.88	10.12
PBpr1 -1-2	Ap2	85.7	12.7	5.56	7.14
PBpr1 -2-1	Ap3	86.9	15.0	3.67	11.33
PBpr1 -2-2	Ap4	89.2	17.5	6.38	11.12
PBpr1 -3-1	Ap5	73.2	11.8	3.14	8.66
PBpr1 -3-2	Ap6	82.3	17.0	7.81	9.19
PBpr1 -3-3	Ap7	80.0	18.8	7.33	11.47
PBpr1 -4-1	Ap8	70.6	8.9	1.10	7.80
PBpr1 -4-2	Ap9	66.3	9.8	1.44	8.36
PBpr1 -4-3	Ap10	86.5	11.6	3.13	8.47
PBpr1 -4-4	Ap11	69.6	9.9	1.85	8.05
PBpr1 -4-5	AP12	69.2	12.5	3.34	9.16
PBpr1 -4-6	Ap13	72.5	8.5	2.78	5.72
PBpr1 -4-7	Ap14	80.8	13.2	3.37	9.83
PBpr1 -4-8	Ap15	79.2	14.2	4.27	9.93
PBpr1 -4-9	Ap16	74.0	12.4	4.04	8.36
PBpr1 -4-10	Ap17	73.8	17.9	7.33	10.57
PBpr1 -4-11	Ap18	84.3	17.7	10.01	7.69
PBpr1 -4-12	Ap19	87.2	20.3	8.31	11.99
PBpr1 -4-13	Ap20	85.0	17.8	7.01	10.79
PBpr1 -4-14	Ap21	78.5	12.2	4.62	7.58
PBpr1 -4-15	Ap22	78.0	12.1	3.49	8.61
PBpr1 -4-16	Ap23	79.1	13.8	4.00	9.80
PBpr1 -4-17	Ap24	86.5	17.2	6.54	10.66
PBpr1 -4-18	Ap25	67.3	13.5	4.25	9.25
PBpr1 -4-19	Ap26	80.2	15.9	5.51	10.39
PBpr1 -4-20	Ap27	85.4	11.7	4.16	7.54
PBpr1 -4-21	Ap28	86.0	17.4	7.33	10.07
PBpr1 -4-22	Ap29	76.0	21.4	7.50	13.9
PBpr1 -4-23	Ap30	82.1	11.6	3.60	8.00
PBpr1 -4-24	Ap31	83.2	11.9	3.10	8.80
PBpr1 -4-25	Ap32	76.2	10.6	2.85	7.75
PBpr1 -4-26	Ap33	79.4	8.7	1.80	6.90
PBpr1 -4-27	Ap34	79.8	13.2	3.78	9.42
PBpr1 -4-28	Ap35	78.5	13.0	3.41	9.59

PBpr1 -4-29	Ap36	90.1	16.0	6.20	9.80
PBpr1 -4-30	Ap37	71.0	8.1	2.07	6.03
PBpr1 -4-31	Ap38	84.8	10.2	1.51	8.69
PBpr1 -4-32	Ap39	71.2	11.6	2.37	9.23
average		79.2	13.6	4.46	9.17
standard deviation(SD)		6.4	3.38	2.19	1.65
Lines		Plant height (cm)	Total above ground biomass (g)	Seed weight (g)	Biomass without seeds (g)
Nipponbare 1	NA	79.4	12.1	4.78	7.32
Nipponbare 2	NA	80.9	8.6	2.14	6.46
Nipponbare 3	NA	77.1	16.4	7.23	9.17
Nipponbare 4	NA	80.3	12.8	5.16	7.64
Nipponbare 5	NA	75.3	11.1	3.78	7.32
Nipponbare 6	NA	64.2	9.3	1.96	7.34
Nipponbare 7	NA	61.5	11.6	2.28	9.32
Nipponbare 8	NA	73.2	10.1	2.10	8.00
Nipponbare 9	NA	76.0	10.4	2.59	7.81
Nipponbare 10	NA	71.4	7.1	2.31	4.79
Nipponbare 11	NA	76.6	10.9	5.42	5.48
Nipponbare 12	NA	78.0	11.3	4.74	6.56
Nipponbare 13	NA	68.2	8.9	3.36	5.54
Nipponbare 14	NA	85.5	10.4	4.29	6.11
Nipponbare 15	NA	77.6	10.5	4.13	6.37
Nipponbare 16	NA	78.5	13.5	6.29	7.21
Average		75.2	10.9	3.91	7.03
SD		6.3	2.17	1.63	1.25

Table 2.2: T_2 and T_3 Ap lines screened for homozygosity using hygromycin. Germination frequency was derived by assaying 12 seeds from each package, germinated on MS media without hygromycin to determine seed viability. The germination frequency is then used to determine the proportion of plants that fail to germinate that was not attributed to the lack of hygromycin resistance. Only Ap17 and Ap19 T_3 lines were tested on media without hygromycin. Homozygous lines have been highlighted

	Germination frequency	Total germinated on hygromycin	Total seeds plated for germination	Total seeds expected to germinate	Corrected % germinated on hygromycin
Ap7-1	-	25	36	-	69
Ap7-3	-	26	36	-	72
Ap7-4	-	25	36	-	69
Ap7-6	-	26	36	-	72
Ap7-10	-	25	36	-	69
Ap7-12	-	36	36	-	100
Ap7-13	-	26	36	-	72
Ap7-15N	-	0	36	-	0
Ap7-17N	-	0	24	-	0
Ap7-20	-	30	36	-	83
Ap17-10-1	100%	36	36	36	100
Ap17-10-3	100%	19	36	36	53
Ap17-10-5	80%	24	37	30	81
Ap17-10-6	75%	19	37	28	68
Ap17-10-7	67%	19	36	24	79
Ap18-2N	-	0	12	-	0
Ap18-6	-	25	36	-	69
Ap18-11	-	35	36	-	97
Ap18-12	-	26	36	-	72
Ap18-17	-	18	36	-	50
Ap18-19	-	30	36	-	83
Ap19-1	-	29	36	-	80
Ap19-3	-	30	36	-	83
Ap19-4	-	32	36	-	88
Ap19-5	-	32	36	-	88
Ap19-7N	-	0	36	-	0
Ap19-10	-	10	12	-	83
Ap19-12	-	20	36	-	55

Ap19-16	-	26	36	-	72
Ap19-17	-	25	36	-	69
Ap19-17-1	100%	30	36	36	83
Ap19-17-2	100%	36	36	36	100
Ap19-17-3	91%	29	36	33	89
Ap19-17-4	91%	30	36	33	92
Ap19-17-6	91%	31	40	36	85
Ap28-2	-	36	36	-	100
Ap28-3	-	35	36	-	97
Ap28-7	-	28	36	-	78
Ap28-8	-	16	24	-	67
Ap28-9	-	27	36	-	75
Ap28-10	-	30	36	-	83
Ap28-31	-	26	36	-	72
Ap28-15N	-	0	36	-	0
Ap28-18	-	31	36	-	86
Ap28-20	-	24	36	-	67
AGR1/7-16-6	100%	12	12	12	100
AGR1/7	-	12	12	-	100
NB-7-6	100%	0	14	14	0
NB-20(hyg)	-	0	24	-	0
NB-20 (no hyg)	100%	12	12	12	100

Table 2.3: The NUE of T_2 homozygous Ap lines, AGR1/7 and Nipponbare (NB) plants derived from seed yield per gram of nitrogen applied per plant and the above ground biomass without seed. Each data point is taken from at least 4 replicates. Ap28-2 and Ap28-3 are siblings of the same line.

NUE= Grain yield/ N applied

	Above ground biomass without seed (g)	Seed yield (g)	NUE (yield .unit applied^{-1})
Ap17-10	18.26	8.55	43.63
Ap18-11	18.03	8.29	42.27
Ap19-17	21.58	6.73	34.33
Ap28-2	24.20	8.83	45.07
Ap28-3	17.38	6.15	31.36
NB	19.75	7.48	38.16
AGR1/7	19.94	7.89	40.23

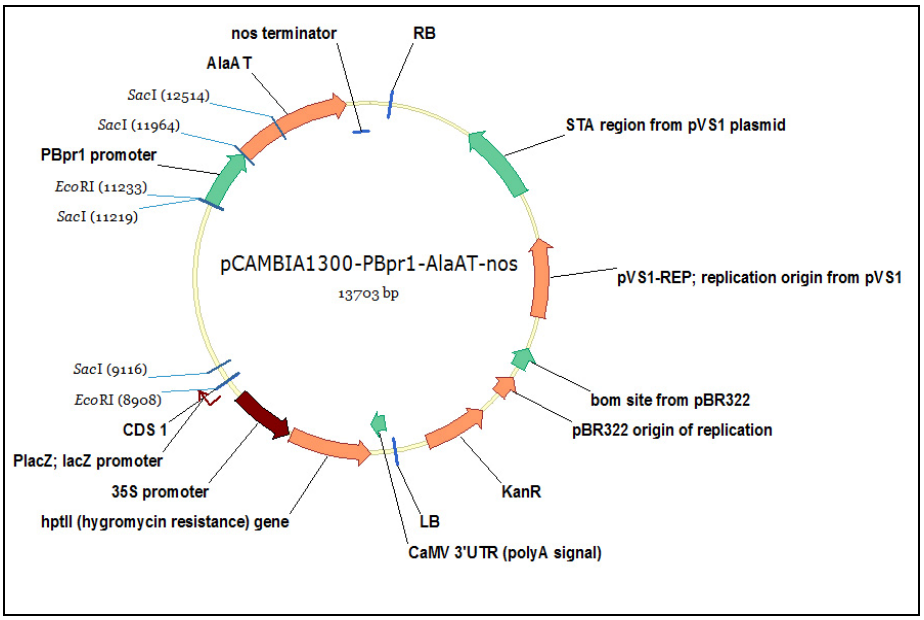


Figure 2.1: Construct map of *PBpr1:: HvAlaAT* in pCAMBIA1300. The HvAlaAT is driven by the *PBpr1* promoter and terminated by a nos terminator. The selection marker is kanamycin for plasmid selection and hygromycin for plant selection.

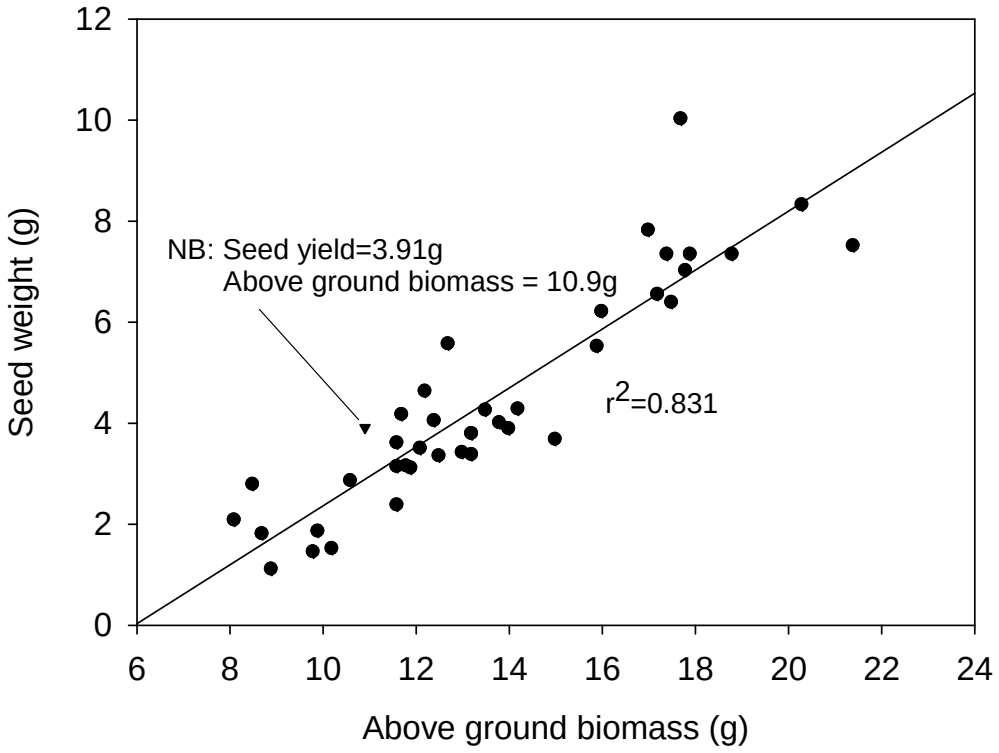


Figure 2.2: Correlation between seed yield (g) and above ground biomass (g) of 39 T_0 Ap plants at maturation. Each data point refers to an individual T_0 plant and NB is the average of wild-type NB plants.

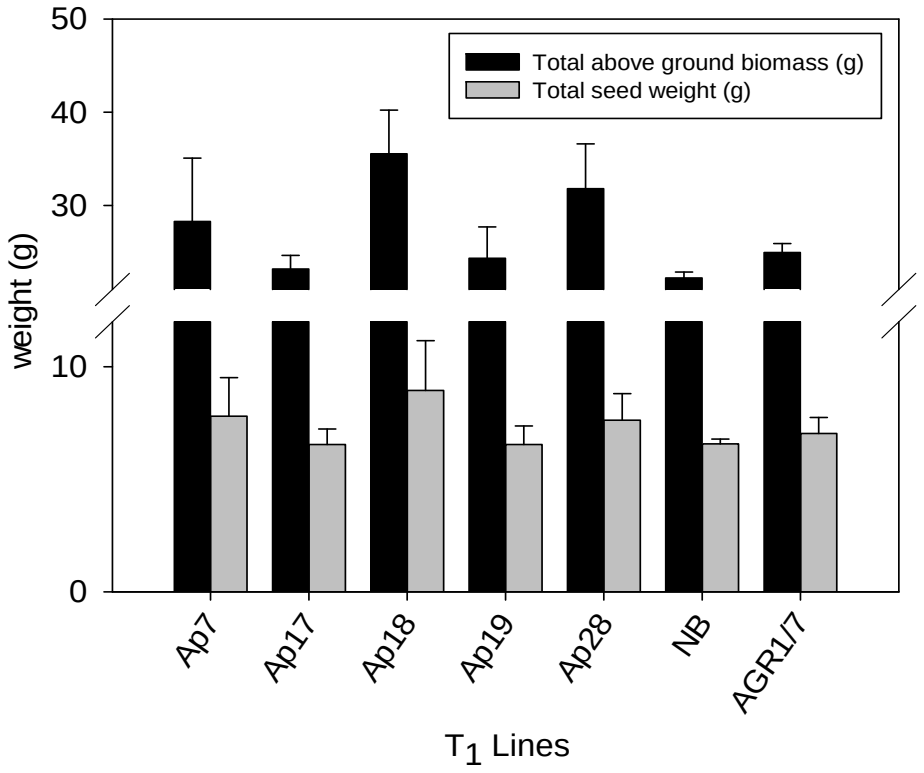


Figure 2.3: Comparison of total above ground biomass (g) and seed yield (g) of T_1 Ap lines, NB and AGR1/7 at maturation. Each measurement is from 5 to 7 replications and error bars show standard deviations.

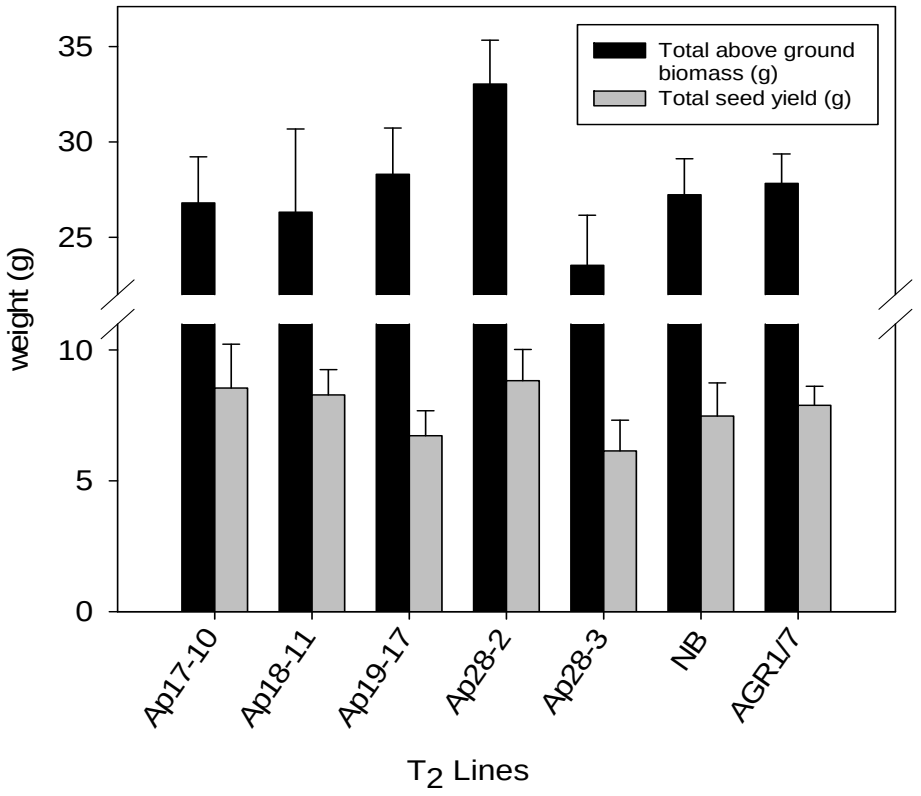


Figure 2.4: Comparison between T_2 Ap plants, NB and AGR1/7 above ground biomass (g) and seed yield (g) at maturity. Values from each line are from 7 replications and error bars represent SD.

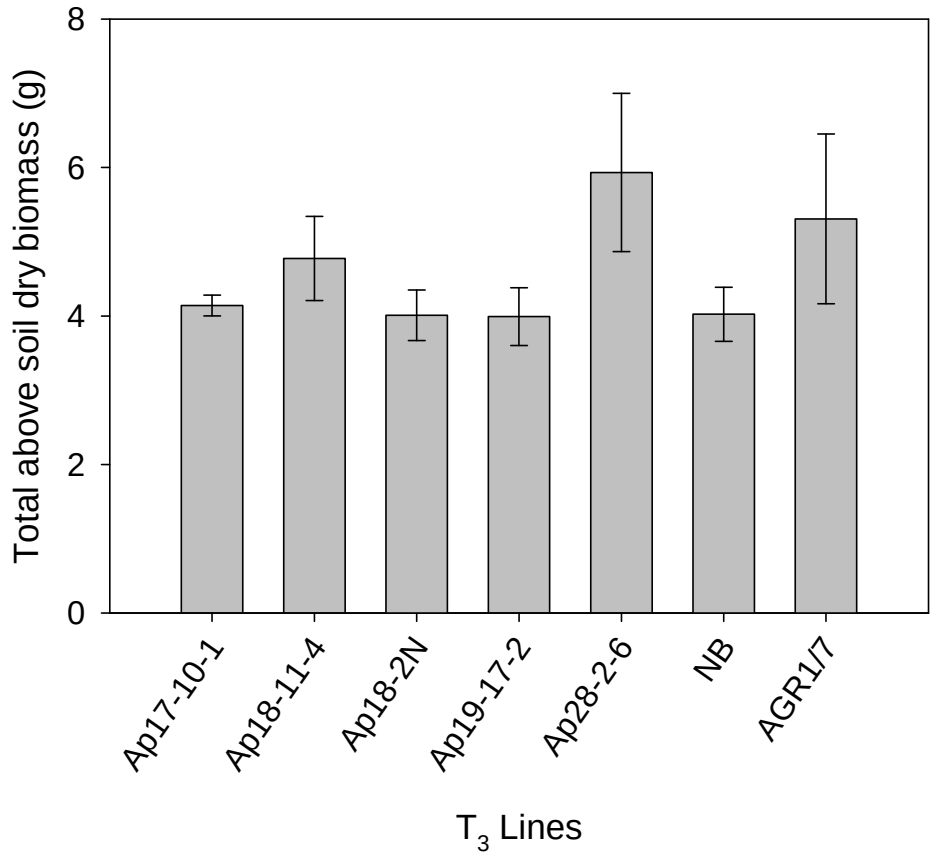


Figure 2.5: Dry above ground biomass (g) of T_3 Ap lines at 52 DAG. Each line was represented by 5 replication plants and error bars show SD.

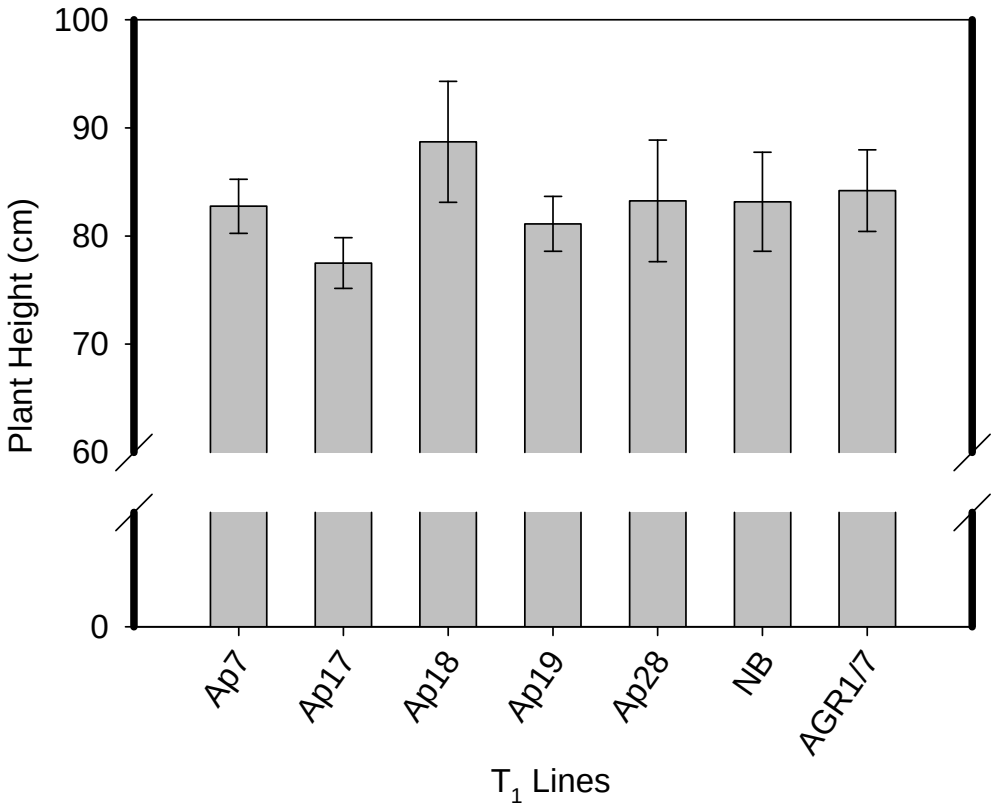


Figure 2.6: Plant height (cm) of select T_1 Ap plants compared to NB and AGR1/7. Each value is represented by 5 replications, and error bars show SD.

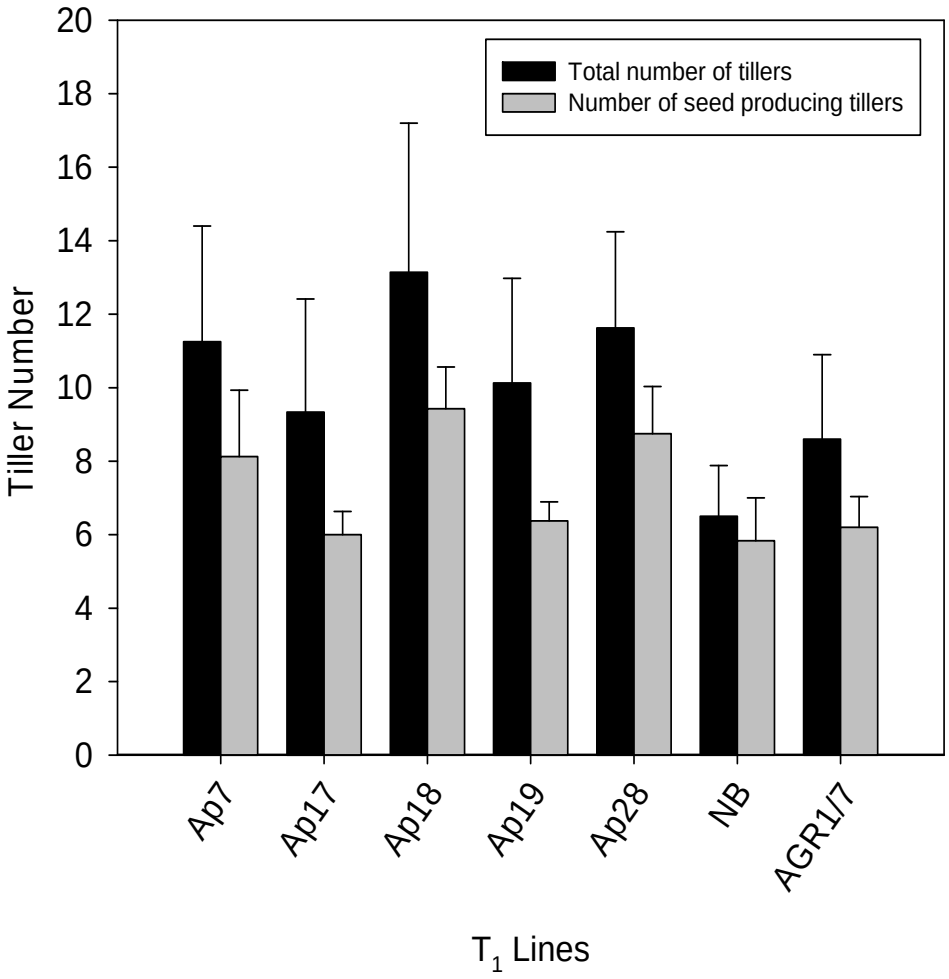


Figure 2.7: Number of tillers and productive tillers produced by T_1 Ap plants compared to NB and AGR1/7 at maturation. Each measurement refers to an average of at least 4 replications and error bars show SD.

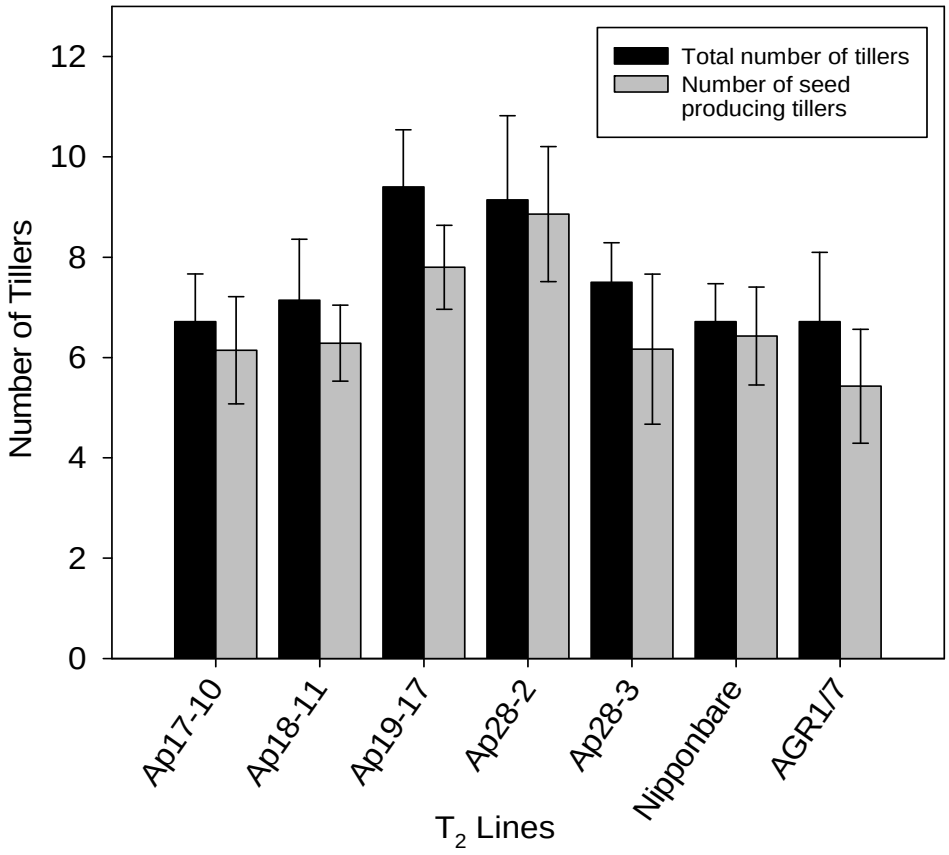


Figure 2.8: Total number of tillers and seed producing tillers of T_2 Ap plants in comparison to AGR1/7 and NB at maturation. Ap28-2 and Ap28-3 are siblings of the same line. Each data point represents an average of 7 replicates and error bars show SD.

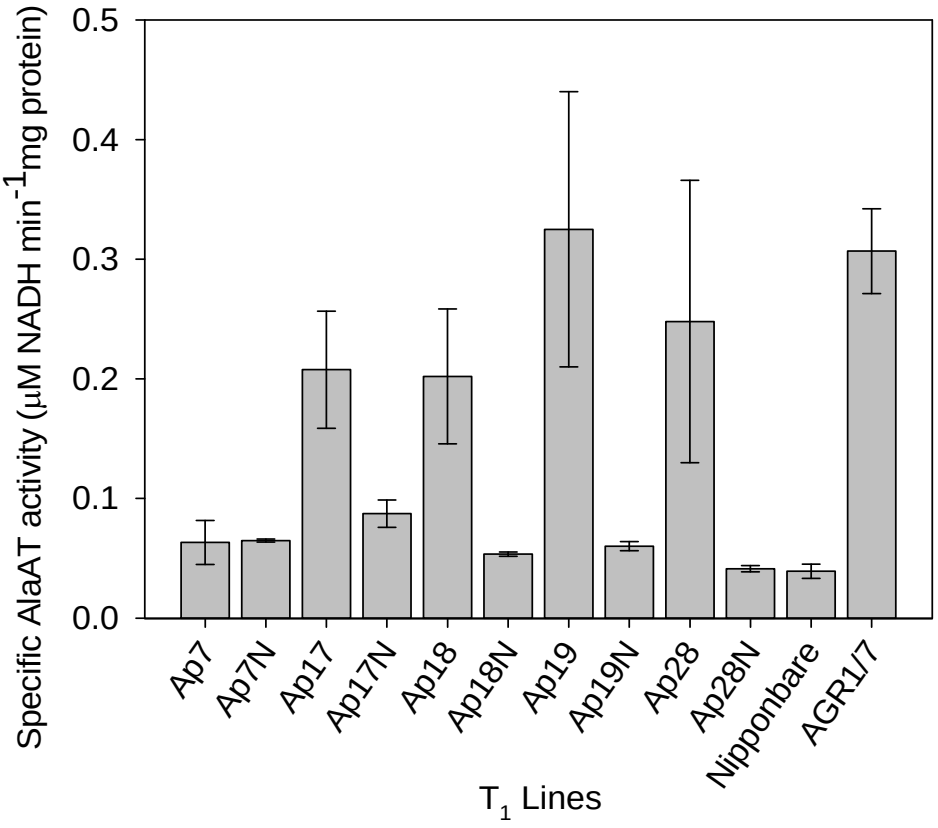


Figure 2.9: Shoot specific AlaAT activity of T_1 Ap lines grown in soil at 40 days after germination (DAG) compared to NB and AGR1/7. All lines labeled with N are null segregants. Each data point for the transgenic plants was taken from an average of 3 replications and error bars show the SD. Only 1 null plant was tested, and here error bars refer to the SD of technical replicates of the enzyme assays.

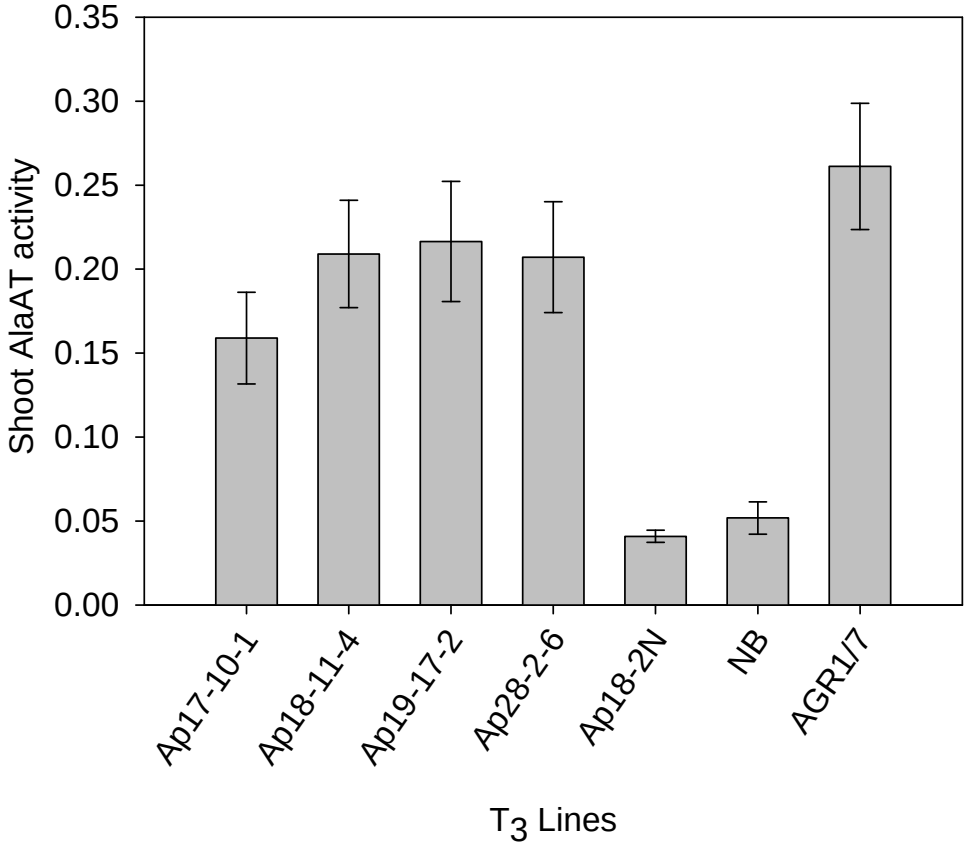


Figure 2.10: AlaAT activity of soil grown T_3 Ap, NB and AGR1/7 shoots at maximum tillering stage (52 DAG). The same tissue was evaluated for transcript profiles in Figure 2.17. Each data point refers to an average of 5 replications and the error bars refer to the SD.

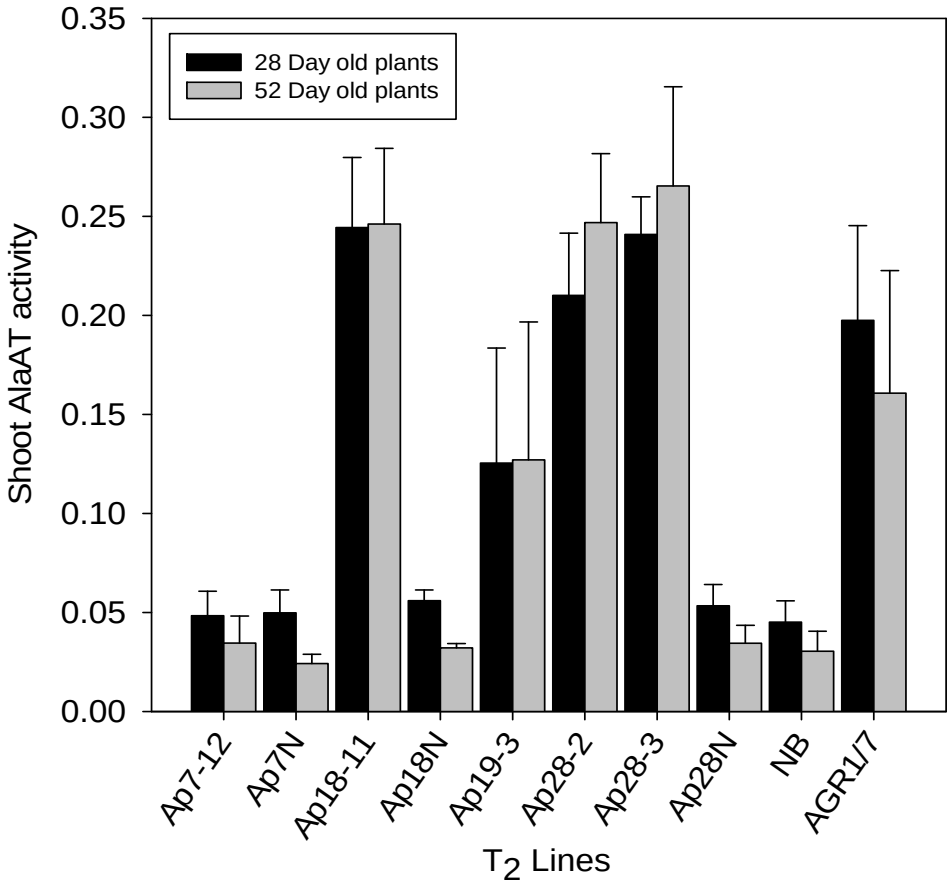


Figure 2.11: Shoot AlaAT activity of T_2 Ap lines, NB and AGR1/7 at 28 and 52 DAG (days after germination). Plants were grown hydroponically in Trostle nutrient solution. Ap 28-2 and Ap28-3 are siblings of the same line. Each data point referres to 5 replications and error bars refer to their SD. The same tissue was tested for transcript profiles in Figure 2.18

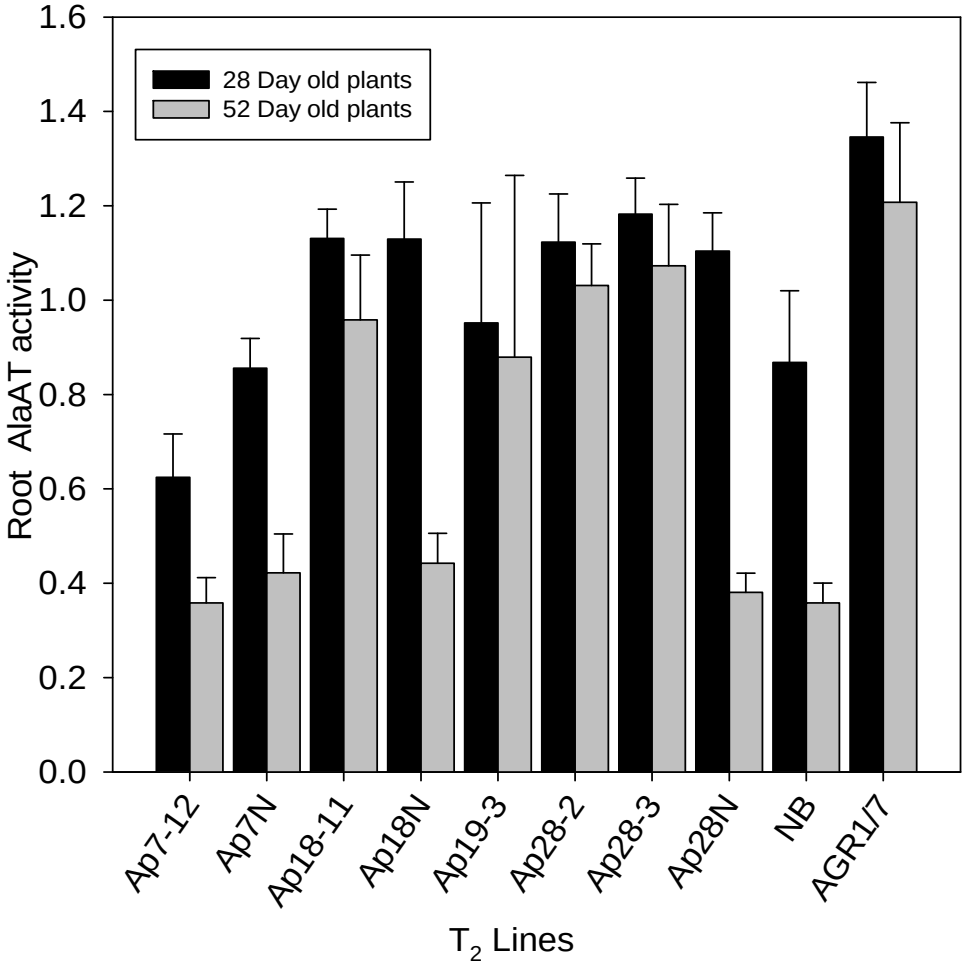


Figure 2.12: Root AlaAT activity of T_2 Ap lines, NB and AGR1/7 at 28 and 52 DAG (days after germination). Plants were grown hydroponically in Trostle nutrient solution. Ap 28-2 and Ap28-3 are siblings of the same line. Each data point referres to 5 replications and error bars refer to their SD. The same tissue was tested for transcript profiles in Figure 2.18

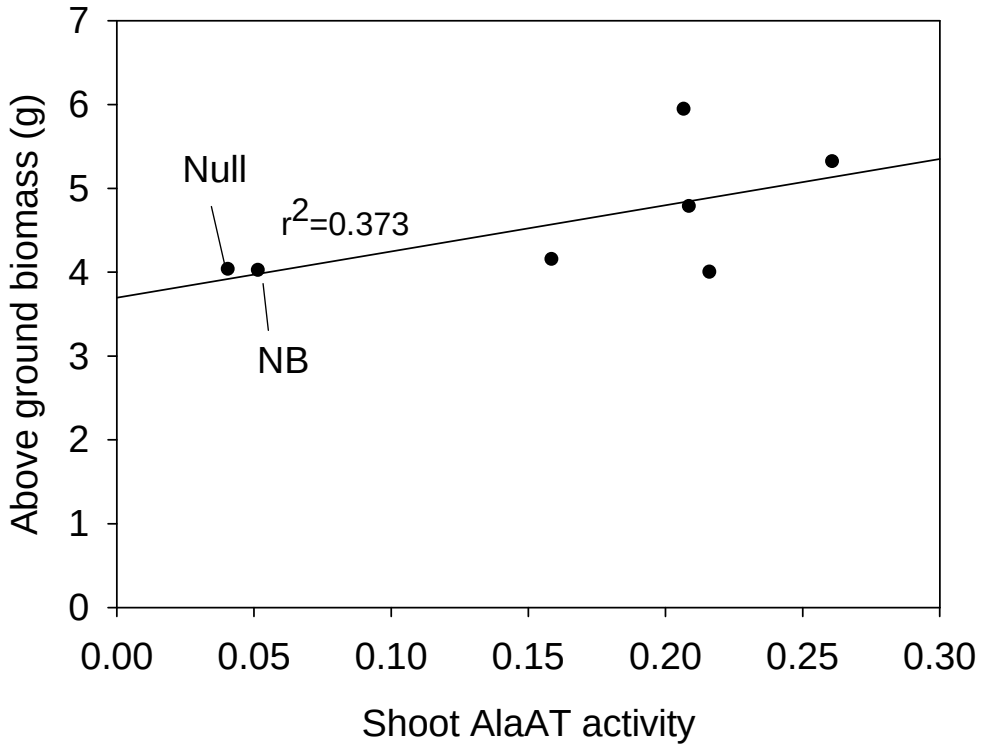


Figure 2.13: Correlation between shoot AlaAT activity and above ground biomass at 52 DAG of T_3 Ap plants, AGR1/7 and NB. Each data point for above ground biomass and AlaAT activity referrs to an average of 5 replications. SDs of above ground biomass is visualized in Figure 2.5 while SDs of shoot AlaAT activity is visualized in Figure 2.10.

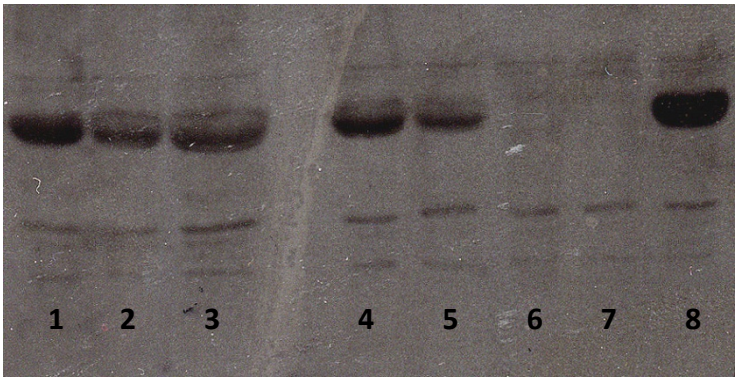


Figure 2.14: Immunoblot analysis of protein extracts of 40 DAG T_1 Ap rice lines detected with HvAlaAT specific ary antibody. Lane 1 to 3 are siblings of line Ap19: Ap19-5. Ap19-12 and Ap19-15. Lane 4 and 5 are siblings of Ap28: Ap28-3 and Ap28-20. Lane 6 is the null segregant, Ap19N, while lane 7 is Nipponbare and lane 8 is AGR1/7. Protein concentration was standardized across all lanes by using the Bradford protein quantification assay.

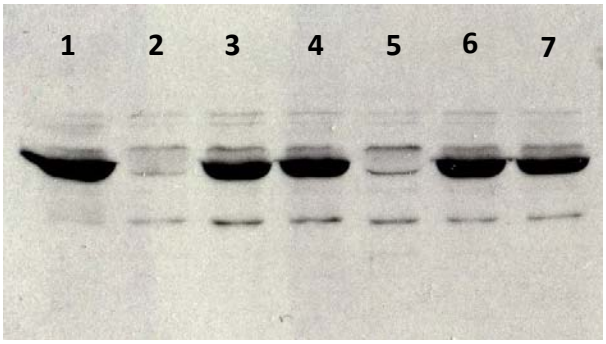


Figure 2.15: Immunoblot analysis of protein extracts of 52 day old T_3 Ap lines detected with HvAlaAT-2 specific 1^o antibody serum. Lane 1: AGR1/7, lane 2: wild-type Nipponbare, lane 3: Ap 17-10-1, lane 4: Ap18-11-4, lane 5: Ap18-2N, lane 6: Ap19-17-2 and lane 7: Ap 28-2-6. Protein concentration was standardized across all lanes by using the Bradford protein quantification assay.

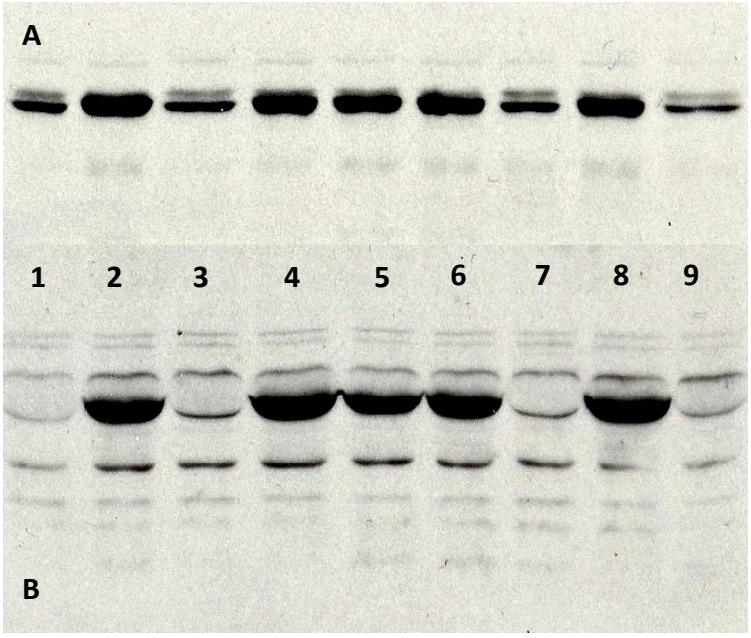


Figure 2.16: Western blot of protein extract from T_2 hydroponically grown roots (A) and shoots (B) of 28 DAG rice lines with HvAlaAT 1^{o} antibody detection to show amounts of *HvAlaAT* protein in each sample. Lane 1: Ap7-12, lane 2: Ap18-11, lane 3: Ap18-2N, lane 4: Ap28-2, lane 5: Ap28-3, lane 6: Ap28-3 replicate, lane 7: Ap28-18N, lane 8: AGR1/7 and lane 9: wild-type Nipponbare. Protein concentration was standardized across all lanes by using the Bradford protein quanitification assay.

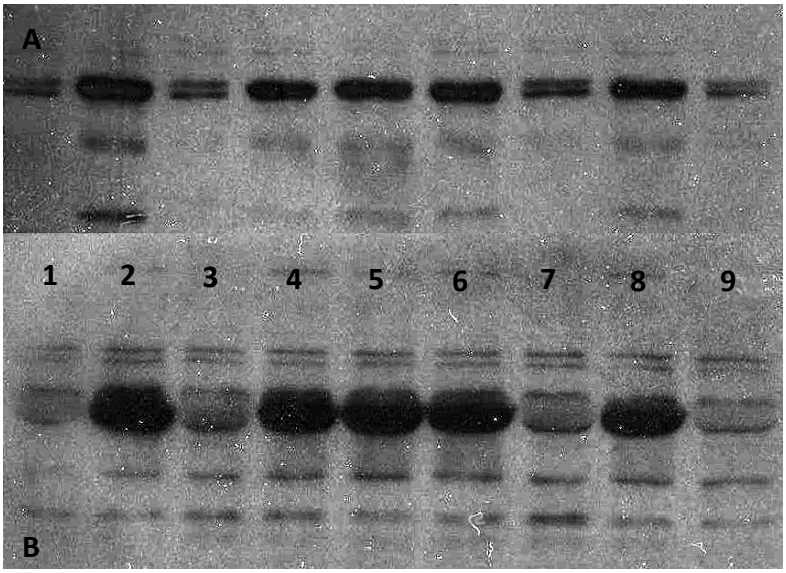


Figure 2.17: Immunoblot of protein extract from T_2 hydroponically grown roots (A) and shoots (B) of 52 DAG rice lines with *HvAlaAT* 1^o antibody detection to show amounts of *HvAlaAT* protein in each sample. Lane 1: Ap7-12, lane 2: Ap18-11, lane 3: Ap18-2N, lane 4: Ap28-2, lane 5: Ap28-3, lane 6: Ap28-3 replicate, lane 7: Ap28-18N, lane 8: AGR1/7 and lane 9: Nipponbare. Protein concentration was standardized across all lanes by using the Bradford protein quanitification assay.

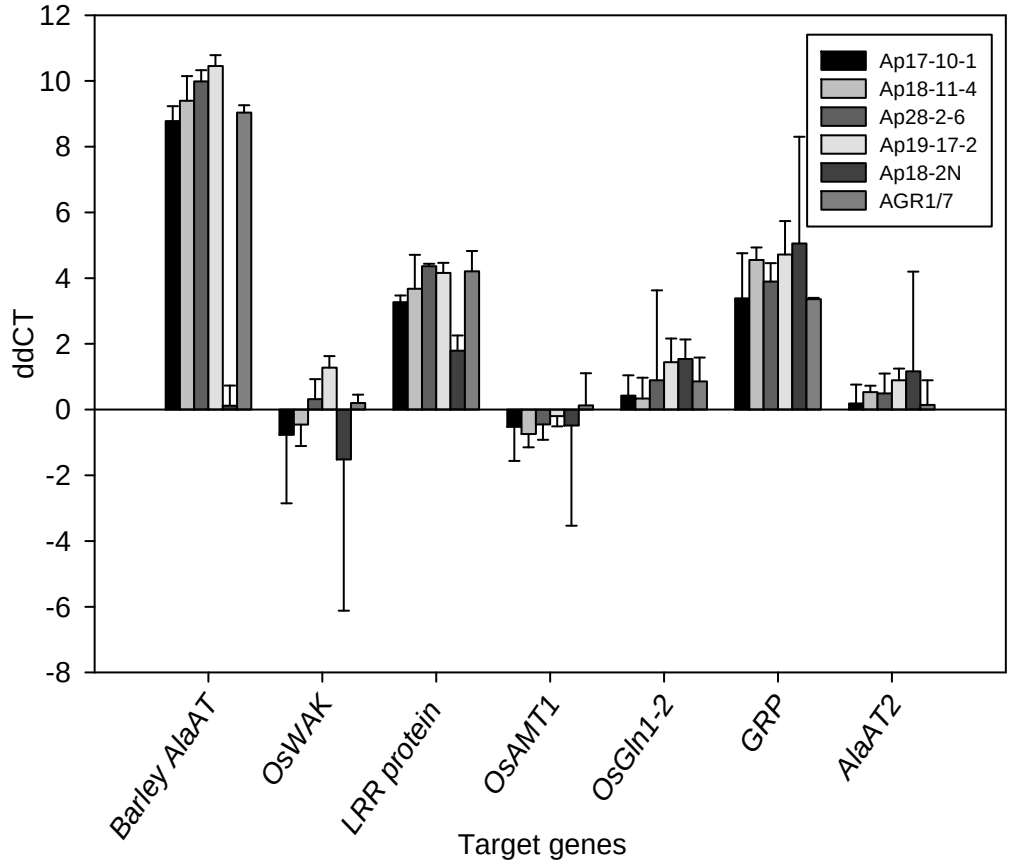


Figure 2.18: Log 2 of the relative quantification (ddCT) of shoots of 4 transgenic T_3 Ap lines, AGR1/7 and null segregants of Ap lines relative to NB plants. 7 transcripts (*HvAlaAT :* Barley AlaAT*, GRP:* Glycine rich protein*, AlaAT2* = rice nascent alanine aminotransferase2*, LRR:* Leucine rich repeat*, OsWAK101:* rice wall associated kinase*, OsGln1-2*: rice cytosolic glutamine synthetase 1 and *OsAMT* : rice ammonium transporter) were measured by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). Plants were raised in hydroponic conditions and harvested at 52 days afters germination. Tissue samples used were the same as those of the AlaAT activity assays in Figure 2.12. (2 fold change represents 1 ddCT. $Log_2 2=1$) All samples that had less than 2 fold change were considered not significant.

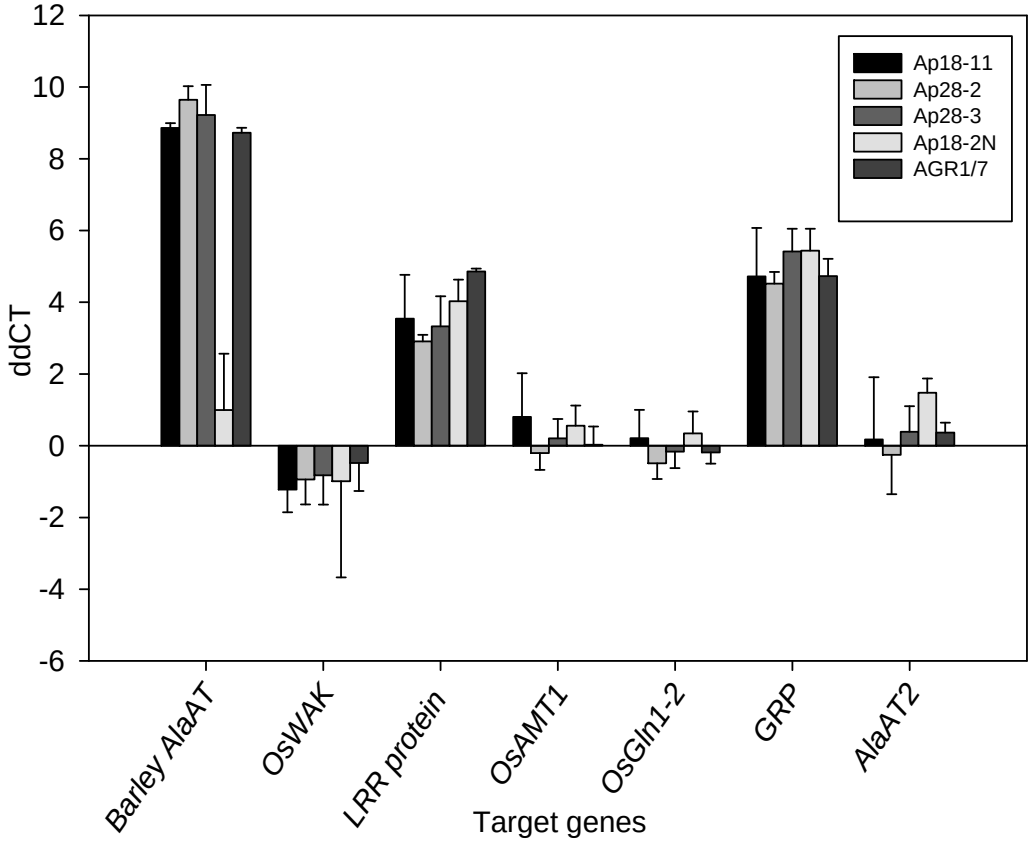


Figure 2.19: Log 2 of the relative quantification (ddCT) of shoots of 2 transgenic T_2 Ap lines (one of the two lines has two siblings analyzed), AGR1/7 and null segregants of Ap lines relative to NB plants. 7 transcripts as mentioned in Figure 2.18 measured by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). Plants were raised in hydroponic conditions and harvested at 52 days afters germination. Tissue samples used were the same as those of the AlaAT activity assays in Figure 2.12. (2 fold change represents 1 ddCT. $Log_2 2=1$) All samples that had less than 2 fold change was considered not significant.

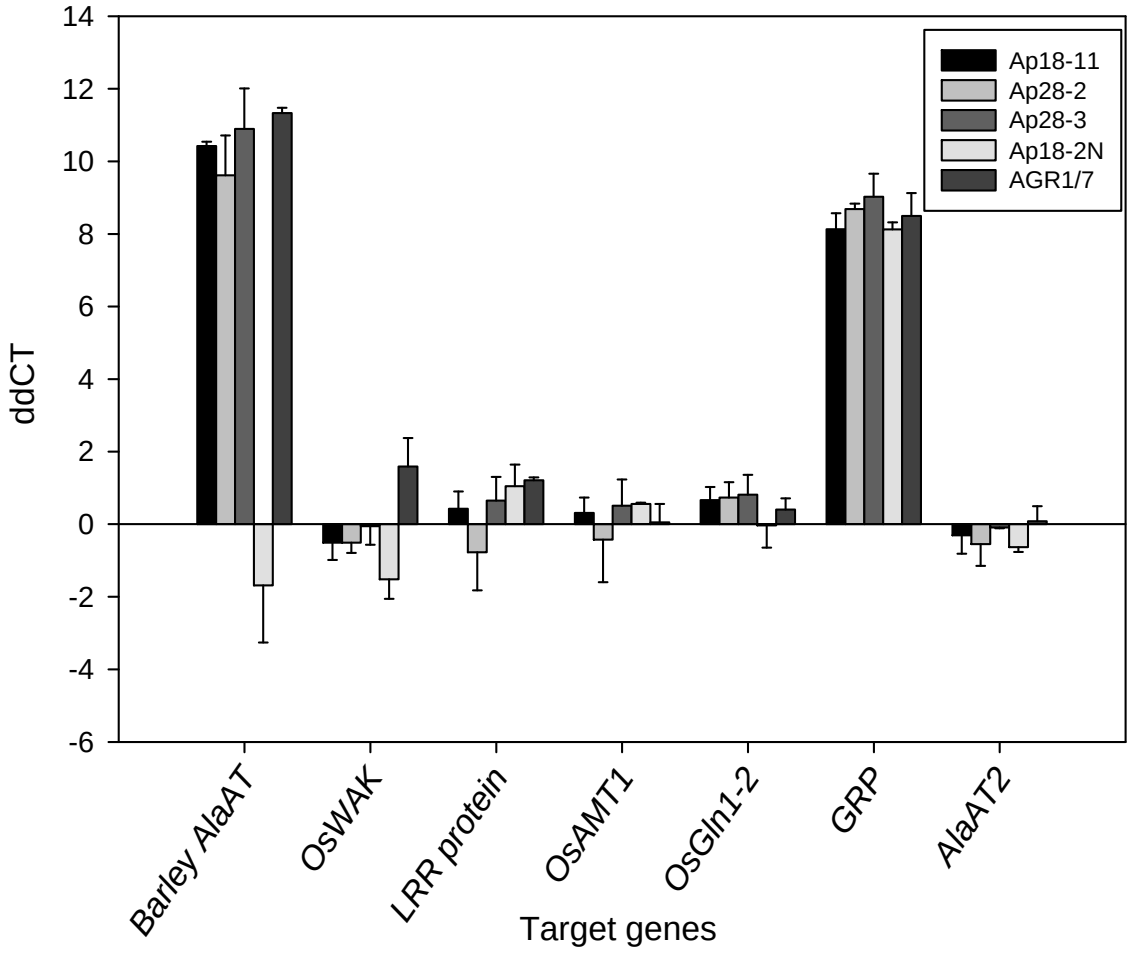


Figure 2.20: Log 2 of the relative quantification (ddCT) of roots of two transgenic T_2 Ap lines (one of the two lines have two siblings analysed), AGR1/7 and null segregants of Ap lines relative to NB plants. 7 transcripts as mentioned in Figure 2.18 measured by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). Plants were raised in hydroponic conditions and harvested at 52 days after germination. Tissue samples used were the same as those of the AlaAT activity assays in Figure 2.12. (2 fold change represents 1 ddCT. $Log_2 2=1$) All samples that had less than 2 fold change were considered not significant.

Chapter 3: The *PBpr1* promoter bioinformatics and pattern expression analysis

3. Introduction

The strength and efficacy of gene expression is often dependent on the promoter. While plant promoter studies have become common in recent years, there are still no "rules" that dictate a promoter's strength or efficacy in driving gene expression. The function of specific promoter elements and motifs are still largely unknown and conditional upon many other factors such as position in the genome, copies of other elements nearby and the organisms' genetic background.

Many studies to date commonly have used a constitutive promoter to drive gene expression. This could result in abnormal development of the plants or frequently produce no phenotype at all. In recent years, tissue specificity in a promoter is often desired because it allows for the controlled expression of genes at a specific organ or developmental stage which may drastically affect the phenotype produced.

The *PBpr1* and *OsANT1* belong to a family of aldehyde dehydrogenases. By bioinformatic studies and gene annotations, *PBpr1* was identified to be the promoter of a methyl malonate semialdehyde dehydrogenase (MMSDH) gene. MMSDH mRNA has been found at high levels in roots and leaf sheaths while protein accumulation was highest in roots, followed by leaf blades (Oguchi *et al.,* 2004). *PBpr1* consistently over-expressed *HvAlaAT* and increased NUE in rice plants as described in Chapter 2 of this thesis.

This chapter describes the *in silico* analysis of the *PBpr1* promoter for promoter core elements or motifs and cis-acting regulatory elements using detection software available on the internet. In addition, an attempt to elucidate *PBpr1*'s tissue specificity and analysis of pattern expression is also described in the later part of this chapter by placing a GUS reporter construct under the influence of *PBpr1*.

3.1. Materials and Methods

3.1.1. Bioinformatics analysis of promoter

The *PBpr1* gene was selected based on homology to the *OsANT1* promoter using NCBI's BLASTn program, and it was further investigated using the gene bioinformatic tools of NCBI Genbank and Refseq. The *PBpr1* promoter was analyzed for promoter motifs using PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html), the Plant promoter database (PPDB) 2.1 (http://ppdb.agr.gifu-u.ac.jp/ppdb/cgi-bin/index.cgi), Softberry TSSP (http://linux1.softberry.com/berry.phtml?topic=tssp&group=programs&subgroup=promoter), TSSP-TCM (http://mendel.cs.rhul.ac.uk/mendel.php?topic=fgen) and Plant Cis-acting Regulatory Elements (PLACE) (http://www.dna.affrc.go.jp/PLACE/).

3.1.2. GUS histochemical staining

T_1 seeds of *PBpr1::GUSplus* transgenic lines, named ApG lines, were sterilized and germinated in sterile liquid MS (4.4 g L^{-1}, pH 5.8). Due to the

heterozygosity of the T_1 seeds, a total of 20 seeds were germinated for staining. From each line, five seeds were selected at each sampling time for staining. Seedlings were sampled at 3 DAG and 7 to 10 DAG. This allowed for some degree of characterization of *PBpr1*'s temporal expression. Seedlings were removed from the Magenta jar and immersed in 90% acetone to permeabilize plant samples for 1 hour. They were washed twice in 100mM $NaHPO_4$ pH 7.5, then immersed in GUS staining buffer (100mM $NaHPO_4$, 0.5mM Potassium Ferricyanide, 0.5mM Potassium Ferrocyanide, 2mM X-Gluc (Rose Scientific, Edmonton, AB)) for up to 24 hours. Stained seedlings were fixed in 3:1 glacial acetic acid: 100% ethanol to prevent GUS stain diffusion and also to remove all the chlorophyll in the shoots. Additional changes of 3:1 glacial acetic acid : 100% ethanol were used until all the chlorophyll was removed. Then, seedlings were stored in 70% ethanol at 4^oC. Stained seedlings were viewed under the light microscopeat varying magnification to determine the expression pattern of the *PBpr1* promoter. In order to clear tissues, 8:3:1 chloral hydrate : water : glycerol was used to visualize internal structures if necessary.

3.2. Results

3.2.1. Bioinformatics analysis of PBpr1 promoter

The *PBpr1* promoter was analyzed using PlantCARE, PLACE, Softberry TSSP, TSSP-TCM and Plant promoter DB (PPDB) for promoter elements detection. Resulting from the promoter analysis, none of the elements predicted were based on known rice promoter elements, however, the elements identified

were homologous to regulatory elements identified in other cereal crops or *Arabidopsis*. Elements that were identified by both programs were considered as a strong indication for the presence of a promoter element, but the importance of these predictions can only be proven with further experimentation.

3.2.1.1. Core promoter elements

TSSP-TCM, PPDB and Softberry TSSP make use of algorithms and a known list of transcription start sites (TSS) motifs to detect the presence of any TSS in promoters (TSSP). Only Softberry TSSP predicted the presence of a TSS while both PPDB and TSSP-TCM did not yield any reliable hits. The TSS predicted by Softberry showed transcription initiation at the 128 bp position (Figure 3.1) and is associated with a predicted TATA box 14 bp upstream of the predicted transcriptional start site (Table 3.1).

TATA boxes are known to be the most conserved functional signal in eukaryotic promoters and are generally believed to be involved in the assembly of the RNA polymerase complex and dictating the site for the start of transcription. A functional TATA box is usually located 25-50 bp upstream of a TSS. A total of eight other TATA boxes were predicted by PlantCARE, two predicted by PPBD, two predicted by PLACE in *PBpr1* (Table 3.1). However most of these predictions (e.g. PlantCARE detected a TATA box in the sense strand and PPBD on the antisense strand at 462 bp of the promoter and therefore ~300 bp upstream of the *HvAlaAT*'s ATG), were sufficiently far upstream to make them unlikely to be the core TATA box.

PPDB was the only software used that was able to detect Y-patches (also known as pyrimidine patches). The Y-patch was recently identified and designated to be a core promoter element by Yamamoto *et al.* (2007). The Y-patch is a T/C rich motif that is commonly found in Arabidopsis and rice. The function of the Y-patch is still unknown but it is speculated to be highly involved in transcription initiation due to its prevalence in plant promoter regions. Two Y-patches were found, one about 10 bp upstream of the closest TATA box to the ATG while the other was found at 82 bp position on the antisense strand (Figure 3.1).

Eight unique CAAT boxes were predicted by both PLACE and PlantCARE while other software did not detect the presence of them (Table 3.1). CAAT boxes may be closely related to the strength and efficacy of the promoter and are frequently found ~70 bp upstream of the TSS (Klug and Cummings, 1991). *Arabidopsis* and *Brassica napus* CAAT boxes are scattered throughout the *PBpr1* promoter and are often within ~100 bp of a predicted TATA box or TATA like element: a CAAT box was found to be ~58 bp upstream of the TATA box closest to the ATG (Figure 3.1).

3.2.1.2. Environmental responsive and enhancer elements

Based on the promoter analysis performed by Softberry, there is a rice CGACG element known to be involved in α-amylase expression, 1 bp upstream of the designated TATA box. A rice Pyr-box CCTTTT responsive to gibberellic acid (GA) was predicted at 30 bp position. The same Pyr-box element was also

predicted by PLACE. By Softberry TSSP analysis all the enhancer elements were found on the first 150bp of the 5' end of the promoter.

Two major regions of enhancer elements were found at ~586 bp and ~675 bp (~50 bp upstream of the ATG) in the *PBpr1* promoter by PLACE and PlantCARE. Both PlantCARE and PPBD predicted light responsive cis-acting regulatory element GCCACGTC， abscisic acid responsive element (ABRE), a CGTCA methyl jasmonic acid (MeJA) responsive element, an ACGT core, and a TGA box, predicted to be part of an auxin responsive element by both PlantCARE and PLACE. A TC rich repeat was also predicted by PlantCARE and PLACE at the ~200 bp upstream region of the ATG.

By analyzing the *PBpr1* promoter using PlantCARE, there are 10 elements categorized into five motifs in the promoter that were predicted to be light responsive elements. There are also six elements categorized into two motifs that were identified as MeJA responsive cis-regulatory elements. Similar results were also obtained when analysis was carried out using PLACE. By using PPDB analysis, there were four PCNAII elements found in the entire promoter. PLACE also predicted *PBpr1* to have seven GATA boxes which are known to be light regulated and involved in tissue specific expression in petunias when placed between a CAAT box and a TATA box (Gidoni *et al.,* 1989).

3.2.2. GUS histochemical staining of ApG plants

ApG plants were initially screened by GUS staining at day 7 to determine lines that exhibit GUS expression. Of the 25 lines, 19 lines were positive for GUS

staining and four lines were selected to be further tested. Three and 7 to 10 day old ApG plants were stained for GUS expression to determine the expression pattern of the *PBpr1* promoter at the seedling stage (Figure 3.2; 3.3). The original staining protocol allowed for seedlings to stain under GUS staining buffer conditions slightly below pH 7.0. At this pH, GUS stains appeared at the tip of the newly emerged shoots in 3 day old plants (Figure 3.2, A.2). In 7to 10 day old ApG plants, staining was observed in the interface between the shoots and the roots, within the remnant seed coat (Figure 3.3, E.2). Faint GUS staining was also found in the veins of the leaf blades and tillers as well as exterior of the tiller of ApG plants (Figure 3.3 C.2 and E.2). All of the GUS staining observed was faint compared to the *OsANT1::GUSplus* lines used as a positive control.

When a different GUS protocol with conditions > pH7.5 was attempted, no GUS staining was observed in any organs in any of the four selected lines in 3 or 7 to 10 day old seedlings (Figure 3.2; 3.3, A.1 to F.1). When tissue was cleared with chloral hydrate solution and viewed under the microscope, no staining was observed and tissue parts were difficult to visualize since the entire tissue was clear. On the other hand, with the *OsANT1*::*GUSplus* lines used as a positive control to determine the efficacy of the protocol under conditions <pH7.0 or >pH7.0, GUS staining appeared within one hour of incubation in GUS staining buffer and GUS stains appeared in the root hairs, root tips and veins of the leaves.

3.3. Discussion

3.3.1. Bioinformatic analysis of the PBpr1 promoter

3.3.1.1. Core promoter elements of the PBpr1 promoter

The *in silico* analysis of the *PBpr1* promoter was carried out as part of selecting it as a potential promoter to drive *HvAlaAT* over-expression. Various different promoter software prediction programs were used to determine promoter motifs or elements that were found on *PBpr1*, and to determine common predicted elements, since promoter element detection is based on slightly different algorithms. Of the software used, only Softberry TSSP, TSSP-TCM and PPDB provided information on transcription start sites (TSS), while PlantCARE, PPDB, Softberry TSSP and PLACE provided detection for plant promoter elements and other binding sites that could affect gene expression. Generally, only one of the four core promoter elements (TATA box, CAAT box, TSS and GC box) is needed to drive gene expression and it is rare to find all four elements in one promoter (Ren *et al.,* 2005; Weaver, 2002).

The detection of TSS is a challenging process, and like any other *in silico* analysis, many motifs predicted are not necessarily functional *in vivo*. While the use of more than one software program might bring more accuracy to its detection, the only way for the identification of a true TSS is by experimentation. Only one TSS was predicted by Softberry TSSP while the other software programs failed to determine a reliable TSS in *PBpr1*. Since the relative positions of elements are important with regards to the TSS and translation start site (ATG), a weak TSS hit

makes it difficult to deduce if any core promoter elements predicted are valid hits. Therefore for the purpose of this thesis, the ATG of HvAlaAT, which was fused to the promoter, is referred to as a reference point. In order to truly identify a functional TSS in *PBpr1*, rapid amplification of cDNA ends (RACE) can be carried out. This is discussed in this thesisin the "Future work" section.

By the four motif analysis software programs nine unique TATA boxes and TATA like elements were predicted. These unique TATA motifs were in the 31 to 462 bp region of the *PBpr1* promoter and only three of them were predicted by two or more software programs. The closest TATA box to the ATG is 271 bp upstream of the ATG where two software programs predicted the presence of this TATA box. However, the validity of this TATA box cannot be concluded since no TSS is predicted and their relative position is unknown until further experimentation can be carried out. Although TATA boxes are known to be the most conserved functional signal in eukaryotic promoters, only ~30% of *Arabidopsis* promoters contain a TATA box and in rice plants only 19% of the promoters have a TATA box (Civáň and Švec, 2008). In a recent study, TATA like elements (although their sequence wasn't highly conserved) were found in abundance either upstream and, less commonly, downstream of the TSS (Civáň and Švec, 2008). These TATA like elements have yet to be characterized and their role in transcription initiation is still unclear. The authors however suggested that the presence of high numbers of these TATA like elements may decrease transcription binding protein pools and cause incorrect assembly of the transcription machinery (Civáň and Švec, 2008).

PBpr1 studies suggest that TATA-less promoters are usually linked to housekeeping processes that are not tightly regulated while promoters that contained TATA boxes are usually highly regulated by stress or biotic stimuli (Yang *et al.,* 2007). In agreement with this, the *btg26* promoter, a homologue of *PBpr1* in canola, is a drought stress induced promoter and was found to have a TATA box (Stroeher *et al.,* 1995). As well, the *OsANT1* promoter, known in previous studies to drive high levels of *HvAlaAT* expression, also had a TATA box. In the case of *PBpr1*, it seemed to drive high *HvAlaAT* over-expression at all times in shoots and after 52 days in roots, suggesting that it is does not tightly regulate gene expression.

CAAT boxes are scattered throughout the *PBpr1* promoter. CAAT boxes are core promoter elements thought to determine the strength and efficiency of the promoter (Klug and Cummings, 1991). They are usually positioned between -70 to -80 of the TSS. Since no apparent TSS is predicted, an assumption was made that the TATA box was positioned at -20 to -30 of the TSS, therefore the CAAT box should be ~50 bp upstream from the TATA box. The closest TATA box to the ATG has a predicted CAAT box ~58 bp upstream of it (Figure 3.1). Another TATA box predicted at the 65 bp position of *PBpr1* also seemed to have a CAAT box ~45 bp upstream of it (Table 3.1).

Y-patches are found to be more abundant in rice promoters; 50% of rice promoters contain Y-patches whereas only 19% contain a TATA box. This suggests that Y-patches may play a more important role in transcription initiation than the TATA box in rice (Civáň and Švec, 2008). The only software used in this

study that was able to detect Y-patches was PPDB. This software found one 5' of the ATG while the other is located 18 bp upstream of the TATA box closest to ATG in *PBpr1*. It has also been suggested that Y-patches may aid in the accessibility of TATA like elements in TATA-less promoters (Civáň and Švec, 2008). Studies on the parasite *Toxoplasma gondii* also suggest that Y-patches may be involved in transcriptional regulation (Yamagishi *et al.,* 2010). However, the exact role of Y-patches in gene expression and regulation is still unknown.

3.3.1.2. Environmental and enhancer response elements in PBpr1

All the software programs predicted two regions, ~50 bp and ~147 bp upstream of the ATG, in *PBpr1* to be an enhancer or responsive element, including auxin, methyl-jasmonic acid, abscisic acid and salicylic acid responsive element. Such concentrated numbers of predicted elements suggest that the *PBpr1* promoter may be regulated by multiple plant hormones and may be developmentally regulated, since levels of plant hormones change over different developmental stages.

One of the elements predicted, the abscisic acid responsive element (ABRE) is known to be needed in close proximity to the TSS to be functional (Mehrotra and Mehrotra, 2010). No strong TSS was found in *PBpr1,* therefore it is difficult to predict if the ABRE plays a pivotal role in *PBpr1*. But it has been found in previous studies that ABRE dependent genes respond to cellular dehydration during senescence and osmotic stress during vegetative growth (Fujita *et al.,* 2010).

The ABRE predicted in *PBpr1* also contained the ACGT core, which has been reported to be recognized by plant bZIP proteins (Choi *et al.,* 2000: Uno *et al.,* 2000), which in our analysis was also found to be a predicted enhancer element (Table 3.1). The ACGT core by itself is usually not sufficient to confer ABA mediated induction of transcription (Mehrotra and Mehrotra, 2010). Multiple ACGT core elements and their distance from each other have been shown to make the promoter responsive to abscisic acid or salicylic acid (Fujita *et al.,* 2011; Mehrotra and Mehrotra, 2010). *PBpr1* had more than one ACGT core but may not be in close enough proximity to confer responsiveness to abscisic or salicylic acid. However, PLACE also predicted a salicylic acid responsive element in the same region as the ABRE, suggesting a possibility of the ACGT core functioning for abscisic acid and salicylic acid responsiveness. The light responsive element predicted in the same region could be linked to the ABRE, since abscisic acid accumulates in a diurnal pattern and controls circardian period in a light dependent manner (Hanano *et al.,* 2006; Mizuno and Yamashino, 2008). GATA boxes involved in light responsiveness were also found to be scattered throughout *PBpr1*.

A methyl-jasmonate (MeJA) response element was also predicted in the ~675 bp region concentrated with enhancer elements. MeJA is a regulator that modulates physiological and developmental processes in plants. It is involved in seed germination, root growth, fertility, fruit ripening, and senescence and also induces stress related genes (Creelman and Mullet, 1997; Creelman and Mulprin. 2002; Wasternack and Hause. 2000: Wasternack and Parthier, 1997). This again

suggests that *PBpr1* may be developmentally regulated or is highly up-regulated when biotic or abiotic stress is applied.

Previous studies on *PBpr1* in its native form have reported that auxin treatment drives high levels of MMSDH and the elevated levels remain high over time (Oguchi *et al.,* 2004). Therefore, the presence of an element that is a part of an auxin response element in *PBpr1* was expected. Auxin regulates plant developmental processes by determining plant polarity and apical dominance (Casimiro *et al.,* 2001). It also controls lateral root initiation and emergence, cell elongation, division and differentiation (Abel and Theologis, 1996).

From the *in silico* analysis of *PBpr1*, there is little indication that *PBpr1* is tissue specific. There is a strong possibility, however, that *PBpr1* drives developmental regulation because of the number of plant hormone response elements observed in two regions of *PBpr1*. In the transgenic lines where *PBpr1* drives *HvAlaAT* over-expression, the increase in AlaAT activity was only tested and observed after 28 days in roots indicating a temporal regulation of *HvAlaAT* by *PBpr1*. In order to better characterize this promoter, Ap lines would have to be monitored through different developmental stages for AlaAT activity in the different tissues beginning at germination.

With the detection of many plant hormone responsive elements in the *PBpr1* promoter, it may be interesting to investigate the response of the promoter driving a reporter gene under treatments of different levels of plant hormones. Ap plants could be tested for their levels of MeJA, auxin, ABA and salicylic acid at

development stages such as active tillering and maximum tillering to further understand the developmental regulation of *PBpr1*. In addition, the application of drought, light and nutrient stress may allow us to determine the stress responsiveness of *PBpr1* in terms of driving gene expression. Promoter deletion and mutagenic studies can also determine the minimal promoter of *PBpr1*, its essential elements to drive gene expression and specific enhancer elements that may drive more efficient and stronger expression of the target gene.

3.3.1.3. Differences in PBpr1 analysis between software programs

In silico analysis of a promoter is extremely dependent on the algorithm used by each of the software programs. In this study, software programs were used to perform the *PBpr1* promoter analysis. PlantCARE and PLACE are less stringent therefore detecting a larger list of enhancers. Softberry TSSP and TSSP-TCM on the other hand show more stringency and the enhancers predicted in their output is usually coupled to a TSS. PPDB detects for motifs and elements in the promoter's native form in the genome and is therefore useful at determining promoter function in its native form but not when genetically modified. Interestingly, Softberry TSSP predicted a reliable TSS in the 5' end of the *PBpr1* promoter, therefore designating a ~600 bp 5'UTR. PLACE and PlantCARE shared a large proportion of the elements predicted while PPDB had few elements in common with them. TSSP-TCM which claimed to be successful at predicting ~85% of the TSS promoter did not detect any reliable TATA boxes or elements in *PBpr1* (Shahmuradov *et al.,* 2005). Therefore, to accurately predict elements found in a promoter of interest, it is relatively important to not only look at the

promoter in context of the entire genome but also analyze the promoter with multiple algorithms.

3.3.2. Pattern analysis of PBpr1 with GUS histochemical staining

PBpr1:: GUSplus transgenic plants were analyzed for the tissue specific expression of the *PBpr1* promoter. The *GUSplus* gene was originally isolated from *Staphylococcus spp.,* but later optimized for plant codon bias (pCAMBIA). GUS (β-glucuronidase) staining showed that at incubation conditions below pH 7.0, the *PBpr1* construct exhibited GUS activity in newly emerged shoots of seedlings, leaf veins, stems and basal region of shoots in ApG and NB plants. The staining observed below pH 7 may have been attributed to endogenous GUS activity and were not caused by the *PBpr1* over-expression of the *GUSplus* gene in the plants. Initially, it was believed that the GUS enzyme was absent in higher plants because it has been used as a selection marker for transformed plants and any GUS activity observed was deemed to be low or sometimes undetectable (Gilissen *et al.,* 1998). Most bacterial GUS genes are found to be most active at conditions above pH 7.0 (Gilissen *et al.,* 1998; Sudan *et al.,* 2006). However, recently, endogenous plant GUS activity was found to be high at about pH 4.0 to 6.0 and the pH condition is critical for endogenous GUS expression (Sudan *et al.,* 2006). Rice plants below neutral pH exhibit endogenous GUS activity in the all cell types of the shoots including vascular tissue, mesophyll and sclerenchymatous cells of the leaves and predominantly in the basal region of the inter-nodal stem segments (Sudan *et al.,* 2006). The results observed are consistent with the staining observed for the ApG and NB plants below pH7.0.

Once the pH of the staining conditions was adjusted to above pH 7.0 in our experiments, no GUS staining was observed in any of the tested ApG lines suggesting the lack of or low levels of GUS activity in the ApG plants. The lack of visible levels of GUS activity indicates that the *PBpr1* promoter in the ApG lines tested does not drive high levels of GUS activity at the early seedlings stage. With the lack of GUS activity, no definitive conclusion can be made about *PBpr1*'s expression pattern using GUS staining. However, analysis of the *PBpr1::HvAlaAT* lines suggests that PBpr1 may be developmentally regulated and only drives high levels of expression in roots after active tillering and is constitutively expressed in shoots. The *OsANT1:: GUSplus* lines served as a positive control for the GUS protocol tested and their expression pattern agrees with the work of Shrawat *et al.* (2008).

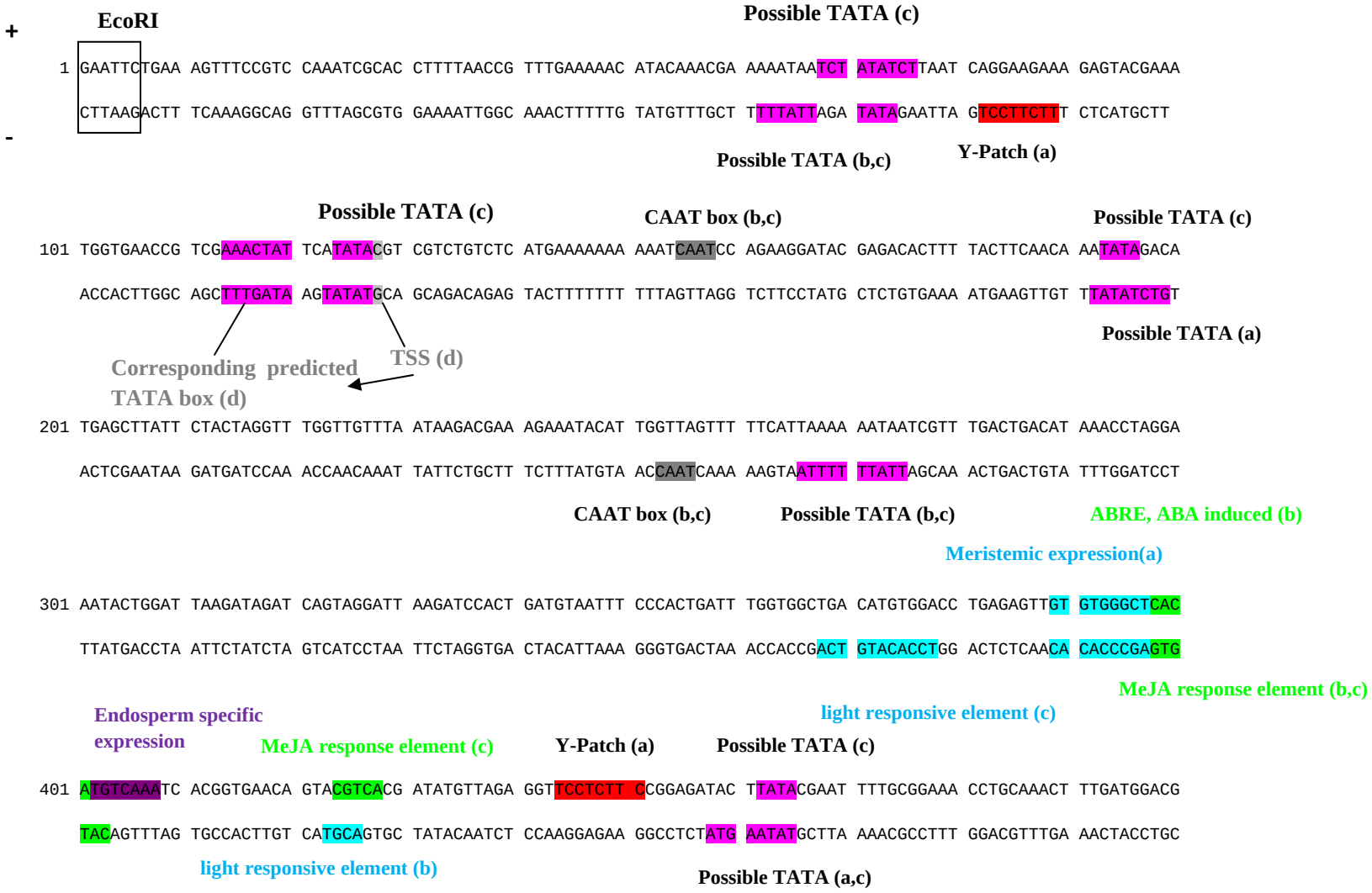
To successfully and more concisely visualize the expression pattern of the *PBpr1* promoter, it should be cloned into another reporter construct that is not of the pCAMBIA backbone. pCAMBIA constructs contain one or more enhancer element in the CAMV35S driving the hygromycin resistance gene. The element has been reported to bidirectionally drive specific transcription or increase the ectopic expression of nearby genes, causing false identification of promoter strength and tissue specificity (Xie *et al.,* 2001; Yoo *et al.,* 2005). A reporter construct using another constitutive promoter such as an actin promoter (Thilmony *et al.,* 2006) or the *nos* promoter to drive the selection marker could more accurately analyze the expression pattern of *PBpr1*.

An interesting observation is that the *PBpr1* promoter drives the over-expression of *HvAlaAT*, but when coupled with the GUS construct, exhibits no GUS activity. Additionally, these ApG plants are hygromycin resistant, indicating the presence of the trangene insertion. The explanation for these obtained results is still unknown but could be attributed to the temporal expression of the *PBpr1* promoter. In all the experiments for AlaAT activity characterization, AlaAT activity was only tested between active tillering and maximum tillering and not during the seedling stage. However, GUS activity was only tested at the seedling stage. The lack of GUS expression in the seedling stage could indicate that *PBpr1* only drives high levels of expression in later stage of development. In addition, there is a possibility that the threshold of over-expression is not sufficient to produce detectable levels of GUS activity. In order to validate the presence of GUS expression, qPCR analysis could be carried out to determine if any GUS mRNA is transcribed.

Table 3.1: Base pair position of promoter elements and motifs detected by plant promoter database (PPDB), Plant Cis-regulatory elements (PLACE), PlantCARe and Softberry TSSP of the *PBpr1* promoter. The grey and blue highlighted elements represents the cis-acting elements that are part of the two strong regulatory elements predicted at ~587 bp and ~675 bp. * represents elements common among software.

	PPDB	PLACE	PlantCARE	Softberry
Core Promoter Elements				
TATA box	*192 bp (-) *462 bp (-)	*62 bp (-) 270 bp (-)	31 bp (+) *67 bp (+), *69 bp (+) 123 bp (+) 177 bp (+) *192 bp (+) 228 bp (+) 265 bp (-) *460 (-),* 461 bp (+)	114 bp (+)
CAAT box		*155 bp(+) *249 bp (-) *501 bp (-) *563 bp(+),*569 bp (+)	20 bp (+) *154 bp (+) 188 bp (+) *248 bp (+) 404 bp (+) *500 bp (-) *562 bp (+), *568 (+) 579 bp (+)	
Y-patch	82 bp (-) 444 bp (-)			
Plant Hormone responsive elements				
ABRE related		589 bp (+) *675 bp (-) , *677 bp (-)	*674 bp (-)	113 bp (-)
Auxin responsive element		424 bp (-) 587 bp (+) *675 bp (-)	*674 bp (-)	
Methyl Jasmonic acid (MeJA) responsive element			CGTCA-motif: 423 bp (+) 586 bp (-) 678 bp (+)	

MeJA cont'		TGACG-motif: 424 bp (-) 587 bp (+) 679 bp (-)	TGACG-motif: 423 bp (-) 586 bp (+) 678 bp (-)	
Other plant hormone responsive elements GA responsive element Salicylic acid responsive element		*30 bp (+) 425 bp (-) 586 bp (+) 424 bp (-) 587 bp (+) *675 bp (-)		*30 bp (+)
Other elements				
Meristemic expression element	389 bp (+) 660 bp (-)		15 bp (+)	
Light responsive elements, G-box, bZIP binding motifs, leucine zipper motif	*675 bp (+,-)	*363 bp (-) 423 bp (+) 488 bp (+) *586 bp (-) 664 bp (+), 665 pb(-) *674 bp (-),*675 (+,-), *678bp (+)	*367 bp (-),*368 bp (+) *587 bp (-) *673 bp (-), *675 bp (-), *676 (+)	
Defense, stress and disease response		44 bp (+) 59 bp (+) 143 bp (+) 258 bp (-) 368 bp (+), 371 bp (+) 389 bp (+) 525 bp (-) 545 bp (+)	519 bp (+),524 bp (+),	45 bp (-)


+
-
EcoRI
Possible TATA (c)
1 GAATTCTGAA AGTTTCCGTC CAAATCGCAC CTTTTAACCG TTTGAAAAAC ATACAAACGA AAAATAATCT ATATCTTAAT CAGGAAGAAA GAGTACGAAA
CTTAAGACTT TCAAAGGCAG GTTTAGCGTG GAAAATTGGC AAACTTTTTG TATGTTTGCT TTTTATTAGA TATAGAATTA GTCCTTCTTT CTCATGCTT
Possible TATA (b,c)
Y-Patch (a)
Possible TATA (c)
CAAT box (b,c)
Possible TATA (c)
101 TGGTGAACCG TCGAAACTAT TCATATACGT CGTCTGTCTC ATGAAAAAAA AAATCAATCC AGAAGGATAC GAGACACTTT TACTTCAACA AATATAGACA
ACCACTTGGC AGCTTTGATA AGTATATGCA GCAGACAGAG TACTTTTTTT TTTAGTTAGG TCTTCCTATG CTCTGTGAAA ATGAAGTTGT TTATATCTGT
Possible TATA (a)
Corresponding predicted TATA box (d)
TSS (d)
201 TGAGCTTATT CTACTAGGTT TGGTTGTTTA ATAAGACGAA AGAAATACAT TGGTTAGTTT TTCATTAAAA AATAATCGTT TGACTGACAT AAACCTAGGA
ACTCGAATAA GATGATCCAA ACCAACAAAT TATTCTGCTT TCTTTATGTA ACCAATCAAA AAGTAATTTT TTATTAGCAA ACTGACTGTA TTTGGATCCT
CAAT box (b,c)
Possible TATA (b,c)
ABRE, ABA induced (b)
Meristemic expression(a)
301 AATACTGGAT TAAGATAGAT CAGTAGGATT AAGATCCACT GATGTAATTT CCCACTGATT TGGTGGCTGA CATGTGGACC TGAGAGTTGT GTGGGCTCAC
TTATGACCTA ATTCTATCTA GTCATCCTAA TTCTAGGTGA CTACATTAAA GGGTGACTAA ACCACCGACT GTACACCTGG ACTCTCAACA CACCCGAGTG
MeJA response element (b,c)
light responsive element (c)
Endosperm specific expression
MeJA response element (c)
Y-Patch (a)
Possible TATA (c)
401 ATGTCAAATC ACGGTGAACA GTACGTCACG ATATGTTAGA GGTTCCTCTT CCGGAGATAC TTATACGAAT TTTGCGGAAA CCTGCAAACT TTGATGGACG
TACAGTTTAG TGCCACTTGT CATGCAGTGC TATACAATCT CCAAGGAGAA GGCCTCTATG AATATGCTTA AAACGCCTTT GGACGTTTGA AACTACCTGC
light responsive element (b)
Possible TATA (a,c)

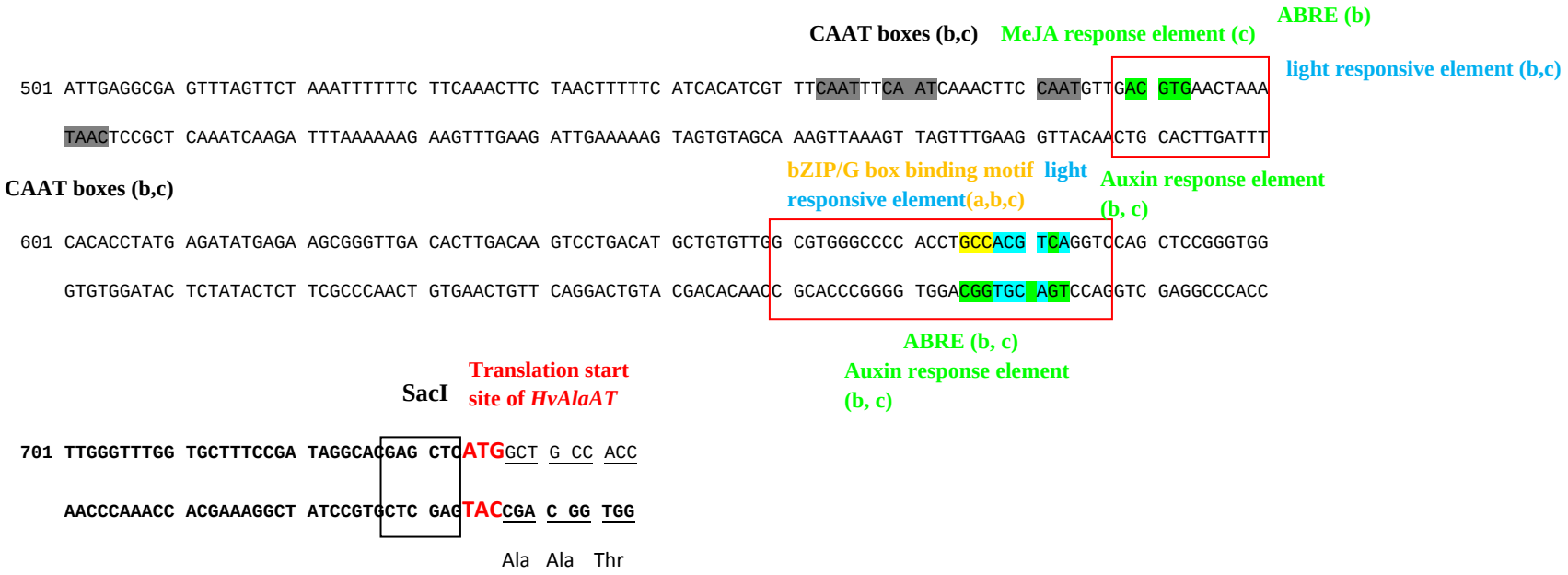


Figure 3.1: *In silico* promoter motif analysis of *PBpr1* by PPDB(a), PLACE(b), PlantCARE(c) and (Softberry TSSP (d). No element was detected by TSSP-TCM.

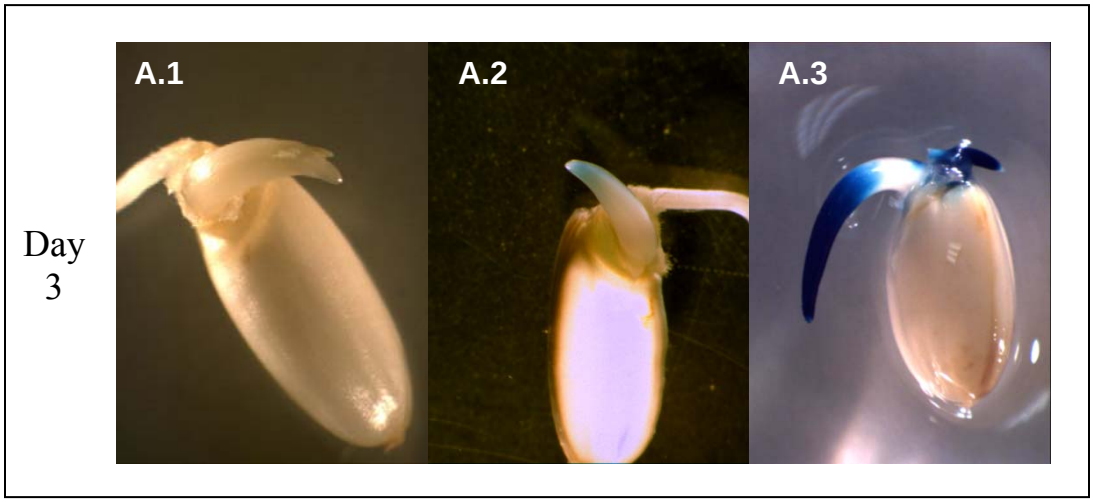
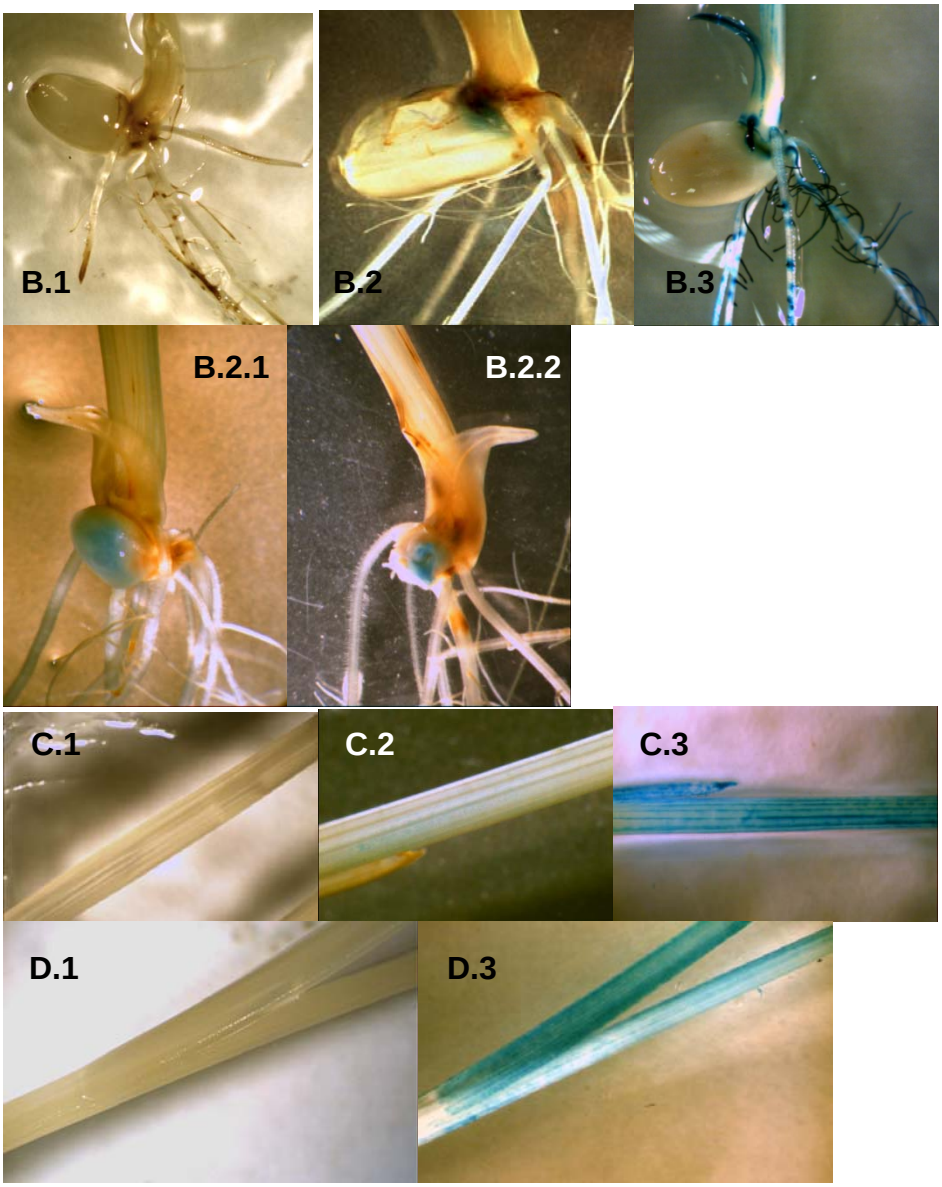


Figure 3.2: GUS staining of 3 day old seedlings that were transformed with *PBpr1::GUSplus* (ApG lines) incubated under conditions at >pH 7.0 and <pH 7.0. *OsANT1::GUSplus* (OsANT1::GUS lines) were used as a positive control. Numbering after the alphabet refers to the treatment and the lines. 1 and 2 were ApG lines while 3 were OsANT1::GUS lines. 1 and 3 were treated under >pH 7.0 while 2 was treated under <pH 7.0 GUS staining conditions.

Day 7 to 10



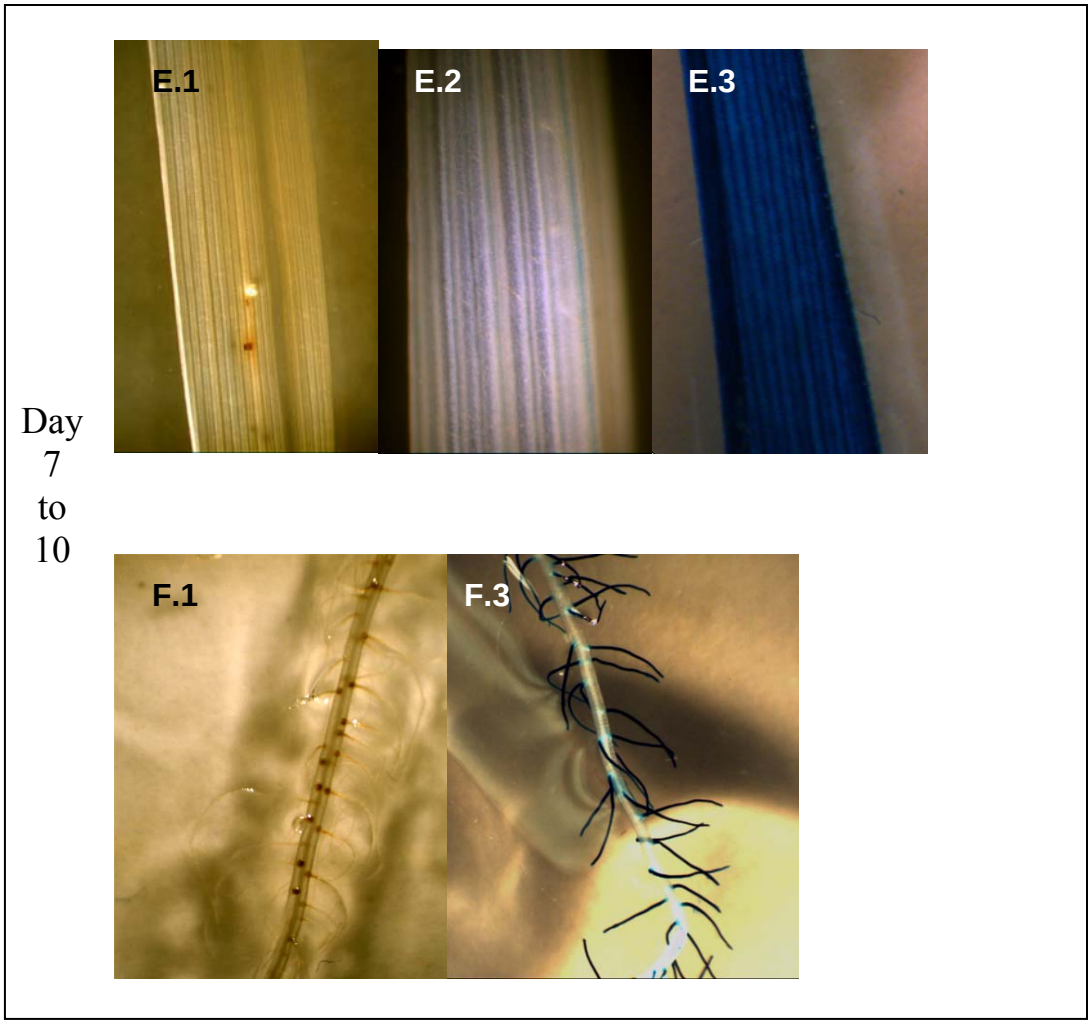


Figure 3.3: GUS staining of 7 to 10 day old seedlings that were transformed with *PBpr1::GUSplus* (ApG lines) incubated under conditions at >pH 7.0 and <pH 7.0. B: basal region of the shoot/shoot-root interface containing remnant seed, C: stem, D: leaf sheath and leaf blade interface, E: leaf blades and veins and F: roots and root hairs. Numbering after the alphabet refers to the treatment and the lines. 1 and 2 were ApG lines while 3 were OsANT1 GUS lines. 1 and 3 were treated under >pH 7.0 while 2 was treated under <pH 7.0 GUS staining conditions.B.2.1 and B.2.2 were staining in the interior of B.2 under <pH 7.0.

General Discussion

Nitrogen is the major limiting nutrient to plant growth and development (Good and Beatty, 2011a). With the increasing food production costs, the need for the development of NUE crop plants has become unequivocal. Literature has shown that frequently the over-expression of a gene itself is not sufficient to affect the NUE of a plant but when coupled with a tissue specific promoter, can produce a strong NUE phenotype. Previous studies in our laboratory have successfully developed NUE canola by over-expression of *HvAlaAT* driven by a tissue specific *btg26* promoter, and its phenotype has persisted in field conditions (Good *et al.,* 2007). By homology, the rice *OsANT1* promoter was developed and, when coupled with *HvAlaAT*, also produced higher NUE rice (Shrawat *et al.,* 2009).

This thesis was undertaken to evaluate several hypotheses to determine if different rice promoters could be used to over-express *AlaAT* and what the phenotypic effect of these genes constructs would be. Specifically, I tested the following hypotheses:

1. Can *PBpr1* drive over-expression of *HvAlaAT* in *O. sativa*.
2. Can *PBpr1* regulate transgene expression similar to *OsANT1* lines.
3. Are plants carrying the promoter gene fusion *PBpr1* ::*HvAlaAT* capable of producing an NUE phenotype.
4. Additionally, there are regulatory elements that are present in the *PBpr1* promoter and they have the potential to affect tissue specific expression or temporally regulate gene expression.

Multiple promoters previously selected and designed in our lab were studied in a preliminary analysis. Of these, only one promoter, *PBpr1,* showed consistent over-expression of *HvAlaAT*.

Nitrogen efficient Ap lines.

Chapter 2 describes the development of homozygous *PBpr1::HvAlaAT* lines and their characterization for over-expression of *HvAlaAT* and growth enhancements. From this analysis, *PBpr1* is indeed over-expressing *HvAlaAT*. First, high levels of AlaAT activity were observed in all the Ap lines consistently throughout all generations analyzed. Second, immunodetection showed that there is a high level of HvAlaAT protein in transgenic plants and finally, qPCR analysis demonstrated an increase in *HvAlaAT* mRNA in the Ap lines. Morphologically, two of the *HvAlaAT* over-expressing Ap lines consistently produced higher seed yield and biomass compared to NB indicating the increase in NUE in the preliminary generations. In addition, they also exhibited increased tillering compared to NB.

In shoots, AlaAT activity appears to be high at all times using the *PBpr1* promoter, while in roots its activity is developmentally regulated. The HvAlaAT protein was shown to be produced at higher levels (based on immunoblotting), however, this did not result in an increase in AlaAT activity until after active tillering. This was distinctly different from the *OsANT1* promoter which highly over-expressed *HvAlaAT* at all times. By comparing transcript and enzyme

activity, our study also suggests the presence of post transcriptional control of *HvAlaAT* in rice.

Transcriptomic studies of seven different genes demonstrated the similarity in transcript profile between the Ap lines and the *OsANT1*::*HvAlaAT* lines, with a nitrogen metabolism gene (*OsGln1-2*) and an ammonium transporter gene (*OsAMT1*) being unaffected by the over-expression of AlaAT, compared to NB. GRP, which was shown to be up-regulated in the *OsANT1* lines, was also up-regulated in the null lines, suggesting that it is not directly linked to the over-expression of *HvAlaAT,* but may instead be linked to the transformation process. The transcript profiling matched those of *OsANT1* lines done by Beatty *et al.* (2009), suggesting a similarity in transcript changes in both the Ap lines and the *OsANT1*::*HvAlaAT* lines.

Promoter characterization

Chapter 3 provides the promoter studies of *PBpr1*. The *PBpr1* promoter motif studies show no strong TSS and TATA boxes in close proximity to the ATG site but at least nine TATA like elements and CAAT boxes scattered throughout the promoter. Two of the TATA like elements were in close proximity to the CAAT boxes and one was closest to the ATG site. The presence of TATA boxes is not instrumental in plant promoter activity and the lack of a TATA box usually relates to the lack of tight regulation. Two T and C rich regions known as pyrimidine Y-patches were also predicted, and since more than 50% of rice promoters contain Y-patches, this suggests the importance of Y-patches as plant

regulatory elements in rice (Civáň and Švec, 2008). Further motif analyses identified two regions with strong prediction for plant hormone regulatory elements including ABRE, salicylic acid responsive element, auxin response element and MeJA responsive element. These strong hits for element prediction suggest that *PBpr1* is developmentally regulated. *PBpr1::GUSplus* lines pattern analysis showed no GUS expression at the seedling stage (from 3 to 10 DAG) yet *HvAlaAT* over-expression is detected in *PBpr1::HvAlaAT* lines. GUS staining at non-optimal pH conditions also demonstrated the presence of endogenous GUS activity in rice plants. The results obtained were not definitive in terms of the expression pattern of *PBpr1* and further experiments need to be conducted to elucidate the tissue specificity of *PBpr1*.

Future work

Our laboratory is continuing research on the over-expression of *HvAlaAT* using *PBpr1* and its regulation of the transgene. The development of the homozygous Ap lines developed in this thesis allows for their further study.

Future work could include the following. First, in order to clearly determine the number of transgene inserts in each line, a Southern blot with a transgene probe should be carried out. Further characterization of the developmental regulation of *PBpr1* should also include AlaAT activity, immunodetection and qPCR analysis of Ap lines, from the seedling stage to senescence. Hydroponic experiments of the Ap lines can determine the total nitrogen uptake of the Ap lines, allowing the determination of NUpE.

Additionally, Ap plant samples grown in soil can be harvested to determine the total N in the plants and calculate the NUtE from its grain yield. Ap lines can also be grown under different nitrogen sources and regimes (low or high N) to determine its phenotypic response to different environments. Also, metabolite profiles by High Performance Liquid Chromatography can be carried out to determine changes in amino acid profiles from the over-expression of *HvAlaAT* by *PBpr1*. N^{15} labelling studies can also determine the proportion of N partitioned into grain and biomass. For transcript profiling more genes should be studied to provide a better understanding of the impact of *HvAlaAT* over-expression using *PBpr1* or a microarray study of Ap lines could be carried out. In our study, transcript profiles were analyzed at 52 DAG but not at 28 DAG due to limited time. The analysis of 28 DAG Ap plant samples that were already collected will provide us with more insight on the regulation and over-expression of *HvAlaAT*. Since nulls differ significantly from the Nipponbare plants, they should be used in all studies as negative controls.

Since no expression of GUS activity is observed in the ApG lines, qPCR analysis of GUS mRNA should be done to determine if *PBpr1* drives the over-expression of GUS. In order to successfully and more concisely visualize the expression pattern of the *PBpr1* promoter, it should be cloned into another reporter construct that is not of the pCAMBIA backbone to avoid the potential bidirectional and ectopic expression caused by the CAMV35S multiple enhancer element. A reporter construct using another constitutive promoter such as an actin

promoter or the *nos* promoter to drive the selection marker may allow for more accurate analysis of the expression pattern for *PBpr1*.

In order to determine the TSS of the PBpr1 promoter, rapid amplification of cDNA ends (RACE) should be carried out. Other promoter studies such as promoter bashing can be done to determine the minimal promoter and regulatory elements essential to drive high levels of target gene expression. Also, plant hormone stimuli can be applied to Ap plants to determine the validity of the regulatory elements predicted and if it enhances *PBpr1* promoter over-expression. Levels of different plant hormones such as abscisic acid, auxin, salicylic acid and methyl-jasmonate can be measured in rice plants to determine at which developmental stage they are highest and therefore causing the regulation of the *PBpr1* promoter.

Finally, all the studies conducted to date have been in growth chamber conditions. The key step in elucidating NUE of crop plants is to test for the transgenic phenotype in the field and whether it will prevail under field conditions and if so, what conditions specifially. Evaluation of transgenic phenotype in the field has been a key stumbling block to the development of NUE plants. To date, only a few transgenic lines with increased NUE in the growth chamber have maintained their NUE phenotype in the field (Good *et al., 2007*; Fan *et al.,* 2010; Brauer *et al.,* 2011). Therefore, the NUE phenotype of the Ap lines should be further tested in field conditions. Generally, if possible, transgenic plants should be tested in soil directly, ideally in the field, since it is in the field environment that a plant needs to exhibit its NUE phenotype.

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4. Appendix

Appendix 1: PCR screens of transgenic T_1 rice plants Ap7, Ap17, Ap18, Ap19 and Ap28. All lines carrying the *PBpr1::AlaAT* construct using primers specific to *hptII*. PCR that yielded no band at ~800bp refer to plants that are null segregants while PCR reactions that yielded a ~800bp band are plants that contain the transgene *PBpr1::AlaAT*. A fraction of the plants did not germinate and therefore PCRs could not be carried out due to lack of tissue. Negative control of wild type Nipponbare and positive control of AGR 1/7 were also included.

	Positive	Negative	Fail to germinate	Total
Ap7	16	3	1	20
Ap17	5	4	11	20
Ap18	9	7	4	20
Ap19	10	6	4	20
Ap28	16	2	2	20
NB	0	20	0	20
AGR1/7	16	0	4	20