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Identification of the gene responsible for fragrance in rice and characterisation of the enzyme transcribed from this gene and its homologs

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The flavour or fragrance of Basmati rice is associated with the presence of 2-acetyl-1- pyrroline. This work shows that a gene with homology to betaine aldehyde dehydrogenase (BAD) has significant polymorphisms in the coding region of fragrant genotypes relative to non fragrant genotypes. Accumulation of 2-acetyl-1-pyrroline in fragrant rice genotypes may be explained by the presence of mutations resulting in loss of function of the fgr gene product. The fgr gene corresponds to the gene encoding BAD2 in rice while BAD1 is encoded by a gene on chromosome 4. Development of an allele specific amplification (ASA) based around the deletion in the gene encoding BAD2 allows, perfect, simple and low cost discrimination between fragrant and non-fragrant rice varieties and identifies homozygous fragrant, homozygous non-fragrant and heterozygous non-fragrant individuals in a population segregating for fragrance. The cDNAs transcribed from rice chromosomes 4 and 8, each encoding an enzyme with homology to betaine aldehyde dehydrogenase were cloned and expressed in E. coli. The enzyme responsible for fragrance, encoded from chromosome 8, had optimum activity at pH 10, showed low affinity towards betaine aldehyde (bet-ald) with Km value of approximately 63μM but a higher affinity towards γ-aminobutyraldehyde (GABald) with a Km value of approximately 9μM. The enzyme encoded from chromosome 4 had optimum activity at pH 9.5 and showed generally lower affinity towards most substrates compared to the enzyme encoded from chromosome 8, substrate specificities suggest that the enzymes have higher specificity to aminoaldehydes and as such both should be

Identification of the gene responsible for fragrance in rice and characterisation of the enzyme transcribed from this gene and its homologs

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A thesis submitted to Southern Cross University in fulfillment of the requirements for the degree of Doctor of Philosophy.

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August 2007

Statement of Originality

I certify that the work presented in this thesis is, to the best of my knowledge and belief, original, except as acknowledged in the text, and that the material has not been submitted, either in whole or in part, for a degree at this or any other university. I acknowledge that I have read and understood the Universities rules, requirements, procedures and policy relating to my higher degree research award and to my thesis. I certify that I have complied with the rules, requirements, procedures and policy of the University.

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Louis Michael Thomas Bradbury

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Abstract

The flavour or fragrance of Basmati rice is associated with the presence of 2-acetyl-1-pyrroline. This work shows that a gene with homology to betaine aldehyde dehydrogenase (BAD) has significant polymorphisms in the coding region of fragrant genotypes relative to non fragrant genotypes. Accumulation of 2-acetyl-1-pyrroline in fragrant rice genotypes may be explained by the presence of mutations resulting in loss of function of the *fgr* gene product. The *fgr* gene corresponds to the gene encoding BAD2 in rice while BAD1 is encoded by a gene on chromosome 4. Development of an allele specific amplification (ASA) based around the deletion in the gene encoding BAD2 allows, perfect, simple and low cost discrimination between fragrant and non-fragrant rice varieties and identifies homozygous fragrant, homozygous non-fragrant and heterozygous non-fragrant individuals in a population segregating for fragrance. The cDNAs transcribed from rice chromosomes 4 and 8, each encoding an enzyme with homology to betaine aldehyde dehydrogenase were cloned and expressed in *E. coli*. The enzyme responsible for fragrance, encoded from chromosome 8, had optimum activity at pH 10, showed low affinity towards betaine aldehyde (bet-ald) with K_m value of approximately 63μM but a higher affinity towards γ-aminobutyraldehyde (GABald) with a K_m value of approximately 9μM. The enzyme encoded from chromosome 4 had optimum activity at pH 9.5 and showed generally lower affinity towards most substrates compared to the enzyme encoded from chromosome 8, substrate specificities suggest that the enzymes have higher specificity to aminoaldehydes and as such both should be renamed as an aminoaldehyde dehydrogenase (AAD). The inactivation of AAD2 (BAD2)

in fragrant rice varieties likely leads to accumulation of its main substrate GABald which then cyclises to Δ^1-pyrroline the immediate precursor of 2AP.

List of publications arising from this thesis

Articles

Bradbury LMT, Fitzgerald TL, Henry RJ, Jin QS, Waters DLE (2005) The gene for fragrance in rice. Plant Biotechnology Journal 3: 363-370

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Rice N, Cordeirro G, Shepherd M, Bundock P, Bradbury LMT, Pacey-Miller T, Furtado A, Henry RJ (2006) DNA Banks and their role in facilitating the application of genomics to plant germplasm. Plant Genetic Resources 4: 64-70

Bradbury LMT, Gillies S, Brushett D, Waters DLE, Henry RJ (2008) Inactivation of an aminoaldehyde dehydrogenase is responsible for fragrance in rice. Plant Molecular Biology 68: 439-449

Book Chapters

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International Patents

Henry RJ, Jin QS, Waters DLE, Bradbury LMT, Fitzgerald TL (2005) Method of producing fragrance by inactivation or reduction of a functional protein with betaine aldehyde dehydrogenase (BADH) activity. International Patent Application number PCT/AU2005/001458, Publication number WO/2006/032102

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Appendix ii: Bradbury LMT, Henry RJ, Jin QS, Reinke RF, Waters DLE (2005) A perfect marker for fragrance genotyping in rice. Molecular Breeding 16: 279-283

Appendix iii: Rice N, Cordeirro G, Shepherd M, Bundock P, Bradbury LMT, Pacey-Miller T, Furtado A, Henry RJ (2006) DNA Banks and their role in facilitating the application of genomics to plant germplasm. Plant Genetic Resources 4: 64-70

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List of Abbreviations and Symbols

2AP	**2-acetyl-1-pyrroline**
AAD	**amino aldehyde dehydrogenase**
ASA	**allele specific amplification**
BAD	**betaine aldehyde dehydrogenase**
bet-ald	**betaine aldehyde**
bp	**base pair**
cDNA	**complimentary DNA**
cM	**centimorgan**
CMO	**choline monooxygenase**
Da	**Dalton**
DAO	**diamine oxidase**
DNA	**deoxyribonucleic acid**
GABA	**γ-aminobutyric acid**
GABald	**γ-aminobutyraldehyde**
GC	**gas chromatography**
GCMS	**gas chromatography mass spectrometry**
GGBald	**γ-guanidinobutyraldehyde**
GT	**gelatinisation temperature**
HPLC	**high pressure liquid chromatography**
IPTG	**isopropyl β-D-thiogalactoside**
kbp	**kilo base pair**

kDa	**kilo dalton**
KOME	**Knowledge-based Oryza Molecular Biological Encyclopaedia**
LCMS	**liquid chromatography mass spectrometry**
mRNA	**messenger RNA**
MS	**mass spectrometry**
NAGABald	**N-acetyl- γ-aminobutyraldehyde**
NIL	**near isogenic lines**
PCR	**polymerase chain reaction**
PTS1	**type 1 peroxisomal targeting signal**
RFLP	**restriction fragment length polymorphism**
RNA	**ribonucleic acid**
SNP	**single nucleotide polymorphism**
SSIIa	**soluble starch synthase IIa**
SSR	**simple sequence repeat**

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CHAPTER 1: Flavour Development in Rice

Introduction:

There are many distinct yet subtle flavours and textures that influence rice eating quality. Rice consumers are aware of these flavours and often demand what they perceive to be the best quality rices. The most desirable combination of traits can be unique to particular markets. Some consumers prefer the flavours and qualities of older stored rice, while other consumers have a preference for fresh rice flavours (Zhou *et al*., 2002). There are many chemicals that contribute to the aroma and flavour of rice (Table 1.1) and these are often associated with long term storage. Methods have been developed to remove or mask flavours which are characteristic of stored rice (Arai and Watanabe, 1994; Fukai and Ishitani, 2004) in order to meet consumer preferences.

Old flavours of rice

Differing levels and combinations of various chemicals explain the flavours and fragrances associated with long term rice storage. (Masumoto *et al*., 2004) determined 1-butanal, 1-hexanal, 1-heptanal, methyl ethyl ketone, 1-pentanal and propanal are responsible for the what is known as the “old” or “stale” aroma of stored rice while 1-butanal and 1-heptanal are involved in the aroma of “fresh” rice. Many of these old flavours or fragrances, particularly hexanal, have been noted elsewhere (Zhou *et al*., 2002; Lam and Proctor, 2003; Masumoto *et al*., 2004).

Table 1.1. Concentration, thresholds, odour unit and odour descriptions of significant volatile aroma compounds in fragrant and non-fragrant rices. – Adapted from Wilkie *et al*. (2004).

			Jasmine	Basmati	Non-fragrant
Aroma compound	Threshold (ppb)	Odour description	Conc (ppb)	Conc (ppb)	Conc (ppb)
Hexanal	5	Green, grass	853	751	1960
Butanol	500	Medicinal	5	1	9
Heptan-2-one	140	Fruit, spicy	23	22	40
Heptanal	3	Fruit, fatty	25	34	26
(E)hex-2-enal	17	Green, fruity	7	5	15
2-Pentyl furan	-	Nutty, beany	35	21	78
Pentan-1-ol	4000	Sweet, strong	84	139	104
Octanal	0.7	Citrus, fatty	26	40	29
(E)hept-2-enal	13	Fatty, green	45	22	80
2-acetyl-1-pyrroline	0.1	Sweet, popcorn	49	7	3
6-methylhept-5-en-2-one	50	Herby, green	11	3	3
Hexan-1-ol	2500	Sweet, green	51	45	59
Nonanol	1	Floral, fatty	28	25	42
(E)oct-2-enal	3	Green, herby	47	27	95
Oct-1-en-3-ol	1	Herby, earthy	34	25	58
2-ethyl hexanol	-	Oily, sweet	0	-	44
Benzaldehyde	350	Nutty, sweet	36	27	49
(E)non-2-enal	0.08	Fatty, waxy	14	6	28
(E)dec-2-enal	0.4	Fatty, green	11	9	15
(E,E)deca-2,4-dienal	0.07	Fatty, citrus, powerful	13	8	31
4-vinylguaicol	3	Spicy, fruity	15	23	42
Indole	140	Faecal, floral	12	3	17

Methods such as the addition of aromatic rice to old rice have been developed in an effort to mask or dilute old rice flavours (Fukai and Ishitani, 2004). Protease treatment followed by washing in water is another method of old flavour reduction in stored rice (Arai and Watanabe, 1994).

The amount of hexanal in rice grain has been reported to be linearly proportional to the concentration of oxidised linoleic acid in the grain (Shin *et al*., 1986). When stored at 35 ºC for longer than two weeks, several rice varieties have been shown to have markedly reduced pentanal, hexanal and pentanol levels compared to other rice varieties (Suzuki *et al*., 1999). Analysis of these varieties found reduced levels of one of the three lipoxygenase (LOX) isozymes, an enzyme known to be involved in peroxidation of some polyunsaturated fatty acids including linoleic acid (Suzuki *et al*., 1999). Introduction of the LOX-3-less character into many rice varieties using molecular breeding methods is now occurring in many breeding programs (Suzuki *et al*., 1999) in an effort to reduce the levels of old flavours of rice.

Rice texture

While the texture of the rice grain may not be directly linked to flavour, it is one of the most important eating quality traits of rice. In addition, cooking temperature is known to affect the flavour of many foods (Bhandari *et al*., 2001), and so the flavour compound profile of cooked rice will be altered by reducing the cooking temperature. The texture and cooking temperature of rice is directly influenced by the properties of rice starch.

Starch is composed of a mixture of two forms of glucose polymer, amylose and amylopectin. Amylose is principally a linear polymer of α(1-4) linked glucose with some α(1-6) linkages. Amylopectin is a more complex mixture due to the extensive branching introduced by many more α(1-6) linkages of the α(1-4) linked chains of glucose. Starch is synthesised by the activity of several enzymes, each of which occur as a number of different isoforms that display tissue specific expression (Ball and Morell, 2003; Fitzgerald, 2004).

In its native state, rice starch has a semi-crystalline structure which is disrupted by cooking, transforming the starch into a softer edible gel like material. Because it is associated with the cooking time and texture of cooked rice, the temperature at which rice starch gelatinises is an important component of rice eating quality (Maningat and Juliano, 1978). A link between the gelatinisation temperature (GT) of rice starch and enzymes of starch bio-synthesis was made when it was found that the major gene that controls rice starch gelatinisation temperature via amylopectin structure, genetically maps to a genomic region that codes for soluble starch synthase IIa (SSIIa) (Umemoto *et al*., 2002). Analysis of near-isogenic rice lines (NIL) in which a narrow genomic region surrounding the SSIIa encoding gene of the high GT variety Kasalath was introgressed into the low GT variety Nipponbare (Umemoto *et al*., 2004) and Western analysis for SSIIa in two rice varieties that differed by starch disintegration in both urea and alkali (Jiang *et al*., 2004), provided further support for the hypothesis that SSIIa is the enzyme responsible for natural variation in GT. DNA sequence analysis of the gene that codes for SSIIa found a number of single nucleotide polymorphisms (SNP) in the gene, two of which

were associated with GT class (Umemoto and Aoki, 2005; Waters *et al*., 2006). Rice cultivars belonging to the high GT class had haplotype G/GC while rice cultivars in the low GT class was either A/GC or G/TT at the key SNP sites. The presentation of two GT classes is consistent with the finding that the fine structure of Asian rice varieties falls into one of two categories, either the L-type which has more long cluster chains or the S-type with more short cluster chains (Nakamura *et al*., 2002). In cultivated rice, it is possible that the combination of 'G' (valine) at SNP3 and 'GC' (leucine) at SNP4 is required for the production of L-type rice starch and this has a higher GT relative to the S-type starch. Changing the valine to methionine or the leucine to phenylalanine may change starch from the L-type to the S-type which in turn reduces the GT of the starch.

Fragrant rice

The most important rice flavours are often considered to be the aromatic or fragrant flavours associated with the Basmati and Jasmine style rices. The demand for fragrant rice has increased markedly in recent years to the extent that consumers are willing to pay a premium price for fragrant rices. In 2006, premium non-fragrant rice traded for US$250 to US$300 per metric ton, premium Jasmine rice traded for US$400 per metric ton, while premium Basmati rice traded at US$850. The higher price obtained for Basmati rice is due not only to the demand for the aroma and unique characteristics of the rice grain but also to the limited supply of the Basmati grain. Despite India, the largest supplier of Basmati rice (Bhattacharjee *et al*., 2002), contributing to about one-third of the worlds rice acreage, production levels of Basmati rice are relatively low, reflecting the low yields of Basmati varieties in comparison with non-Basmati rice varieties (Singh *et al*.,

2000; Aggarwal *et al*., 2002; Bhattacharjee *et al*., 2002; Nagaraju *et al*., 2002; Garg *et al*., 2006). Premium Jasmine rice also suffers from low yields (Berner and Hoff, 1986; Sriboonchitta and Wiboonpongse, 2005) though usually due to environmental rather than genetic factors alone. Low yield in fragrant rice varieties is due in part to its susceptibility to insect pests (Lorieux *et al*., 1996; Ghareyazie *et al*., 1997; Tanasugarn, 1998; Sriboonchitta and Wiboonpongse, 2005; Toojinda *et al*., 2005), poor disease resistance (Tanasugarn, 1998; Sriboonchitta and Wiboonpongse, 2005; Toojinda *et al*., 2005) and compounded by management practices which exploit the enhanced aroma response of Jasmine rices to abiotic stresses such as drought and high salinity (Yoshihashi *et al*., 2004). Because fragrant rices exhibit higher aroma levels when grown in sub-optimal conditions, virtually all premium quality Thai Jasmine rice is grown in the Isan areas of north-eastern Thailand, a region characterised by saline sandy soils that are prone to flooding and drought (Rigg, 1985; Itani *et al*., 2004 and references therein). The soil structure combined with the sub-optimal rainfall of this area make the application of nitrogenous fertiliser economically impractical, further reducing yield in fragrant rice varieties (Rigg, 1985). The combined effects of environmental and genetic factors inducing both low yield and enhanced aroma in rice is probably a major contributor to the US and other non-traditional rice growing countries being unable to produce rice varieties with sufficient aroma. Most non-traditional rice growing countries utilise intensive farming practices which are focused on high yield through irrigation and addition of nitrogenous fertilisers, practices which probably enhance yield to the detriment of rice aroma. Many countries that are net exporters of rice, such as the US, import large amounts of rice from Thailand, India and Pakistan because they can not produce fragrant

varieties with grain qualities equal to those from these countries (Boriss, 2006).

Since the start of the green revolution, most rice breeding programs have focused on improving disease and insect resistance and most importantly grain yield. Because fragrant varieties have low yield, farmers stopped growing specific local fragrant varieties and replaced them with the new, fast growing, disease resistant, high yielding, non-fragrant varieties (Bhattacharjee *et al*., 2002; Itani *et al*., 2004). This has been to the detriment of the genetic diversity of fragrant rice varieties (Berner and Hoff, 1986; Ghareyazie *et al*., 1997; Singh *et al*., 2000; Bhattacharjee *et al*., 2002; Garg *et al*., 2006) as many local fragrant types were out-competed and lost.

Due to an increasing worldwide interest in fragrant rice, biotechnological and breeding efforts later focused on increasing the yield of these fragrant varieties while retaining their aromatic qualities (Ghareyazie *et al*., 1997; Bhattacharjee *et al*., 2002; Garg *et al*., 2006). This has been particularly difficult with Basmati styles as there are many qualities, other than aroma, such as grain elongation with little swelling in breadth on cooking, soft texture and fine cooking quality (Bhattacharjee *et al*., 2002) that make Basmati rice distinctive and popular. These desirable traits may be linked to genes for, or may be the causal factor of, low yield in Basmati rice.

Basmati rices belong to a genetically distinct cluster, known as *group V* (Glaszmann, 1987) also known as *aromatic* (Garris *et al*., 2005). Molecular genetic evidence suggests the aromatic Basmati rices have been through a recent or severe bottleneck event (Nagaraju *et al*., 2002; Garris *et al*., 2005). The occurrence of a bottleneck in the fragrant

Basmati rice group means that there is very little genetic diversity to be utilised for crop improvement within this group. Attempts at introgressing traits influencing yield into Basmati rice from the other rice groups often result in sterile progeny, due to inter-group incompatibility (Glaszmann, 1987; Pinson, 1994; Garris *et al*., 2005). Despite the inter-group sterility issues facing Basmati breeding programs, some breeding programs have enjoyed limited success using conventional breeding in enhancing yield, disease, insect and lodging resistance in Basmati varieties (Bhattacharjee *et al*., 2002; Shobha Rani *et al*., 2002; Khan *et al*., 2003). Others have resorted to mutation breeding (Awan and Cheema, 1999; Soomro *et al*., 2003) or using transgenic approaches (Ghareyazie *et al*., 1997; Garg *et al*., 2006), though no genetically modified fragrant rice breeding lines are currently available.

Fragrant Jasmine rice from Thailand fall within the *indica* group (Khush, 2000; Garris *et al*., 2005), while a few fragrant cultivars from countries such as China and the Philippines fall into the *japonica* group. The green revolution initially greatly enhanced yields in the *japonica* group and then later in the *indica* group. Although limited in comparison to non-fragrant rices, efforts aimed at increasing yield in fragrant Thai Jasmine rice have enjoyed some success, (Siangliw *et al*., 2003; Toojinda *et al*., 2005) relative to what has been achieved in the aromatic Basmati rices.

Breeding programs outside of traditional fragrant rice growing countries which have attempted to introduce fragrance into adapted backgrounds (Reinke *et al*., 1991; Bollich *et al*., 1992; Marchetti *et al*., 1998; Wilkie and Wootton, 2004) have met with limited

success. The main barrier to breeding high yielding fragrant rice cultivars, whether Basmati, Jasmine or in adapted backgrounds, is the accurate assessment and selection of the subtle, recessive trait of fragrance within individual plants. To this end many sensory and chemical methods have been developed to enable rice breeders to determine whether or not rice plants or grain are fragrant, each method having various positive and negative attributes. At their most simple, these methods involve smelling or chewing individual grains (Ghose and Butany, 1952; Reinke *et al*., 1991). Unfortunately the objective evaluation of fragrance using these methods is labour intensive and unreliable. A panel of analysts is required as the ability to detect fragrance varies between individuals. For any individual analyst, the ability to distinguish between fragrant and non-fragrant samples diminishes with each successive analysis, as the senses become saturated or physical damage occurs from abrasions to the tongue which often result from chewing the hard grain (Garland *et al*., 2000; Cordeiro *et al*., 2002). Semi-chemical methods for determining the fragrance status of grains or plants involve heating grain or leaf in water or reacting them with solutions of KOH or I_2-KI before smelling the vapours (Sood and Sidiq, 1978). These methods require an objective analysis from a panel of experts, can saturate the senses of the analytical panelists and can cause damage to the nasal passages, leading to inaccurate and unreliable analysis of the fragrance status of an individual plant. The requirement for an accurate and reliable method for determining the fragrance phenotype of rice plants has led to many investigations into the chemical and genetic components of rice fragrance.

The chemistry of rice fragrance

Chemical analysis of a wide range of rice varieties has revealed many compounds that differ in concentration between fragrant and non-fragrant rice varieties. (Yajima *et al*., 1978; Yajima *et al*., 1979; Buttery *et al*., 1986; Paule and Powers, 1989; Petrov and Lorieux, 1996; Widjaja *et al*., 1996; Grosch and Schieberle, 1997; Wilkie and Wootton, 2004) (Table 1.1). Using a combination of sensory panels and gas chromatogram techniques Buttery *et al*. (1983) determined 2-acetyl-1-pyrroline (2AP), although only present in fragrant rice at low concentrations, was the primary chemical responsible for the characteristic aroma of Jasmine and Basmati rice. 2AP is also present in non-fragrant rice varieties but at a concentration in the range of 10 to 100 times lower than that of fragrant rices (Buttery *et al*., 1983; Buttery *et al*., 1986; Widjaja *et al*., 1996; Wilkie and Wootton, 2004). The threshold concentration at which 2AP can be detected by the human nose is around 0.1ppb when diluted in water (Buttery *et al*., 1983), but is probably somewhat higher in the complex rice grain. Fragrant rice grain has 2AP concentrations from about 3000 times this level and upwards, while non-fragrant rice has concentrations of 2AP of only about 30 times this threshold level of 2AP in water (Buttery *et al*., 1983; Buttery *et al*., 1986; Wilkie and Wootton, 2004) (Table 1.1). A wide range of 2AP concentrations have been observed in both fragrant and non-fragrant varieties in different studies. These differences may be due to the different rice varieties studied, differences in extraction procedure or quantification of 2AP, environmental influences on the level of fragrance such as temperature and salt and drought stress (Itani *et al*., 2004; Yoshihashi *et al*., 2004), harvest time or storage conditions of the rice (Bhattacharjee *et al*., 2002; Itani *et al*., 2004; Yoshihashi *et al*., 2005), whether the rice was milled or unmilled (Buttery *et*

al., 1983; Philpot *et al*., 2005) or timing/level of nitrogenous fertiliser application to the growing plants (Wilkie and Wootton, 2004).

Buttery *et al*. (1983b) also discovered that 2AP was the main chemical cause of fragrance of *Pandanus amaryllifolius* leaf. The aroma of fragrant rice is often described as Pandanus like and in some Asian cultures dried Pandanus leaf is added to non-fragrant rice while cooking to impart a characteristic Basmati/Jasmine scent. Since the discovery that 2AP is the major chemical compound involved in fragrance in both rice and Pandanus, it has been found the flavour of a range of foods, including, popcorn (Schieberle, 1995), corn tortillas (Buttery and Ling, 1995), baguettes (Zehentbauer and Grosch, 1998), ham (Carrapiso *et al*., 2002), cheese (Zehentbauer and Reineccius, 2002), mung bean (Brahmachary and Ghosh, 2002), green tea (Kumazawa and Masuda, 2002) and wine (Herderich *et al*., 1995) is associated with the presence of 2AP. Analysis of a diverse range of biological non-consumables such as tiger pheromone (Brahmachary *et al*., 1990) and select bacteria, moulds and yeasts (Rungsardthong and Noomhoom, 2005) have found the characteristic aroma of these sources is also due the presence of 2AP.

Many objective methods of determining the level of 2AP using gas chromatography, have been developed (Lorieux *et al*., 1996; Widjaja *et al*., 1996; Bergman *et al*., 2000). However, these methods often require large tissue samples, are expensive, extremely time consuming and the peak corresponding to 2AP is small compared to the peaks corresponding to other chemicals present in rice, making the results difficult to interpret.

The genetics of rice fragrance

Molecular markers are used widely as a selection tool for specific traits in breeding programs in many species including rice. If the genetic cause of a trait is known or the genomic region that controls a trait is sufficiently narrow, genotypic tests using molecular markers offer many advantages relative to many direct phenotypic tests. The advantages include a requirement for small quantities of tissue, independence from the confounding effects of the environment and a single platform test for multiple traits.

The majority of studies which have focused on the genetics of fragrance in rice determined fragrance is due to a single recessive gene (Sood and Sidiq, 1978; Berner and Hoff, 1986; Ahn *et al*., 1992; Bollich *et al*., 1992; Lorieux *et al*., 1996; Garland *et al*., 2000; Cordeiro *et al*., 2002; Jin *et al*., 2003) while other studies have identified two, three or four genetic loci as having an influence on fragrance (Kadam and Patankar, 1938; Dhulappanavar, 1976; Geetha and Nadu, 1994; Pinson, 1994; Vivekanandan and Giridharan, 1994; Lorieux *et al*., 1996). The contradictory nature of these reports may be due to either the different rice varieties studied or the different methods used to evaluate fragrance. Some methods used a simple binary system of fragrant/non-fragrant, to categorise the fragrance phenotype (Sood and Sidiq, 1978; Pinson, 1994; Jin *et al*., 2003). Other methods measured varying degrees of fragrance using sensory panelists scaling fragrance, usually on a scale of one to ten (Berner and Hoff, 1986) while others used a combination of sensory panelists and gas chromatographic methods to measure the level of 2AP in plant samples (Lorieux *et al*., 1996).

While not ruling out the possibility of multiple genetic loci influencing the level of fragrance in rice, it appears there is one major genetic loci on chromosome 8 that determines if rice is fragrant or not, in essence an on/off switch for fragrance. Berner and Hoff, (1986) determined that aroma in the fragrant cultivar Della was due to a single recessive gene. Ahn *et al*. (1992) then used 126 molecular markers to map the position of this gene, *fgr*, in aromatic Lemont (which derives its aroma from Della), and determined the gene was 4.5 cM from the chromosome 8 RFLP marker (RG28). Linkage of RG28 to aroma was verified using an F_3 population segregating for fragrance with fragrant and non-fragrant Lemont parents. Phenotyping the F_3 population was undertaken both by chewing seeds and by utilising the method developed by Berner and Hoff (1986) which involved placing leaf or grain samples in KOH before smelling the samples and scoring each sample fragrant or non-fragrant in a binary fashion. Using both gas chromatography analysis of 2AP and sensory evaluation panelists in conjunction with linkage analysis of 16 polymorphic markers separated by no more than 25 cM across chromosome 8 in a population of 135 double haploid lines, Lorieux *et al*. (1996) further refined the map position of *fgr,* when they determined *fgr* is flanked by the molecular markers RG28 and RG1 at a distance of 6.4 ± 2.6 and 5.3 ± 2.7 cM, respectively. Lorieux *et al*. (1996) also identified two other QTL on chromosomes 4 and 12 involved in fragrance. However, these QTL were only detected when the analysis accounted for a major gene for fragrance being located on chromosome 8.

In an effort to develop PCR based markers for *fgr,* Garland *et al*. (2000) identified a one base pair polymorphism within the RFLP clone RG28. This marker required the use of

expensive capillary electrophoresis equipment to discriminate between PCR products that differed by one base pair and because it was physically removed from *fgr* was not 100% accurate in discriminating between non-fragrant and fragrant plants. Corderio *et al*. (2002) utilised rice genome sequence data to identify a SSR marker which was approximately 4 cM from *fgr*. This loci was highly polymorphic, 13 alleles were identified, eight were found in the fragrant plants and eight in the non-fragrant plants, three of which were common to both fragrant and non-fragrant varieties. DNA sequence analysis of approximately 500bp of the 5' ends of 14 genes chosen based on their proximity to RG28 revealed only one SNP (RSP04) between Kyeema (fragrant cultivar) and Doongara (non-fragrant cultivar) (Jin *et al*., 2003). This SNP was assessed for linkage to *fgr* by pyrosequence analysis of the SNP in 164 phenotyped F_2 individuals from a Gulfmont/Doongara cross, placing RSP04 approximately 2 cM from *fgr*, making RSP04 the closest known marker to *fgr*.

This study was undertaken to identify the gene responsible for the generation and accumulation of the subtle yet potent fragrance compound 2-acetyl-1pyrroline in Jasmine and Basmati style rice. A combined molecular, enzymatic and metabolomic investigation of this fragrance pathway was undertaken to aid in the breeding of fragrant rice varieties and to gain insights into the potential enhancement of 2-acetyl-1-pyrroline levels in a variety of foodstuffs.

CHAPTER 2: The Gene for Fragrance in Rice

Summary:

The flavour or fragrance of basmati and jasmine rice is associated with the presence of 2-acetyl-1-pyrroline. A recessive gene (*fgr*) on chromosome 8 of rice has been linked to this important trait. This chapter shows that a gene with homology to the gene that encodes betaine aldehyde dehydrogenase (BAD) has significant polymorphisms in the coding region of fragrant genotypes relative to non fragrant genotypes. The accumulation of 2-acetyl-1-pyrroline in fragrant rice genotypes may be explained by the presence of mutations resulting in a loss of function of the *fgr* gene product. The allele in fragrant genotypes has a mutation introducing a stop codon upstream of key amino acid sequences conserved in other BADs. The *fgr* gene corresponds to the gene encoding BAD2 in rice while BAD1 is encoded by a gene on chromosome 4. BAD has been linked to stress tolerance in plants. However, the apparent loss of function of BAD2 does not seem to limit the growth of fragrant rice genotypes. Fragrance in domesticated rice has apparently originated from a common ancestor and may have evolved in a genetically isolated population or may be the outcome of a separate domestication event. This is an example of effective human selection for a recessive trait during domestication.

Introduction:

The flavour of a range of foods, including, popcorn (Schieberle, 1995), corn tortillas (Buttery and Ling, 1995), baguettes (Zehentbauer and Grosch, 1998), ham (Carrapiso *et al*., 2002), cheese (Zehentbauer and Reineccius, 2002), mung bean (Brahmachary and Ghosh, 2002), green tea (Kumazawa and Masuda, 2002) and wine (Herderich *et al*., 1995) has been associated with the presence of 2-acetyl-1-pyrroline. However this compound is most closely associated with the fragrance of basmati rice (Buttery *et al*., 1983; Lorieux *et al*., 1996; Widjaja *et al*., 1996; Yoshihashi *et al*., 2002). Although many other compounds are also found in the headspace of fragrant rice varieties (Widjaja *et al*., 1996), possibly due to secondary effects related to the genetic background of the rice variety, 2-acetyl-1-pyrroline is widely known to be the main cause of the distinctive basmati and jasmine fragrance. The desirability of fragrance would result in strong human preference and selection for this trait. Non-fragrant rice varieties contain very low levels of 2-acetyl-1-pyrroline while the levels in fragrant genotypes are much higher (Widjaja *et al*., 1996). A recessive gene, on chromosome 8 of rice, largely controlling the level of 2-acetyl-1-pyrroline, has been identified in genetic studies. Genetic markers for this gene have been developed to allow selection for this trait in rice breeding. The chromosomal location of the gene was defined by mapping in segregating populations using simple sequence repeat (SSR) or microsatellite (Cordeiro *et al*., 2002) and single nucleotide polymorphism (SNP) (Jin *et al*., 2003) markers. The availability of a rice genome sequence (Goff *et al*., 2002) provided an opportunity to discover the gene responsible by comparison of the sequences of fragrant and non-fragrant genotypes. This

allowed this study to target the re-sequencing of genes and genomic sequences in a fragrant genotype to the most likely parts of chromosome 8.

Materials and methods:

Plant materials

A population of 168 field grown F_2 individuals derived from a cross between Kyeema (Pelde//Della/Kulu) (tall, jasmine-style, long-grain, Australian cultivar) and Gulfmont (Lebonnet//CI9881/PI 331581) (early-maturing, semi-dwarf, non-aromatic long-grain USA cultivar) supplied by Yanco Agricultural Institute, NSW Agriculture were used as the mapping population in this study.

Following genetic mapping, candidate genes were identified in the region between flanking markers in the published genome sequence and re-sequenced in the fragrant rice variety Kyeema. Polymorphisms were identified by a visual inspection of alignments of sequence derived from Kyeema with the published genome sequence of Nipponbare, a non-fragrant variety. Polymorphisms that were considered to be a possible cause of fragrance were genotyped in 14 fragrant and 64 non-fragrant varieties. The fragrant varieties analysed were: YRF203, 00210-0-15, YRF207/1202, Yasmin, Amber, Dumsorhk, Dellmont, YRF207, YRF204, 00210-33, Basmati370, Dragon Eyeball 100, Goolarah and Khao Dawk Mali 105. The non-fragrant varieties analysed were: YRM42/Wakamizu, Bluebelle, M9, Koshi, Nipponbare, YRM62/M103/M201/Calrose, Rexmont, YRL118, Calmochi 202, YRW4, YRM54/Rexmont, Calrose, Rufipogen,

Sakha102, M20/YRI96/Ardito/YRM54, YRM54/Akitakomachi, Haenukai, Vialone Nano, M202, YRM2/M101/M103/Koshihikari, Hungarian no. 1, Wakamizu, Shimuzi mochi, Millin, Teqing, Giza178, Illabong, Gulfmont, RIL266, Somewake, Pi4, BBL/M9/Pelde/YRI30/4/YRF207, YRL118/Inga, Koshihikari, YRM42IRBB59, Giza182, M201/YRM3/Bogan/h989-4s, L202, YRM49, Jarrah, Dawn, Giza176, Haenukai/Illabong, Akitakomachi, Matsuribane, Sakha101, YRL118/Inga/M9/213d.25, Amaroo, M101, YRM62, Pelde, Moroberekan, Sakha103, YRM54/Akitakomachi, Ardito, M102, Kairyo Mochi, Sakha104, YRM54/Wakamizu, Ari Combo, M103, M401, Echuca and BL24-2.

Genetic mapping

Fragrance was evaluated according to Berner and Hoff (1986). The phenotypes of F_2 individuals were classified as fragrant, segregating or non-fragrant by tasting dehulled F_3 seed. At least 12 F_3 seeds from individual F_2 plants were chewed individually. F_2 plants were rated homozygous fragrant or non-fragrant if all 12 F_3 seeds were fragrant or non-fragrant, respectively. F_3 seeds from heterozygous F_2 plants were expected to contain both fragrant and non-fragrant seeds, therefore if the sample from a single F_2 plant was a mixture of fragrant and non-fragrant, the F_2 plant was considered to be heterozygous. The observed segregation ratio of fragrant:segregating:non-fragrant was tested by χ^2 analysis against the expected ratio for a single gene.

Following identification, SSRs were assessed for polymorphism by comparison of parental alleles. Polymorphic SSRs were genotyped in F_2 individuals from the mapping population. The genetic distances between *fgr* and the polymorphic SSRs were estimated

using MAPMAKER V.3.0 and determined as the percentage of recombinant chromosomes (cM).

Bioinformatics and computer programs

The major gene controlling grain fragrance in rice has been located between the RG1 and RG28 restriction fragment length polymorphism (RFLP) markers (Lorieux *et al*., 1996). Fourteen BAC clones were selected based on their proximity between RFLP markers R1 and RG28 and the sequences of BAC were obtained from GenBank (http://rgp.dna.affrc.go.jp). SSRs were identified by using the Simple Sequence Repeat Identification Tool (SSRIT) from http://www.gramene.org/db/searches/ssrtool. Nine mapped microsatellite markers between RG1 and RG28 were selected for the assessment of polymorphism. The primer sequences for these markers are available from http://www.gramene.org/microsat/ssr.html.

Oligonucleotide primers were designed using Primer Premier Version 5.0 (Premier Biosoft International, Palo Alto, CA). Sequence alignments were performed using ChromasPro version 1.15 (Technelysium Pty Ltd, www.technelysium.com.au/ChromasPro.html). BAC AP004463 sequence was obtained from the NCBI web site (www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=24460082). cDNA sequences were obtained from the KOME web site (http://cdna01.dna.affrc.go.jp/cDNA/) using the search term AP004463 and were selected based on their proximity to microsatellite marker SSR-J02 and SNP marker RSP04, and on their predicted functions.

Phylogenetic trees were designed using MacVector™ 7.0 using bootstrap support based on 10,000 replicates. Nucleotide and amino acid alignments were performed using ClustalW.

DNA extraction, PCR, genotyping and sequence analysis

Genomic DNA was extracted using a Qiagen Dneasy® 96 Plant Kit (Qiagen, Doncaster, Victoria, Australia). Oligonucleotide primers were synthesized by Proligo Australia Pty Ltd (Lismore, NSW, Australia). PCR was performed using Platinum® Taq DNA Polymerase (Gibco BRL®, (Invitrogen), Mulgrave, Victoria, Australia) under standard conditions.

SSRs were amplified by PCR and analysed by electrophoresis in either ethidium bromide-stained (0.5 μg/mL) 2.0% agarose or using a Corbett Robotics (Brisbane, QLD, Australia) Gel-Scan 2000™. A 100 bp ladder molecular weight standard (Roche, Castle Hill, NSW, Australia) was used to estimate PCR fragment size.

PCR products were purified using a montage PCR filter device (Millipore Corporation, NorthRyde, NSW, Australia). Sequence reactions were performed using BigDye Terminator version 3.1 (Applied Biosystems, Scoresby, Victoria, Australia), and the completed reactions were purified by ethanol precipitation (Ausubel *et al*., 1998). The reaction products were analysed on an Applied Biosystems 3730 Genetic Analyser.

Results and Discussion:

Genetic mapping of molecular markers and the fragrance phenotype demonstrated that the markers RM515 and SSR-J07 flanked *fgr* (Fig. 2.1). The physical distance between RM515 and SSRJ07 is 386 591 bp. The data suggested that *fgr* was closer to RM515 than to SSRJ07. Careful examination of mapping data suggested that one bacterial artificial chromosome (BAC) (clone AP004463, Fig. 2.1) was most likely to contain the gene. Re-sequencing 17 genes in this BAC revealed significant sequence variations in only one, other genes in this region showed very little polymorphism. This analysis revealed 6 polymorphisms, 3 of which were in exons, including a large polymorphism within an exon of the candidate gene, listed on the Knowledge-based Oryza Molecular Biological Encyclopaedia (KOME) web site as cDNA clone J023088C02 which codes for a betaine aldehyde dehydrogenase homolog. This large polymorphism contained a total of 6 SNPs and 8 deletions within a 25bp region causing the introduction of a stop codon (Fig 2.2). It is highly likely that this mutation would render the protein non-functional, this fits the evidence of fragrance being a recessive trait. Sequence analysis of this region in 14 diverse fragrant and 64 non-fragrant rice varieties found the 14 fragrant varieties showed the identical sequence polymorphism observed in Kyeema while the 64 non-fragrant varieties showed sequence identical to the published Nipponbare sequence, suggesting this is *fgr*.

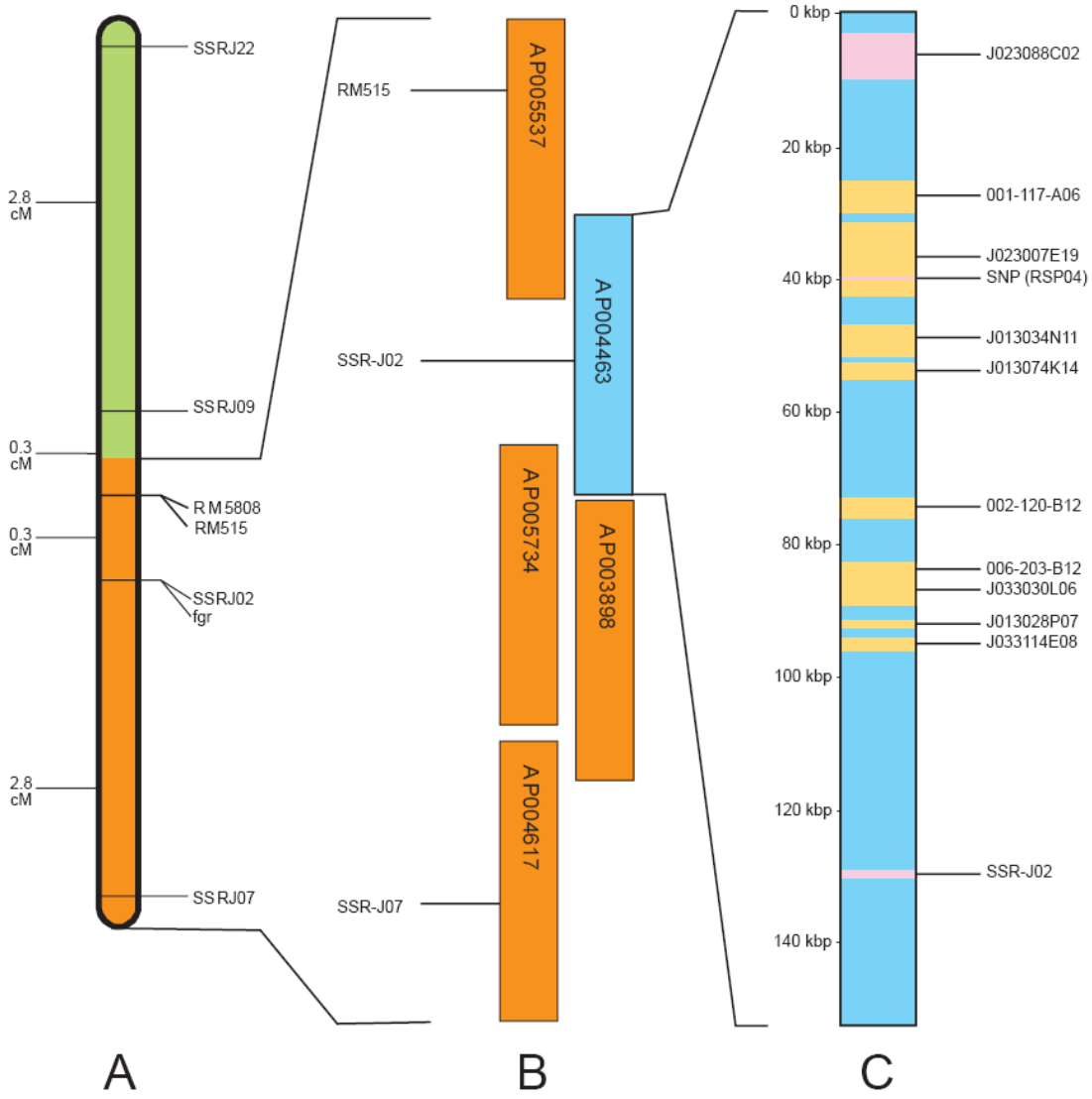

Figure 2.1. Location of fragrance gene in; A) a section of rice chromosome 8, showing the genetic distance between *fgr* and chromosomal markers, B) BAC clones covering the region of rice chromosome 8 flanked by the chromosomal markers RM515 and SSR-J07 where the *fgr* gene was calculated to reside, and C) the AP004463 BAC clone showing the genes in this region (designations assigned by KOME) including the predicted *fgr* gene (J023088C02) and chromosomal markers SSR-J02 and RSP04.

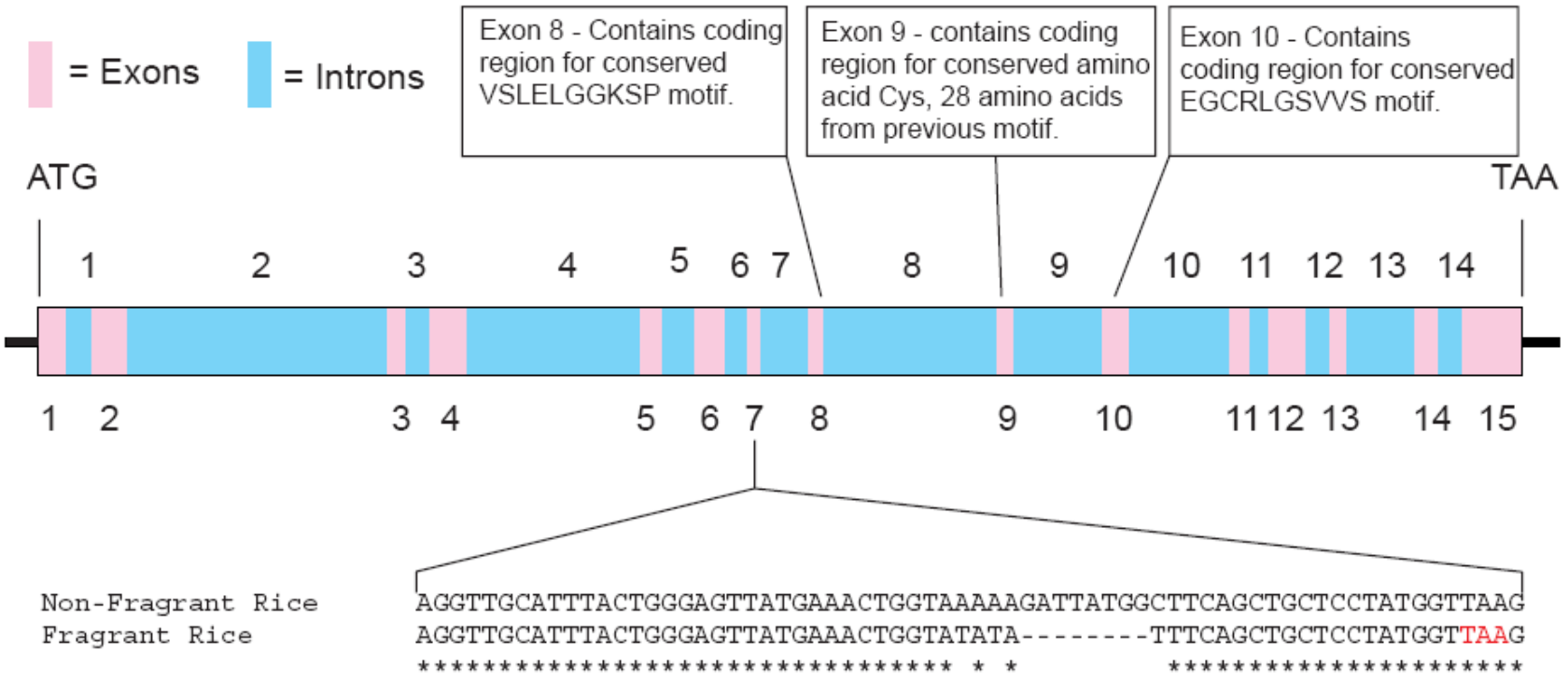

Figure 2.2. Structure of the fragrance gene (*fgr*) (KOME ID: J023088C02) showing initiation codon (ATG), 15 exons, 14 introns and the ATT termination site. The nucleotide sequence of exon 7 is shown for both non-fragrant and fragrant rice varieties. The fragrant variety shows a large deletion and 3 SNPs which then terminates prematurely (stop codon in red), within this exon. The truncated protein encoded in fragrant rice varieties would therefore lack the highly conserved sequences encoded by exons 8, 9 and 10 and which are believed to be required for correct function of this protein.

The predicted amino acid sequence for the protein encoded by the *fgr* gene is shown in Fig 2.3. A peptide sequence (VTELGGKSP) and a cysteine residue [28 amino acid residues away in both betaine aldehyde dehydrogenase 1 (BAD1) and BAD2], found in the genes from non fragrant rice, is highly conserved in aldehyde dehydrogenases (Li *et al*., 2003). These conserved elements are lost in the shorter protein that would be encoded by the gene in fragrant varieties. BADs also contain the conserved peptide EGCRLGSVVS found in the gene from non-fragrant varieties. BAD has been shown to have wide substrate specificity for amino acids and related compounds (Trossat *et al*., 1997; Livingstone *et al*., 2003), many of these compounds are structurally similar to 2-acetyl-1-pyrroline, suggesting that it (or its precursors) is a likely substrate for BAD. Substrates reported for BAD include 3-aminopropionaldehyde, 4-aminobutyraldehyde, 4-guanidinobutyraldehyde (Livingstone *et al* ., 2003) and, most significantly, delta-1-pyrroline, as delta-1-pyrroline exists in equilibrium with 4-aminobutyraldehyde (Trossat *et al* ., 1997). BAD1 in rice is encoded by a gene on chromosome 4. Barley has been shown to contain two BAD isozymes, probably with different substrate specificities (Nakamura *et al*., 2001). The *fgr* gene corresponds to the gene encoding BAD2 from barley. Comparison of the amino acid sequence of the two BAD isozymes from barley revealed that BAD2 lacked the C terminal SKL signal sequence that is present in the BAD1 isozyme (Nakamura *et al*., 2001) and is known to be part of a family of type 1 peroxisomal targeting signals (PTS1) (Reumann, 2004). In contrast, both BAD1 and BAD2 in rice contain the C terminal SKL sequence, suggesting both proteins are targeted to the peroxisome. This indicates that although both the rice and barley BAD2 proteins may have similar *in vitro* activities, they may have a different functional role *in vivo* as

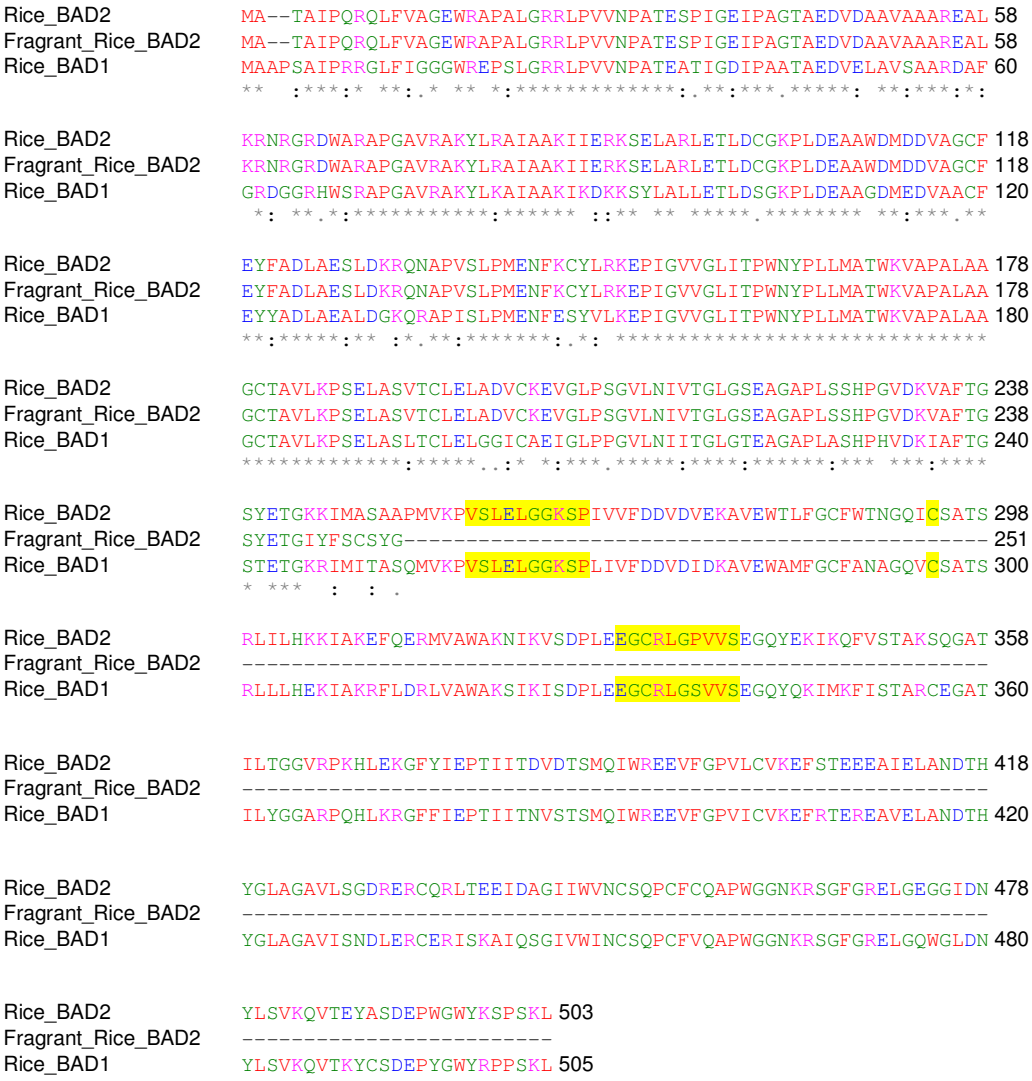

Figure 2.3. Clustal W alignment of the amino acid sequence of the BAD1 protein, encoded for on rice chromosome 4, BAD2 protein from non-fragrant rice, encoded for on rice chromosome 8 and the predicted amino acid sequence of the truncated BAD2 protein from fragrant rice. Highlighted are sequences that are highly conserved in all BADs and are believed to be required for functional activity, these conserved regions are absent from the truncated version present in fragrant rice varieties.

they appear to be targeted to different subcellular locations. However, there are many factors that influence the targeting of PTS1 containing proteins, such as overriding N terminal signal sequences or protein folding that prevents recognition of the PTS1 (Reumann, 2004), these may influence the subcellular targeting of the BAD1 and BAD2 proteins in both rice and barley. The production of two different subunits in the same subcellular compartment allows for the possible formation of heterodimers of the two subunits. The presence of such heterodimers might lead to an altered substrate specificity of BAD in rice. The presence of 2 BAD homologs has been widely reported in many grasses and the BAD2 protein in rice appears to be closely related to the BAD2 protein from wheat, barley, sorghum, mays and the turfgrass *Zoysia tenuifolia* (Fig 2.4). Analysis of the sequences in genes from other species (Fig 2.4) shows that BAD2s from different species are more closely related than different BADs from the same species, suggesting a distinct and important role for each of the two BADs. However, only the BAD2 protein in *Z.tenuifolia* has a similar PTS1 at its C terminus.

Although the biochemical pathway leading to fragrance in rice has not been established, L-proline has been shown to be a precursor of aroma in rice (Yoshihashi, 2002). The *fgr* gene may encode a protein that either catalyses the formation or the removal of 2-acetyl-1-pyrroline. Fragrance is a recessive trait suggesting a loss of function is responsible for the accumulation of 2-acetyl-1-pyrroline. The truncated version of the protein that is encoded by the fragrant genotypes is less likely to be functional and favours this hypothesis. However, fragrance may be due to a loss of function in a competing pathway. A loss of function of an enzyme consuming a precursor of 2-acetyl-1-pyrroline may

explain the elevated levels in fragrant varieties. Other compounds found in fragrant rice may result from the altered metabolic pathways introduced by this single gene change. The different flavours perceived in some genotypes may be due to the modifying influence of other secondary metabolic consequences of this mutation in different genetic backgrounds. This observation suggests that this type of fragrance may be found or produced in other plant species. Pandanus (*Pandanus amaryllifolius* Roxb) is grown as a source of food flavour and also contains 2-acetyl-1-pyrroline (Laohakunjit and Noomhorm, 2004). The Pandanus flavour may have a similar genetic and molecular basis to that in rice. Pandanus is a monocotyledonous plant but the Pandanales are not in the commelinid group that includes the grasses (Poaceae) (Chase, 2005). The origin of 2-acetyl-1-pyrroline in more distant organisms such as yeast (Munch and Schieberle, 1998) might be via another mechanism.

There is strong evidence that cereals have been cultivated and consumed by humans for a very long time (Piperno *et al*., 2004) but the route of domestication of cereals including rice is not understood. Key mutations associated with domestication of barley (Piffanelli *et al*., 2004) and maize (Wang *et al*., 1999) are associated with changes in gene expression at important loci. The *fgr* gene parallels the *sd1* gene (Sasaki *et al*., 2002) for

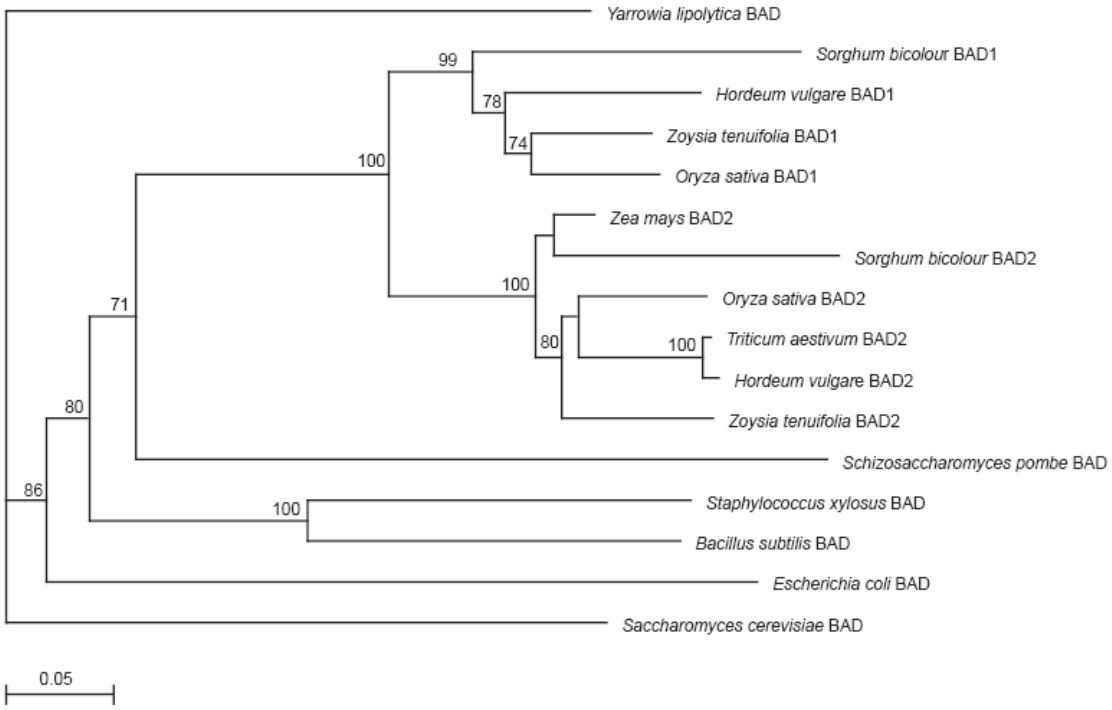

Figure 2.4. Phylogenetic tree showing relatedness of BADs from various species. BAD1 and 2 proteins form distinct groups within monocotyledonous plant species. No full length BAD1 protein or cDNA sequences were available for wheat or mays although there were partial sequences suggesting that BAD1's were present in these species. Bacterial and yeast BAD2's were much less related to any plant BADs. The numbers above nodes indicate bootstrap support based on 10,000 replicates, constructed using MacVector™ 7.0

NCBI accession numbers for protein sequences are *Y.lipolytica* CAG80743, *S.bicolour* BAD1 - AAC49268, *H.vulgare* BAD1 - BAB62847, *Z.tenuifolia* BAD1 - BAD34957, *O.sativa* BAD1 - BAA21098, *Z.mays* BAD2 (translated from mRNA sequence) PCO110216, *S.bicolour* BAD2 - AAC49267, *O.sativa* BAD2 - BAC99806, *T.aestivum* BAD2 - AAL05264, *H.vulgare* BAD2 - BAB62846, *Z.tenuifolia* BAD2 - BAD34947, *S.pombe* BADH - CAA19114, *S.xylosus* GBADH - AAD23900, *B.subtilis* NP_390984, *E. coli* BetB - BAB33781 and *S.cerevisiae* ALDH2 - CAA78962.

semi-dwarf stature in that the trait is due to a mutation resulting in loss of function in one member of a gene family that is not essential for survival whilst the loss of function of other members of the gene family might be lethal. The concentration of 2-acetyl-1-pyrroline in rice is influenced by the environment and reported to be higher in plants subjected to water stress (Yoshihashi *et al*., 2004). BAD has been associated with stress tolerance in plants (Nakamura *et al*., 2001; Li *et al*., 2003; Livingstone *et al*., 2003). A gene that encodes BAD from a halophyte (*Suaeda liaatungenis*) improved salt tolerance when expressed in tobacco (Li *et al*., 2003). Introduction of a barley gene that encodes BAD into rice has been shown to improve tolerance of the plants to salt, cold and heat (Kishitani *et al*., 2000). The mutation in the gene that encodes BAD2 in fragrant rice varieties does not seem to be associated with any loss of plant performance and may have a positive effect under some environmental conditions, as the fragrant variety Khao Dawk Mali 105 was initially selected for drought tolerance (Yoshihashi *et al*., 2004), the concentration of 2-acetyl-1-pyrroline in this variety was found to increase in response to drought and increased salt concentrations (Yoshihashi *et al*., 2004). The presence of one allele of the gene in all fragrant rice genotypes examined is consistent with the trait being inherited from a common fragrant genotype. The modern rice varieties analysed may all have been derived from the same original fragrant parent. This is a difficult trait to breed for because of the recessive nature of the gene and the difficulty of assessing the fragrance of individual grains of rice. The *mlo*-11 allele (Piffanelli *et al*., 2004) was probably selected by biotic stresses in the environment during the domestication of barley. The allele of the *sd1* gene responsible for dwarf stature was introduced into rice by modern plant breeding. The *fgr* gene in basmati rice is likely to be the product of

human selection for flavour long before plant breeding. All three are examples of loss of gene expression leading to a desirable trait.

The unique allele of the *fgr* gene in fragrant rice suggests one of several possibilities, fragrant rice may have been the outcome of a separate domestication event or may have arisen and evolved independently in a wild relative or in a genetically or geographically isolated population. Basmati rice types have previously been described as being a genetically distinct cluster that has poor combining ability with other rice genotypes (Kaushik *et al*., 2003), crosses with non-fragrant varieties often lead to low seed production or sterility (Pinson, 1994).

Evidence for the gene that encodes BAD2 and *fgr* being the same gene comes from many lines of enquiry, including positional, genetic, biochemical and physiological: (i) the gene that encodes BAD2 is in the exact chromosomal location suggested by genetic mapping; (ii) the loss of function is consistent with a recessive trait; (iii) a gene of amino acid metabolism downstream of proline is suggested by metabolic evidence (Yoshihashi *et al* ., 2002) and BAD is a credible biochemical candidate in this pathway; and (iv) elevated fragrance in response to stress has been reported (Yoshihashi *et al* ., 2004), as would be predicted from this biochemistry. The complete association of the deletion in the gene that encodes BAD2 with fragrance in a wide range of unrelated germplasm is especially convincing evidence. Genetic complementation experiments and analysis of the biochemical reactions catalysed by BAD2 and BAD1 may provide further insights into the basis of fragrance in rice.

CHAPTER 3: A perfect marker for fragrance genotyping in rice

Summary:

Allele specific amplification (ASA) is a low-cost, robust technique that can be utilised to discriminate between alleles that differ by SNPs, insertions or deletions, within a single PCR tube. Fragrance in rice, a recessive trait, has been shown to be due to an eight bp deletion and three SNPs in a gene on chromosome 8 which encodes a putative betaine aldehyde dehydrogenase 2 (BAD2). This chapter describes the generation of a single tube ASA assay which allows discrimination between fragrant and non-fragrant rice varieties and identifies homozygous fragrant, homozygous non-fragrant and heterozygous non-fragrant individuals in a population segregating for fragrance. External primers generate a fragment of approximately 580 bp as a positive control for each sample. Internal and corresponding external primers produce a 355 bp fragment from a non-fragrant allele and a 257 bp fragment from a fragrant allele, allowing simple analysis on agarose gels.

Introduction:

The demand for fragrant rice has increased markedly in recent years in both traditional and non-traditional rice growing countries to such an extent that consumers are willing to pay a premium price for fragrant rices. In order to assist in the development of fragrant rice varieties suited to particular local environmental conditions, rice breeders have an interest in gaining access to a simple and inexpensive method for distinguishing between fragrant and non-fragrant rice.

The flavour and fragrance of Basmati and Jasmine style rice have been associated with increased levels of 2-acetyl-1-pyrroline (2AP) (Buttery *et al*., 1983; Lorieux *et al*., 1996; Widjaja *et al*., 1996; Yoshihashi, 2002). A number of sensory methods have been utilised to assist breeders in selecting fragrant rice but they have limitations when processing large numbers of samples. For example, tasting individual grains is one of the original methods for the quality selection of fragrant rice varieties within the Australian breeding program (Reinke *et al*., 1991) and is still the principal means of identifying fragrance in many breeding programs worldwide. However, the objective evaluation of fragrance using this method is labour intensive, difficult and unreliable. A panel of analysts is required as the ability to detect fragrance varies between individuals. For any individual analyst, the ability to distinguish between fragrant and non-fragrant samples diminishes with each successive analysis because the senses become saturated or physical damage occurs from abrasions to the tongue which often result from chewing the hard grain. Chemical methods are available which involve smelling leaf tissue or grains after heating

in water or reacting with solutions of KOH or I_2-KI (Sood and Sidiq, 1978) but these can cause damage to the nasal passages. An objective method of 2AP identification using gas chromatography is available but the assay requires large tissue samples and is time consuming (Lorieux *et al*., 1996; Widjaja *et al*., 1996).

More recently molecular markers, such as SNPs and simple sequence repeats (SSRs), which are genetically linked to fragrance and have the advantage of being inexpensive, simple, rapid and only requiring small amounts of tissue, have been developed for the selection of fragrant rice (Cordeiro *et al*., 2002). However, these markers are only linked with the fragrance gene and therefore do not allow prediction of the fragrant status of any one rice sample with 100% accuracy.

Recently, an eight base pair deletion and three SNPs in exon 7 of the gene encoding betaine aldehyde dehydrogenase 2 (BAD2) on chromosome 8 of *Oryza sativa* was identified as the likely cause of fragrance in Jasmine and Basmati style rice (Chapter 2). Non-fragrant rice varieties possess what appears to be a fully functional copy of the gene encoding BAD2 while fragrant varieties possess a copy of the gene encoding BAD2 which contains the deletion and SNPs, resulting in a frame shift that generates a premature stop codon that presumably disables the BAD2 enzyme. This polymorphism provides an opportunity for the construction of a perfect marker for fragrance in rice. This chapter reports the construction of a PCR assay for fragrance genotyping in rice.

Materials and Methods:

Plant materials

All rice samples were supplied by Yanco Agricultural Institute, NSW Agriculture.

A diverse collection of 14 fragrant and 74 non-fragrant varieties (Chapter 2) in addition to a population of 168 field grown F_2 individuals derived from a cross between Kyeema (Pelde//Della/Kulu) (tall, Jasmine-style, long-grain, Australian cultivar) and Gulfmont (Lebonnet//CI9881/PI 331581) (early-maturing, semi-dwarf, non-aromatic long-grain USA cultivar) was used was used to validate the marker.

Genetic mapping

Fragrance was evaluated according to Berner and Hoff (1986). The phenotype of F_2 individuals were classified as fragrant, segregating or non-fragrant by tasting dehulled F_3 seed. At least 12 F_3 seeds from individual F_2 plants were chewed individually. F_2 plants were rated homozygous fragrant or non-fragrant if all 12 F_3 seeds were fragrant or non-fragrant, respectively. F_3 seeds from heterozygous F_2 plants were expected to contain both fragrant and non-fragrant seeds, therefore if the sample from a single F_2 plant was a mixture of fragrant and non-fragrant, the F_2 plant was considered heterozygous. The observed segregation ratio of fragrant:segregating:non-fragrant was tested by χ^2 analysis against the expected ratio for a single gene.

Primer design

Oligonucleotide primers were designed, using Primer Premier Version 5.0 (Premier Biosoft International, Palo Alto, CA). For non-fragrant varieties the sequence of the gene encoding BAD2 was obtained from the NCBI web site (www.ncbi.nlm.nih.gov) Gen Bank accession number – AP004463 and for fragrant varieties the sequence of the gene encoding BAD2 reported in Chapter 2 was used.

DNA extraction, PCR and genotyping

Genomic DNA was extracted from leaf material using a Qiagen DNeasy® 96 Plant Kit (Qiagen GMbH, Germany) and from whole seeds as described by Bergman *et al* (2001). Rough leaf DNA extractions were performed by boiling 0.1 g of leaf material in 50 µl 10X PCR Buffer (Gibco BRL®) for 10 min. Oligonucleotide primers were synthesised by Proligo Australia Pty Ltd. PCR was performed using 0.2 µl Platinum® Taq DNA Polymerase (Gibco BRL®), 1 µl of genomic DNA 10 ng μl^{-1}, 2.5 µl of 10X buffer (Gibco BRL®), 1 µl of 50 mM $MgCl_2$ (Gibco BRL®), 1 µl of dNTPs [5 mM], 2.5 µl of each primer (ESP, IFAP, INSP and EAP – Table 3.1) [2 µM], in a total volume of 25 µl. PCR was performed using a Perkin Elmer, Gene Amp PCR system 9700. Cycling conditions were an initial denaturation of 94 ºC for 2 min followed by 30 cycles of 5 s at 94 ºC, 5 s at 58 ºC, 5 s at 72 ºC; concluding with a final extension of 72 ºC for 5 min.

PCR products were analysed by electrophoresis in ethidium bromide stained (0.5 ug ml^{-1}) 1.0% agarose gels. A 100 bp ladder molecular weight standard (Roche) was used to estimate PCR fragment size.

Table 3.1. Primers for analysis of fragrance in rice.

Primer name	**5' Primer sequence 3'**
External Sense Primer (ESP)	TTGTTTGGAGCTTGCTGATG
Internal Fragrant Antisense Primer (IFAP)	CATAGGAGCAGCTGAAATATATACC
Internal Non-fragrant Sense Primer (INSP)	CTGGTAAAAAGATTATGGCTTCA
External Antisense Primer (EAP)	AGTGCTTTACAAAGTCCCGC

Results:

Development of the single tube Allele Specific PCR assay

Four primers, two that anneal to sequences common to both fragrant and non-fragrant varieties and external to the area where the mutation occurs and two that are specific to one of the two possible alleles were designed and synthesised (Figure 3.1). The two external primers were designed to act as an internal positive control amplifying a region of approximately 580 bp in both fragrant (577 bp) and non-fragrant (585 bp) genotypes. Individually, these external primers also pair with internal primers to give products of varying size, depending upon the genotype of the DNA sample. The internal primers, IFAP and INSP (Table 3.1), will anneal only to their specified genotype producing DNA fragments with their corresponding external primer pair, ESP and EAP respectively. Using these four primers in a PCR results in three possible outcomes. In all cases a positive control band of approximately 580 bp is produced. In the first case a band of 355 bp is produced indicating a variety or individual is homozygous non-fragrant. In the second case a band of 257 bp is produced indicating a variety or individual is homozygous fragrant. In the third case both bands of sizes 355 bp and 257 bp are produced indicating an individual is heterozygous non-fragrant.

Determination of plant genotype using single tube ASA PCR assay

PCR products were easily separated on an agarose gel. The PCR product of approximately 580 bp serves as a positive control and is present in every sample. Fragrant individuals have a second a product of 257 bp in size while non-fragrant

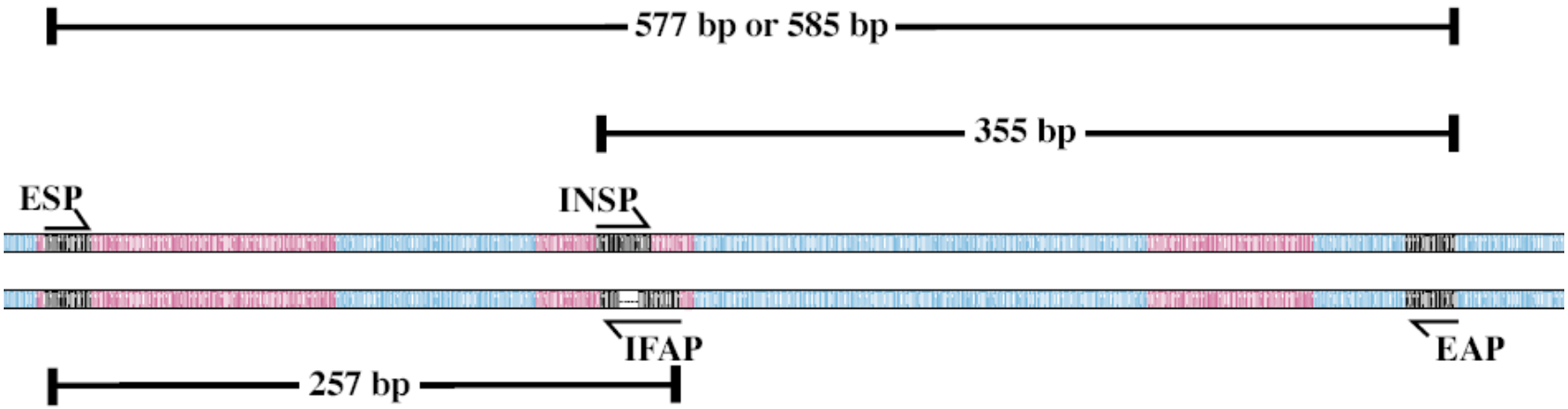

Figure 3.1. Fragrant (Below) and non-fragrant (Above) alleles corresponding to a section of the gene encoding BAD2 [part of intron 5 to 8 (blue), including exons 6, 7 and 8 (pink)] showing the deletion in exon 7 and positions of PCR primers (from table 3.1) used in fragrance assay and size of PCR products.

individuals give a product of 355 bp in size, heterozygotes can also be discriminated by the presence of all three PCR products (Figure 3.2).

The assay predicted the phenotype of 168 F_2 progeny segregating for fragrance with 100% accuracy (46 homozygous fragrant, 80 heterozygotes, 42 homozygous non-fragrant). (Figure 3.3). The assay also allows discrimination between fragrant and non-fragrant grains using DNA derived from rice grains using a simple NaOH extraction protocol (Bergman *et al*., 2001) and leaves using a simple 10 min boiling protocol.

Further evaluation demonstrated the capacity of the assay to work on a broard range of fragrant varieties such as Basmati 370, Kyeema, Khao Dwak Mali 105 and Moosa Tarom (results not shown).

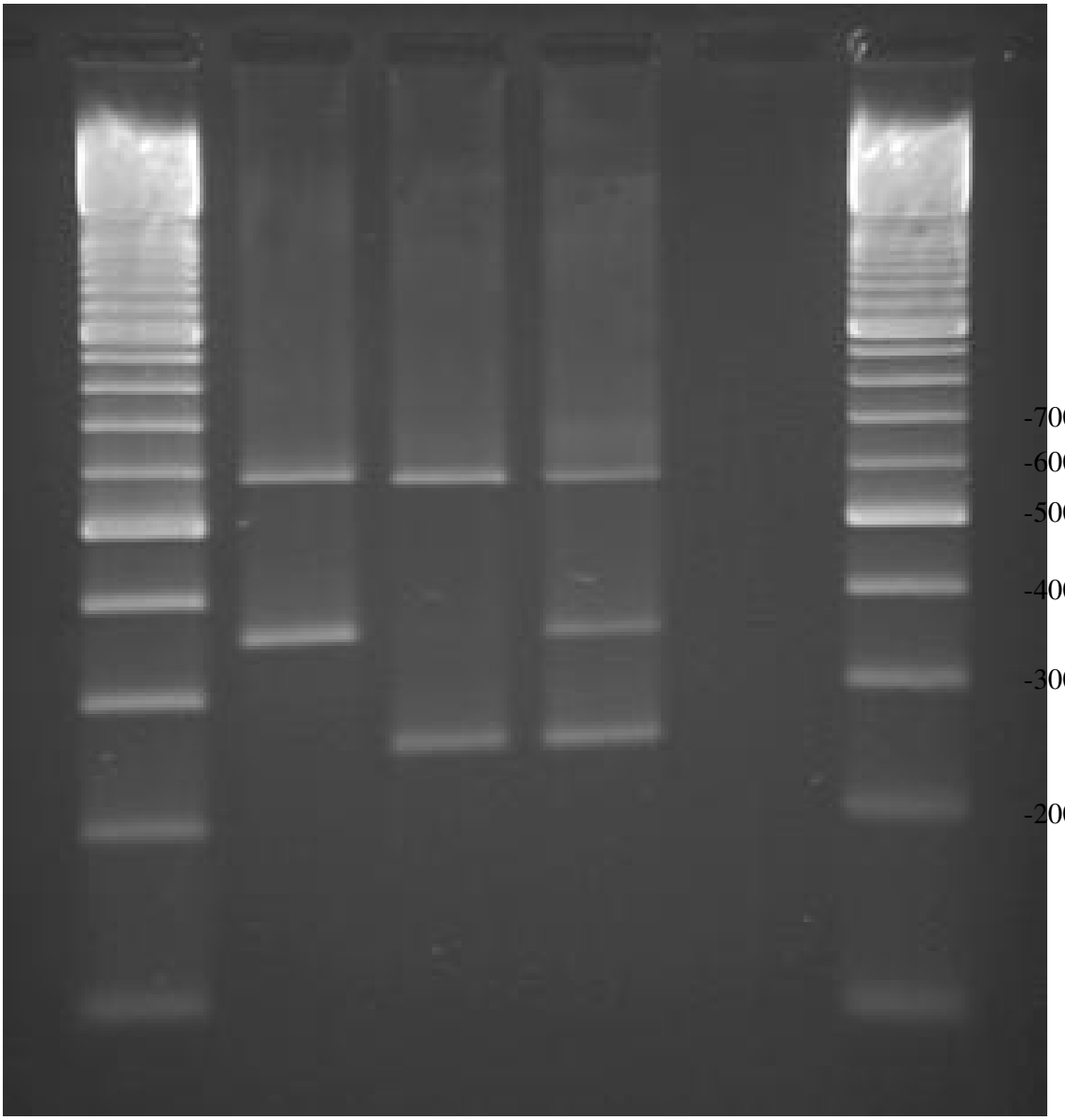

Figure 3.2. Agarose Gel showing (lane 2-5) a non-fragrant variety (Nipponbare), a fragrant variety (Kyeema), a heterozygous individual (Kyeema/Gulfmont) and a negative control (water) flanked by Roche DNA Ladder XIV (100 bp).

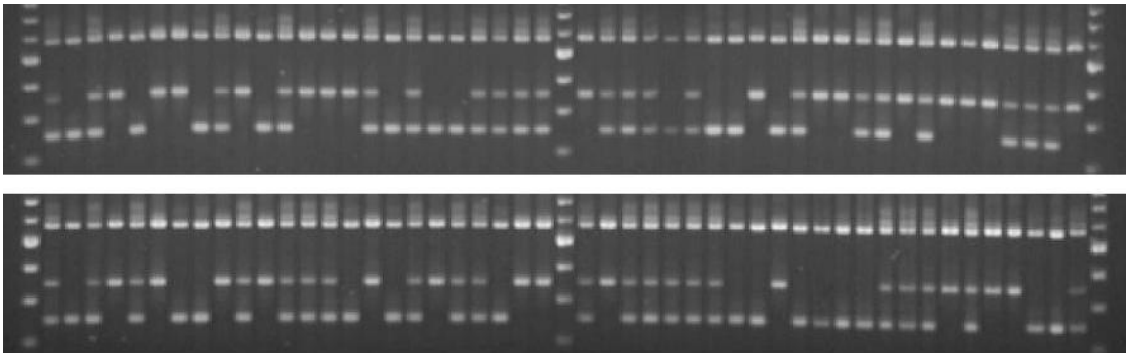

Figure 3.3. Agarose gel showing 96 individuals from an unselected F2 population segregating for fragrance and analysed using single tube ASA. The band of approximately 580 bp corresponds to the positive control amplified by both external primers (ESP and EAP). The 355 bp band corresponds to a PCR product amplified from the non-fragrant allele by the internal non-fragrant sense primer (INSP) and the external antisense primer (EAP). The 257 bp band corresponds to a PCR product amplified from the fragrant allele by the internal fragrant antisense primer (IFAP) and the external sense primer (ESP).

Discussion:

Fragrance in Basmati and Jasmine style rice is a recessive trait (Lorieux *et al*., 1996) which results principally from the presence of elevated levels of the compound 2-acetyl-1-pyrroline (2AP) in the aerial parts of the plant. A deletion in the gene encoding BAD2 on chromosome 8 which disables the BAD2 enzyme is the most likely cause of fragrance (Chapter 2). Functional BAD2 is either responsible for metabolising 2AP which means the presence of the non-functional enzyme results in accumulation of 2AP and hence fragrance, or functional BAD2 is active in a pathway that competes for substrate which would otherwise be used in the production of 2AP and so a non-functional BAD2 enzyme results in increased flux of substrate down the pathway of 2AP production. Knowledge of the most likely genetic cause of fragrance has allowed the development a perfect assay for fragrance in rice. A single tube allele specific PCR which allows determination of the genotypic status of an individual rice plant, either homozygous fragrant, homozygous non-fragrant or heterozygous non-fragrant, has practical utility for rice breeders worldwide. The assay is a simple robust method for screening rice to determine its fragrance status across a wide range of rice varieties and within segregating populations using DNA isolated from rice following simple, inexpensive and rapid extraction protocols.

The PCR products can be analysed easily and inexpensively on agarose gel or alternatively using more sophisticated high throughput equipment, making the assay a very versatile tool.

CHAPTER 4: Inactivation of an aminoaldehyde dehydrogenase is responsible for fragrance in rice.

Summary:

The previous chapters have shown an eight base pair deletion in a gene on chromosome eight encoding a betaine aldehyde dehydrogenase homolog is responsible for fragrance in rice (*Oryza sativa*). In this chapter two rice cDNAs transcribed from chromosomes four and eight, each encoding an enzyme with homology to betaine aldehyde dehydrogenase were cloned and expressed in *E. coli*. The enzyme encoded from chromosome eight had optimum activity at pH 10, little to no affinity towards N-acetyl- γ-aminobutyraldehyde (NAGABald) with a K_m value of approximately 10mM and moderate affinity towards γ-guanidinobutyraldehyde (GGBald) and betaine aldehyde (bet-ald) with K_m values of approximately 260μM and 63μM respectively. A higher affinity towards γ-aminobutyraldehyde (GABald) was observed with a K_m value of approximately 9μM. The enzyme encoded from chromosome four had optimum activity at pH 9.5, showed little to no affinity towards bet-ald with a K_m value of 3mM and had moderate affinity towards GGBald, NAGABald and GABald with K_m values of approximately 545μM, 420μM and 497μM respectively. This enzyme had a half life roughly double that of the enzyme encoded from chromosome eight. This chapter investigates the affinity of these enzymes to a range of substrates and discusses the implications of these findings on the metabolic pathway of fragrance generation in Basmati and Jasmine rice and the potential of rice to accumulate the osmoprotectant glycine betaine.

Introduction:

Jasmine and basmati style rice has a pleasant aroma due to the accumulation of the volatile compound 2-acetyl-1-pyrroline (2AP) (Buttery *et al*., 1983; Lorieux *et al*., 1996; Widjaja *et al*., 1996; Yoshihashi *et al*., 2002) in all aerial parts of the plant including the leaves and grain (Yoshihashi, 2002). As well as being identified as the main chemical cause of fragrance in rice (Buttery *et al*., 1983), 2AP is a significant aroma chemical in a number of organisms and foodstuffs including Pandanus (Buttery *et al*., 1983b; Laohakunjit and Noomhorm, 2004; Thimmaraju *et al*., 2005), popcorn (Schieberle, 1995), corn tortillas (Buttery and Ling, 1995), baguettes (Zehentbauer and Grosch, 1998), ham (Carrapiso *et al*., 2002), cheese (Zehentbauer and Reineccius, 2002), mung bean (Brahmachary and Ghosh, 2002), green tea (Kumazawa and Masuda, 2002), bread flowers (*Vallaris glabra* Ktze) (Wongpornchai *et al*., 2003) some yeasts (Snowdon *et al*., 2006) and select bacteria (Romanczyk *et al*., 1995; Costello *et al*., 2001; Rungsardthong and Noomhoom, 2005; Snowdon *et al*., 2006; Adams and De Kimpe, 2007) including the wine spoilage bacteria *Lactobacillus hilgardii* (Costello *et al*., 2001; Snowdon *et al*., 2006).

Studies into the biological formation of 2AP have shown that either proline or ornithine can be utilised as a precursor in rice (Yoshihashi *et al*., 2002), Pandanus (Thimmaraju *et al*., 2005) and *Lactobacillus hilgardii* (Costello *et al*., 2001). Radiolabelling studies have shown the nitrogen in the pyrroline ring of proline becomes the nitrogen in the pyrroline ring of 2AP while the carboxyl group of proline is removed and replaced with an acetyl group from another source (Yoshihashi *et al*., 2002). Costello *et al*. (2001) have shown

that in *Lactobacillus hilgardii* the acetyl group of 2AP can be derived from fructose when either ethanol or acetaldehyde are supplied in excess. Further, they suggest Δ^1pyrroline, a product of proline catabolism via putrescine oxidation, is the direct precursor of the pyrroline ring of 2AP and that the acetyl group was most likely formed from reaction with acetyl-CoA or acetaldehyde in either a chemical or enzymatic reaction. Additionally, detailed precursor studies have revealed that the formation of 2AP in *Bacillus cerus* proceeds via acetylation of Δ^1pyrroline (Adams and De Kimpe, 2007).

The genetic cause of rice fragrance is most likely due to an eight base pair deletion within a gene encoding a betaine aldehyde dehydrogenase homolog (BAD2) (Chapters 2 and 3). This deletion leads to the generation of a premature stop codon which would, if translated, produce a truncated non-functional gene product. Betaine aldehyde dehydrogenase (BAD) is an enzyme capable of converting betaine aldehyde (bet-ald), a product of choline oxidation, into the osmoprotectant glycine betaine (Ishitani *et al*., 1993; Russell *et al*., 1998; Shirasawa *et al*., 2006), however reports of BADs in non glycine betaine accumulating species, including rice, (Ishitani *et al*., 1993; Nakamura *et al*., 1997; Mohanty *et al*., 2002; Niu *et al*., 2007) suggests BAD enzymes have a role other than the generation of glycine betaine. Many recent studies have identified plant BADs capable of metabolising a range of substrates including omega-aminoaldehydes, often more efficiently than bet-ald (Trossat *et al*., 1997; Incharoensakdi *et al*., 2000; Livingstone *et al*., 2003; Oishi and Ebina, 2005), while others report identification of enzymes with aminoaldehyde dehydrogenase (AAD) activity that have high homology to BADs but no affinity for bet-ald (Sebela *et al*., 2000). It has been suggested that BADs

and AADs are the same enzyme and should be reclassified as such (Sebela *et al*., 2000; Livingstone *et al*., 2003; Reumann, 2004).

Some of the chemicals other than bet-ald that are reportedly substrates of BADs and AADs include; 3-aminopropanaldehyde, 3-dimethylsulfoniopropionaldehyde, γ-guanidinobutyraldehyde (GGBald) and γ-aminobutyraldehyde (GABald) (Trossat *et al*., 1997; Incharoensakdi *et al*., 2000; Sebela *et al*., 2000; Oishi and Ebina, 2005). The latter of these, GABald, is a four carbon aminoaldehyde derived from proline via putrescine oxidation that in solution, exists in equilibrium with its cyclic form, Δ^1-pyrroline (Struve and Christophersen, 2003), a recognised precursor of 2AP in *Bacillus cerus* (Adams and De Kimpe, 2007).

I propose the two cDNAs annotated as encoding enzymes with BAD activity, actually encode enzymes with AAD activity and that inactivation of AAD2 (BAD2), encoded from chromosome eight, leads to the accumulation of GABald / Δ^1-pyrroline, the immediate precursor of 2AP. To that end the two cDNAs transcribed from chromosomes four and eight were isolated, cloned into expression vectors and expressed in *E. coli*, the purified enzymes were then analysed for affinity to a range of substrates including bet-ald and GABald.

Materials and Methods:

Plant Materials and RNA extraction

Oryza sativa cv. Nipponbare was obtained as seed from the Australian Plant DNA Bank (www.biobank.com) and grown in sterile tap water for two to four weeks before

extraction of total RNA using a Qiagen RNeasy® Plant Mini Kit (50) according to the manufacture's instructions. Total RNA quantity and quality was assessed by gel electrophoresis and absorbance at 260 nm. Prior to cDNA synthesis, total RNA was incubated for 30 minutes at 25°C with 1U DNase (Roche)/ µg RNA in a 1/10th volume 10x DNase 1 reaction buffer (Invitrogen).

cDNA synthesis

First strand cDNA synthesis utilised the Transcriptor™ First Strand cDNA synthesis Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Reactions contained 0.5 µg - 1µg total RNA, 10U Transcriptor™ reverse transcriptase and 50 pmol Anchored-oligo$(dT)_{18}$ primer supplied with the kit. Reactions were incubated at 55°C for 30 min and stopped by incubating at 85°C for five min. Subsequent PCR amplification used between 1 µl and 5 µl of the first strand cDNA per 50µl reaction.

PCR Amplification and cloning

As BAD1 and BAD2 have previously been identified as full length cDNAs of known sequence, both were initially amplified in two halves utilising the primers 5`-CCAAGGTCCGGGACAAC -3` and 5`- GCACCGGCACATCTTGCTGT -3` for the first half of BAD1, 5`- TAGCTTCACATCCCCATGTG -3` and 5`-TTCTGTCCGTCCGTTCTG -3` for the second half of BAD1, 5`-GATGGCCACGGCGATC -3` and 5'- TTTCTCCAGATGCTTGGGTC-3' for the first half of BAD2 and 5'-CTGGTAAAAAGATTATGGCTTCA-3' and 5`-CCGTCATACTATGGCCTCTTA -3` for the second half of BAD2. These products were

then used in a PCR for amplification of the full length cDNAs using primers which contained *Nde*1 and *Bam*H1 restriction sites. BAD1 full-length cDNA was amplified by PCR using the sense primer 5'-ATATCCATATGATGGCCGCGCCGTCGGCGATCCC-3' and antisense primer 5'-AATTCGGATCCTGACGTGCTCCGTCGCTCTTG-3'. BAD2 full-length cDNA was amplified by PCR using the sense primer 5'-ATATCCATATGATGGCCACGGCGATCCCGCAG -3' and antisense primer 5'-AATTCGGATCCACGATCAGAACAGATGGGCGTGTC -3'. PCRs were performed using Platinum® Taq DNA Polymerase High Fidelity from Invitrogen™. Reactions contained 0.2 µM each primer, 2 µl cDNA, 2 mM $MgSO_4$, 5% DMSO, 3% glycerol and 2 units of HiFi Taq in 1X buffer to a volume of 50 µl. Thermal cycling parameters were as follows: 94 °C, 2 min for one cycle; 94°C, 30 s; 58°C, 30s; 68°C, 1 min 40 sec for 30 cycles. Analysis of PCR products on a 1% agarose gel revealed single bands of appropriate size. Prior to ligation into pGEM®-T vector (Promega), A overhangs were added by incubation of cDNAs with 5 mM dATP, 1x PCR buffer 5mM $MgCl_2$ and Platinum Taq DNA polymerase at 95ºC for 5 min followed by 70ºC for 30 min. Following ligation into pGEM®-T (Promega) restriction digests with *Nde*1 and *Bam*H1 were performed which allowed directional cloning into pET-19b expression vector (Novagen) following the manufactures instructions.

Sequencing

Cloned cDNAs were sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster, CA., USA) and the labelled DNA fragments separated and analysed with an ABI 3730 48 capillary DNA analyser (Applied Biosystems). All

sequence analysis and alignments were undertaken using ChromasPro software (Technelysium Pty Ltd).

Recombinant protein expression and purification

Full length BAD1 and BAD2 cDNAs were directionally cloned into the *Nde*1 and *Bam*H1 restriction sites of the bacterial expression plasmid pET19b (Novagen) to enable addition of a 10x Histidine tag to the C terminus of both enzymes. *E. coli* strain BL21 (Novagen, Madison, WI., USA) was transformed with the pET19b-BAD1 and pET19b-BAD2 vector constructs and grown in Luria-Bertani medium supplemented with appropriate antibiotics according to the manufacturer's instructions. 100 ml of overnight culture was transferred to 500 ml fresh Luria-Bertani medium and grown for a further three hours. The expression of recombinant protein was then induced by the addition of 1mM isopropyl β-D-thiogalactoside (IPTG) and the cells were allowed to grow for a further three hours at 37°C with shaking (210 rpm) before harvesting by centrifugation at 4°C. The cells were lysed in lysis/bind buffer (300 mM NaCl, 50 mM sodium phosphate buffer (pH 8.0), 10 mM imidazole, 1/10th volume 10x BugBuster® Protein Extraction Reagent (Novagen, Darmstadt, Germany), Complete, EDTA-free protease inhibitor cocktail (Roche, Mannheim, Germany), 100 µM sodium orthovanadate, 100 µM sodium molybdate and 100 µM sodium tartrate. Insoluble material was pelleted by centrifugation at 15000g for 30 min at 4°C and the resulting supernatant was incubated on ice in the presence of 500 µl Ni-NTA His·Bind® Resin (Novagen) for 60 min with gentle shaking. The resin was washed 5x in 10 ml wash buffer (300 mM NaCl, 50 mM sodium phosphate buffer, 20 mM imidazole, pH 8.0) to remove contaminating proteins. Recombinant His-

BAD protein was eluted by 7x 500 µl washes in elution buffer (300 mM NaCl, 50 mM sodium phosphate buffer, 250 mM imidazole, pH 8.0). Elutions were pooled and dialysed against 50 mM HEPES buffer (pH 7.5) for 24 hours at 4°C. Following dialysis the protein was concentrated 10 fold by centrifugation using a 12-14 kDa cut-off spin column and stored at -80°C with the addition of 10% glycerol.

The presence of recombinant protein was confirmed by SDS/PAGE analysis (Laemmli, 1970).

Synthesis of substrates

Betaine aldehyde was obtained from Sigma-Aldrich. GABald/ Δ^1-pyrroline was synthesised to 86% purity by the method of Struve and Christophersen (2003). GGBald was synthesised by a modification of the method of Vanderbilt *et al*. (1975) involving bubbling nitrogen gas through a 20 ml (1.0 mmol) solution of arginine in water for 5 min, heating to 30°C before adding, while mixing, chloramine-T hydrate to a concentration of 1.0 mmol. This mixture was incubated at 30°C for 30 min, cooled on ice and filtered. The filtrate was then purified to 81% by separation on a preparative Phenomenex LunaC18 100A, 5u, 150 x 25.4mm I.D. HPLC column using Gilson 306 gradient pumps, Gilson 155 UV/Vis detector, Gilson 204 fraction collector and controlled using Gilson Unipoint software. The mobile phase was an acetonitrile/water mixture with 0.05% trifluoroacetic acid, at a flow rate of 15mL/min starting at 1% acetonitrile and holding for 5 min before ramping up to 90% acetonitrile over 15 min. Selected fractions were pooled and dried under vacuum on a BÜCHI Rotorvapor R-114 rotary evaporator.

N-acetyl-GABald diethyl acetal was synthesised by modification of the method of Rajabi and Saidi (2005). Briefly, 250µl of GABald diethylacetal was mixed with an equimolar volume of acetic anhydride and microwaved (on high) for 5x 3 seconds. The diethylacetal groups were removed by the method of Struve and Christophersen (2003) to leave N-acetyl-GABald of 73% purity as determined by LCMS.

Purity of GGBald and N-acetyl-GABald was assessed by LCMS using a Phenomenex AquaC18 125A, 5u, 150 x 4.6mm I.D. HPLC column on an Agilent 1100 LC and an Agilent 1100 MSD controlled by chemstation Rev. A.10.02[175] software (Agilent Technologies). The mobile phase was an acetonitrile/water mix with 0.05% trifluoroacetic acid, at a flow rate of 1ml/min starting at 1% acetonitrile and holding for 5 min before ramping up to 90% acetonitrile over 15 min. The MS was run in atmospheric pressure chemical ionisation (APCI) mode, had an ionisation voltage of 150V, capillary voltage of 2000V, drying gas temperature of 345°C, vaporiser temperature of 395°C, nebuliser pressure of 60psig and a gas flow rate of 5L/min.

The purity of Δ^1-pyrroline was assessed on an Agilent 6890 Series GC system with an Agilent 5973Network Mass Selective Detector using an SGE BPX5 capillary column 50.0m x 0.22mm ID x 1µm film thickness. 1µl of sample was injected at an inlet temperature of 280°C and 25:1 split ratio. Helium was used as the carrier gas with a constant flow of 1.5 ml/min. The oven was held at 50°C for 5 min before ramping up to 300°C at a rate of 4°C/min.

Enzyme assay

Enzyme assays mixtures (1mL total volume) were based on the work of Yorifuji *et al*. (1986), Trossat *et al*. (1997), Valenzuela-Soto *et al*. (2003) and Oishi and Ebina (2005) and contained 50mM HEPES, MES or Glycine Buffer, 20mM β-mercaptoethanol, 2mM NAD+, with 10ug of enzyme (BAD1 or BAD2) and varying concentrations of substrates. HEPES and MES buffers were obtained from Sigma, Glycine buffer consisted of 50mM Glycine, 1mM $MgSO_4$, 0.1mM $ZnSO_4$. Buffers were adjusted to the required pH by addition of KOH. The reaction was followed by monitoring the conversion of NAD+ to NADH by an increase in absorbance at 340nm on a Hewlett Packard 8453 UV/vis spectrophotometer controlled by ChemStation software (Agilent Technologies). For determination of optimum pH, 5 mM GABald was used as the final substrate concentration and was confirmed by replacing GABald with 5 mM bet-ald as the final substrate concentration. All enzyme assays were performed a minimum of three times.

Results:

Purification of rice BADs from *E. coli*

SDS gels analysis showed rice BAD1 and BAD2 purified from *E. coli* have subunits of approximately 56kDa (Figure 4.1). Final enzyme concentrations were adjusted to 10µg/µl as determined by Bradford assay (Bradford, 1976).

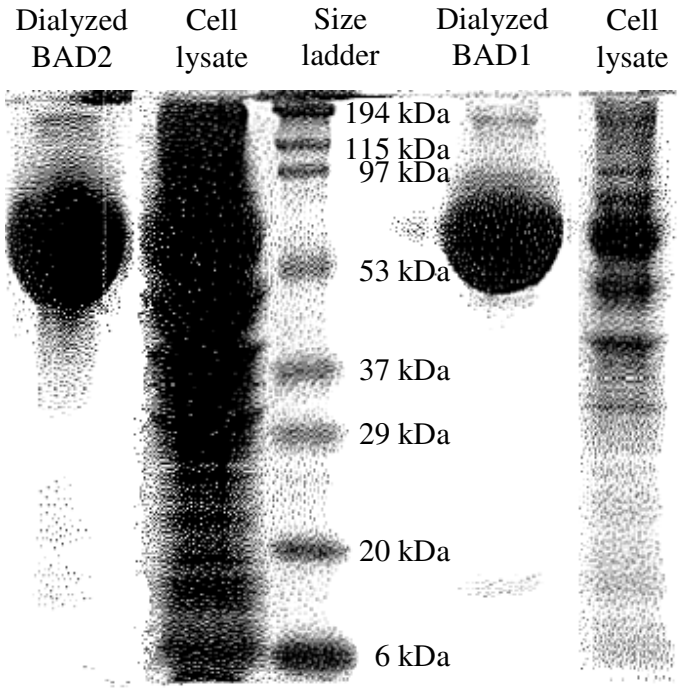

Figure 4.1 Polyacrylamide gel of BAD1 and BAD2 subunits

Synthesis of aldehyde substrates

Purity of synthesised substrates was analysed via GCMS. The GABald preparation displayed two main peaks with molecular weights of 69 and 87 corresponding to the circular Δ^1-pyrroline form and the linear GABald form respectively. Likewise the N-acetyl- γ-aminobutyraldehyde (NAGABald) preparation showed a large peak with a molecular weight of 129 corresponding to the linear form of NAGABald and a smaller peak with a molecular weight of 113 corresponding to the circular form N-acetylpyrroline. Δ^1-pyrroline was taken to be 86% pure based on the method of Struve and Christopherson (2003) while GGBald and NAGABald were determined by LCMS and GCMS analysis to be 81% and 73% pure respectively. (Figure 4.2a & b)

pH optimum and half life of BADs

Optimum activity of BAD1 and BAD2 at room temperature was found to be at pH 9.5 and pH 10 respectively (Figure 4.3). In the absence of beta-mercaptoethanol the half life of BAD1 at pH 9.5 at room temperature was shown to be approximately 10 min while BAD2 at pH 9.5 at room temperature had a half life of approximately 5 min (Figures 4.4 & 4.5).

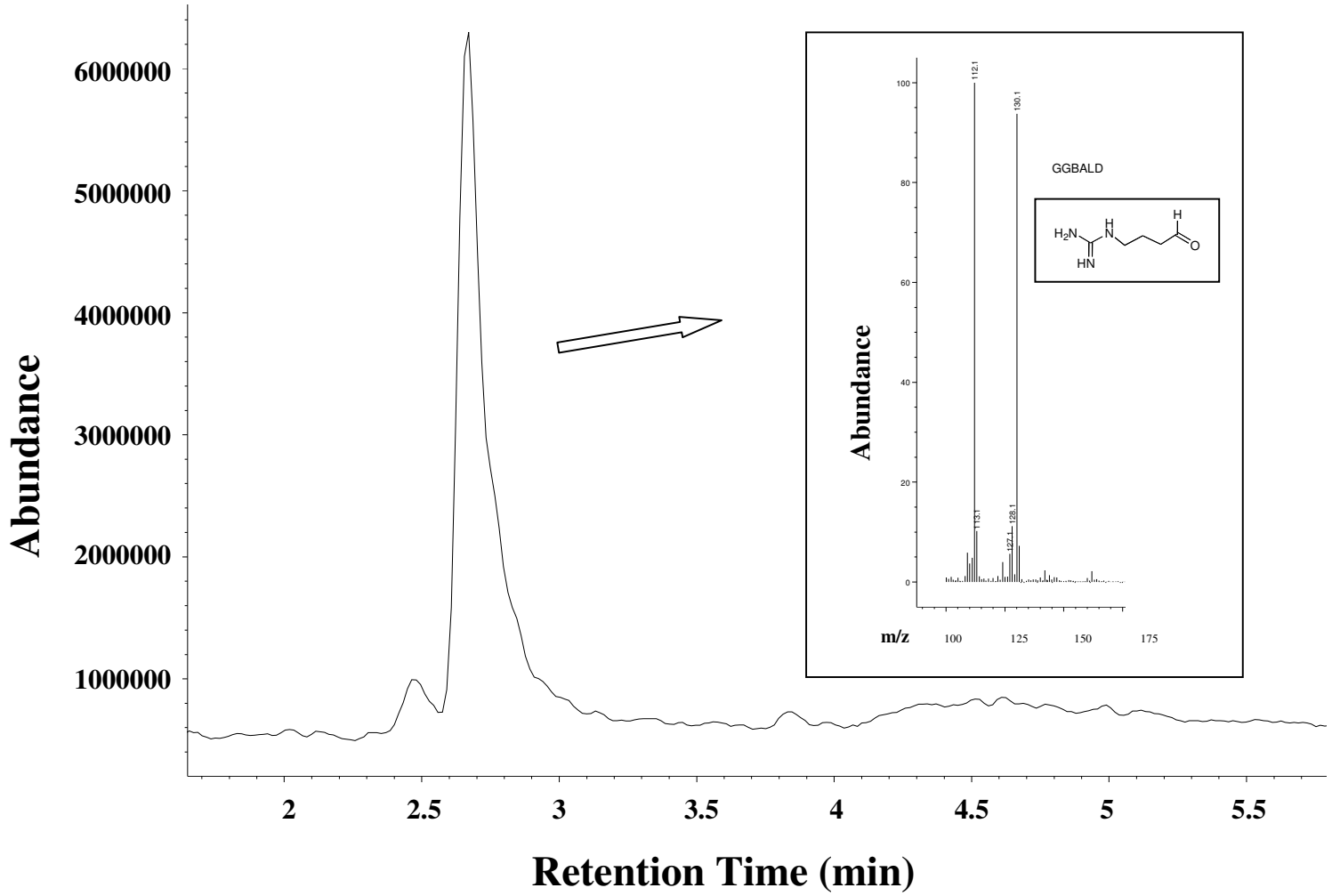

Figure 4.2a) LCMS analysis of γ-Guanidinobutyraldehyde

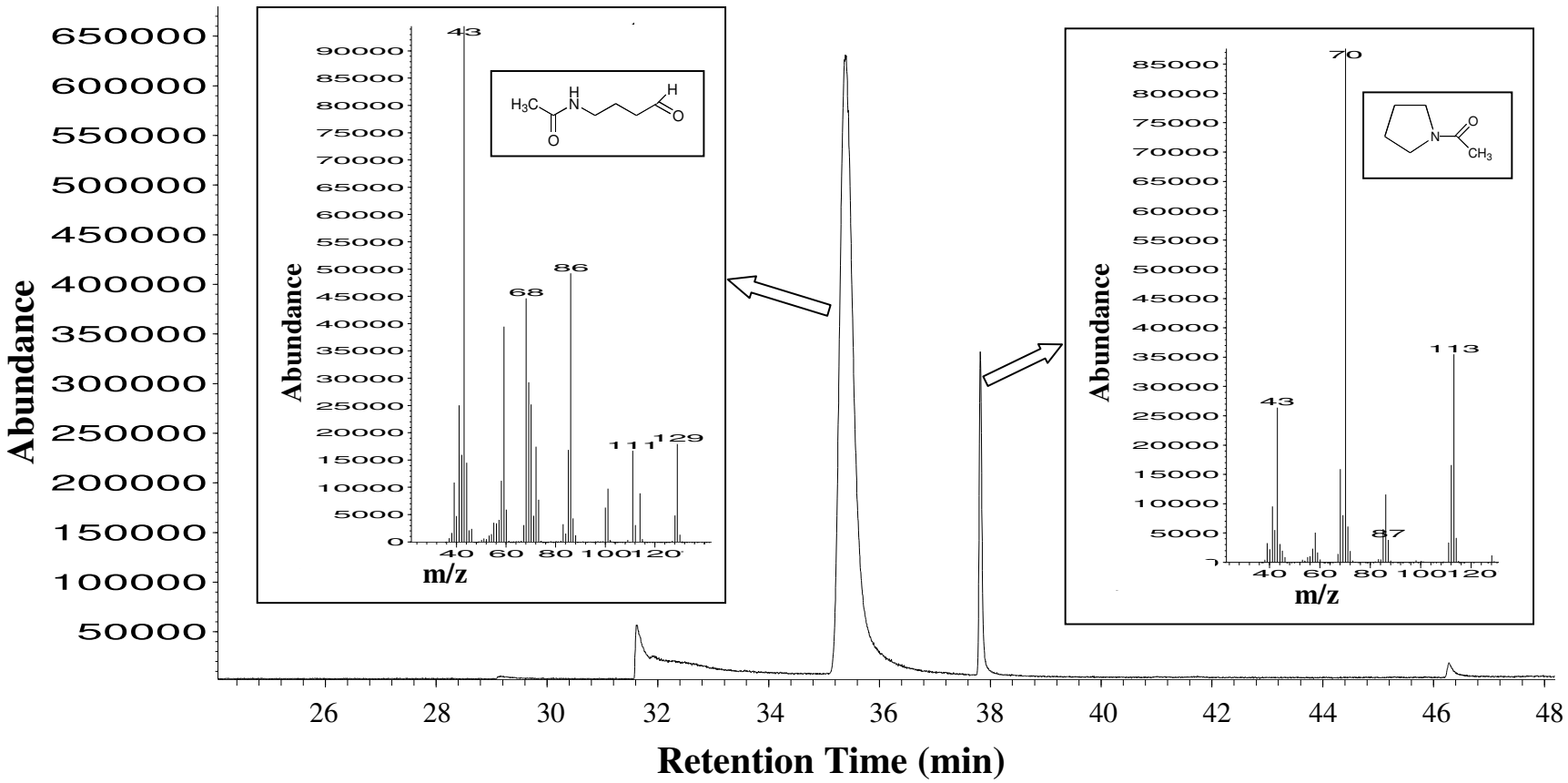

Figure 4.2b) GCMS analysis of N-acetyl-γ-aminobutyraldehyde

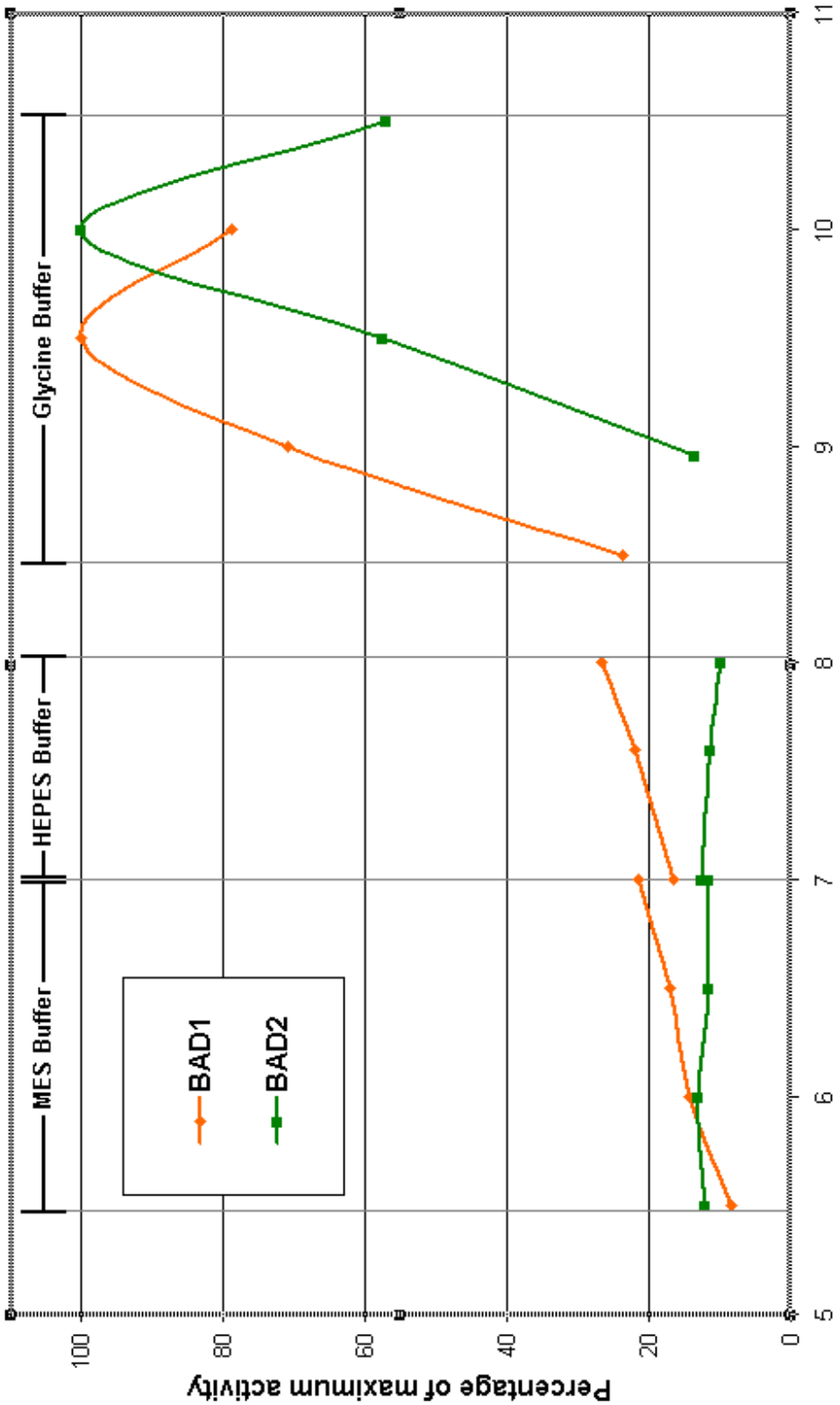

Figure 4.3 Relative activity of BAD1 and BAD2 enzymes.

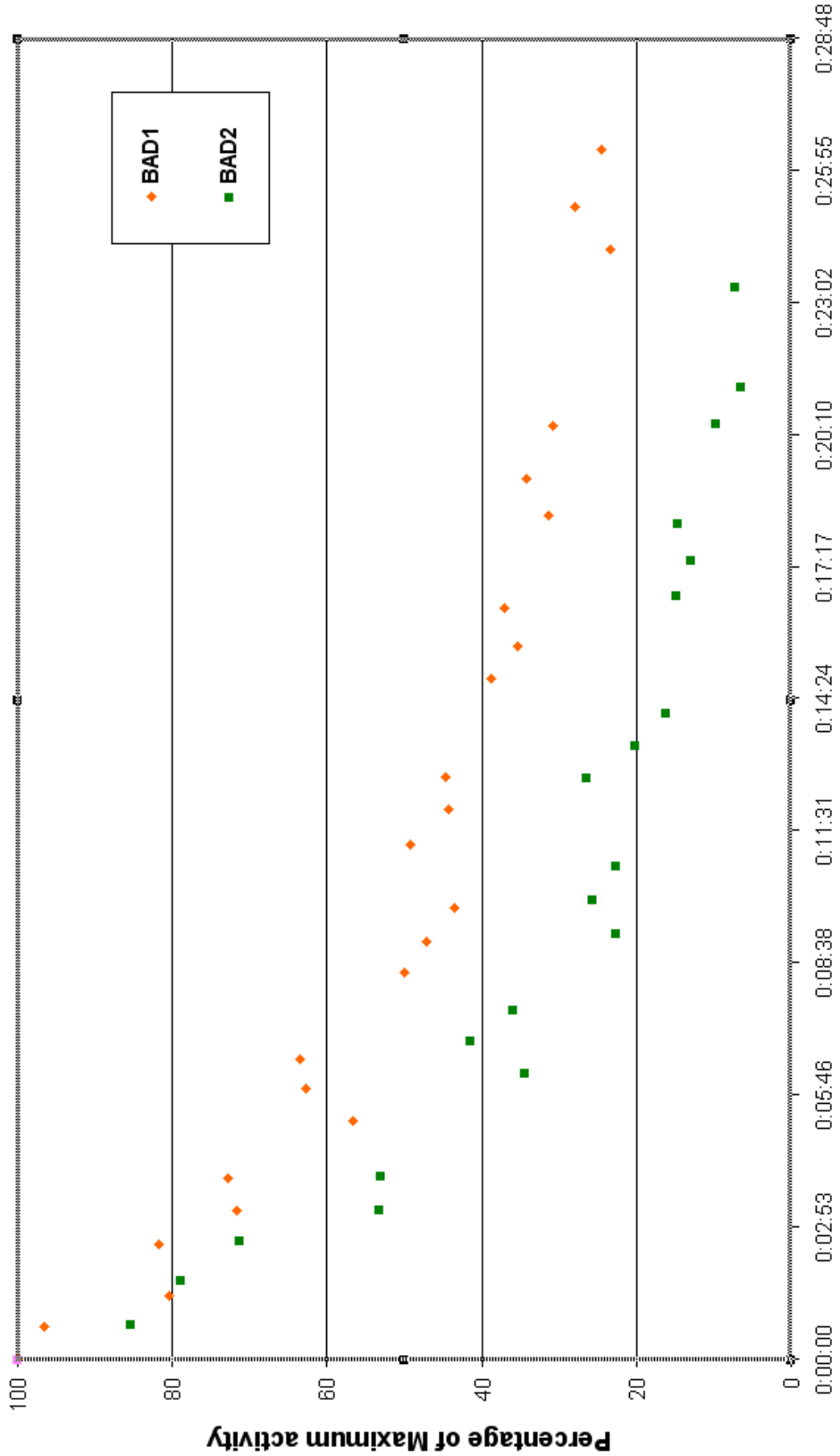

Figure 4.4 Relative activity of BAD1 and BAD2 at pH 9.5 after pre-incubation at room temperature.

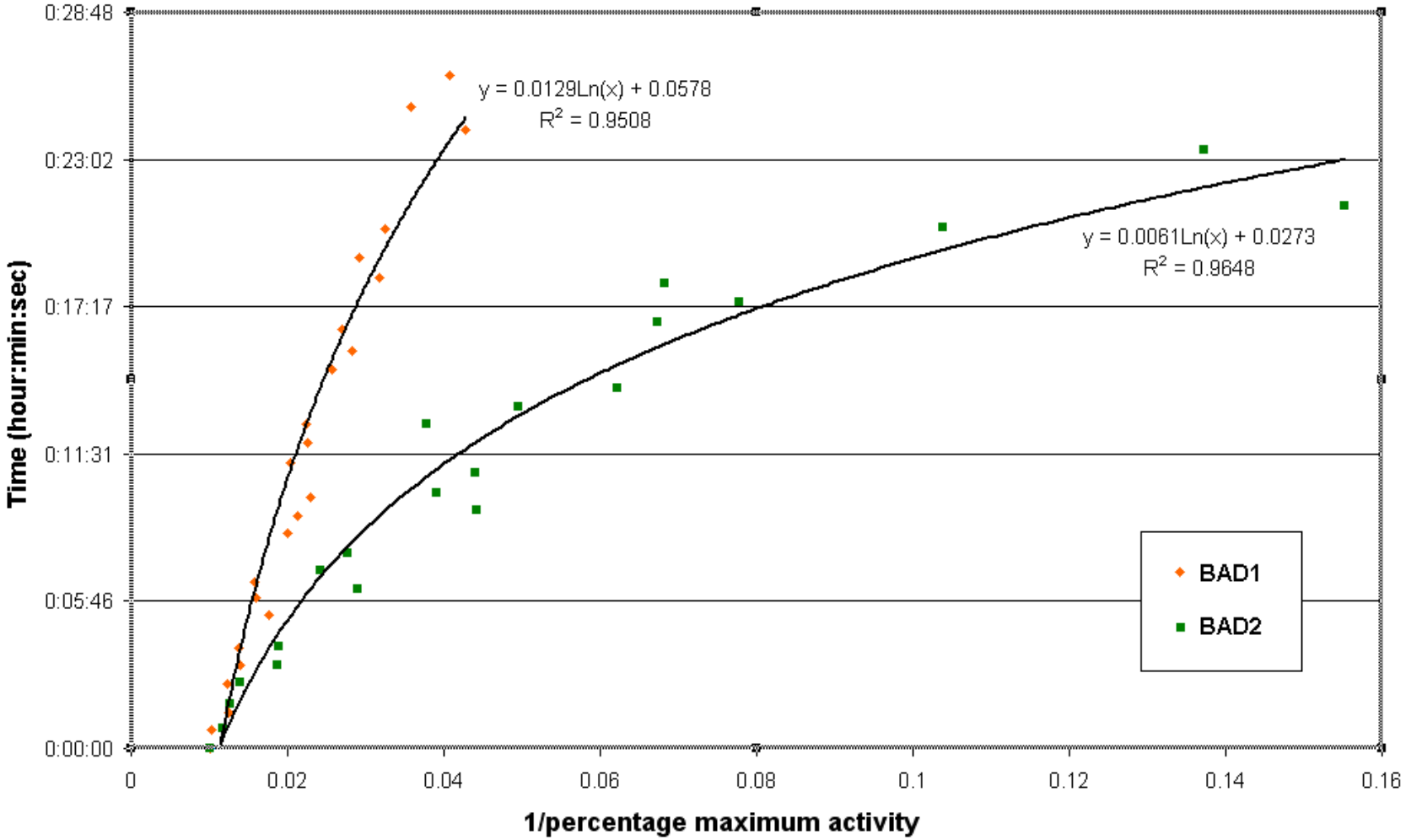

Figure 4.5 BAD1 and BAD2 at pH 9.5 incubated at room temperature before assay versus inverse relative activity.

Substrate specificity and activity of BADs

Both BAD1 and BAD2 showed greatest activity towards GABald, followed by GGBald with kcat/K_m values of 27 $M^{-1}s^{-1}$ and 11 $M^{-1}s^{-1}$ for BAD1 respectively and 68 $M^{-1}s^{-1}$ and 5 $M^{-1}s^{-1}$ for BAD2 respectively (Table 4.1). BAD1 showed minimal activity (2 $M^{-1}s^{-1}$) towards NAGABald and practically no activity (0.1 $M^{-1}s^{-1}$) towards bet-ald while BAD2 showed the reverse. Neither enzyme had activity with 2AP as the substrate. The K_m values generally mirror these results with the exception that BAD1 has a lower K_m value (greater specificity) for NAGABald (420μM) than it does for GGBald (545μM). Compared to BAD2, BAD1 showed higher K_m values (lower specificity) to most substrates with the exception of N-acetyl-pyrroline.

Discussion:

Enzyme half life and pH optima

In the absence of beta-mercaptoethanol the half life of BAD1 was shown to be approximately 10 min while BAD2 had a half life of approximately 5 min. This stability difference could lead to an overestimation of the concentration of active BAD2 enzyme in comparison to BAD1 in corresponding assays, leading to an underestimation of BAD2 activity. This would not affect the reported K_m values but would lead to larger kcat/K_m values suggesting that our estimates of BAD2 kcat/K_m are conservative.

Table 4.1 K_m and kcat/K_m values of AAD1 (BAD1) and AAD2 (BAD2) at optimum pH towards various substrates.

Substrate	**Enzyme K_m (μM)**		**Enzyme kcat/K_m ($M^{-1}s^{-1}$)**	
	AAD1 (BAD1)	**AAD2 (BAD2)**	**AAD1 (BAD1)**	**AAD2 (BAD2)**
betaine aldehyde	3233	63	0.1	2
γ-aminobutyraldehyde /Δ^1-pyrroline	498	9	27	68
γ-guadinoaminobutyraldehyde	545	32	11	5
N-acetyl-aminobutyraldehyde /N-acetyl-pyrroline	420	10,608	2	0.1
2-acetyl-1-pyrroline	-	-	-	-

Optimum activity of BAD1 and BAD2 was found to be at pH 9.5 and pH 10 respectively. High pH optima have been reported for many AAD and BAD enzymes including BAD from oats (Livingstone *et al*., 2003), Zoysia tenuifolia (Oishi and Ebina, 2005) and AAD from pea (Sebela *et al*., 2000).

Both BAD enzymes of rice are believed to be directed to the peroxisome (Nakamura *et al*., 1997; Chapter 2). Although no data are available on the pH of plant peroxisomes, Dansen *et al*. (2000) has shown that human fibroblast peroxisomes have a basic pH of around 8.2 +/- 0.3. Struve and Christophersen (2003) investigated the influence of pH on structural forms of GABald and found that at pHs of 9 to 10 GABald exists almost entirely as the ring form Δ^1-pyrroline and so BAD enzymes may therefore be active toward the ring form of this aldehyde. Alternatively BADs may be active towards the linear form, GABald, which at high pH would have existed in the reaction mixture at very low concentrations. If this is the case, the K_m values of both BADs towards the linear GABald would be significantly lower than those reported here. Studies on 2AP formation from proline in Maillard reactions have shown more 2AP is formed at pH 8 than is formed at pH 6 (Blank *et al*., 2003) suggesting a possible link between BAD location, GABald/Δ^1-pyrroline isomeric form and 2AP formation.

Role of AAD2 (BAD2) and GABald accumulation in fragrance

Previous studies have shown proline is a precursor of 2AP in rice, and radio-labeling studies have shown the nitrogen in the pyrroline ring of proline becomes the nitrogen in the pyrroline ring of 2AP (Yoshihashi *et al*., 2002). The compound Δ^1-pyrroline is identical to the ring component of 2AP and has been shown to be a precursor of 2AP in

Maillard reactions (Hofmann and Schieberle, 1998) and more recently in biological 2AP production by *Bacillus cereus* (Adams and De Kimpe, 2007). Δ^1-pyrroline exists in equilibrium with a linear form GABald which is part of the proline catabolism pathway via oxidation of putrescine. GABald has been shown to be a substrate of BADs and AADs from various plants (Trossat *et al*., 1997; Sebela *et al*., 2000; Livingstone *et al*., 2003; Oishi and Ebina, 2005) and BAD2 is known to be responsible for fragrance in rice (Chapter 2). Because of the associations between Δ^1-pyrroline and 2AP and between GABald and BADs as well as the ability of GABald to spontaneously cyclise into Δ^1-pyrroline, I focused on GABald as the main substrate of interest. The other aldehyde substrates were chosen because of their similarity to GABald and occurrence in related pathways of proline/arginine catabolism. Bet-ald was also chosen because of the original annotation of the gene responsible for fragrance in rice (Chapter 2).

Both BAD enzymes showed greater affinity (K_m) and higher catalytic efficiency ($kcat/K_m$) towards the aminoaldehydes GABald and GGBald than they did towards bet-ald as shown by the lower K_m values and higher $kcat/K_m$ values for GABald and GGBald (Table 4.1). This suggests the annotation of the two enzymes as BADs, which is based on sequence homology to BADs of other plants is not the most appropriate annotation of these enzymes and the annotation of AAD would be more accurate in defining their *in vitro* function. As such, the enzymes encoded from chromosomes four and eight will herein be referred to as AAD1 (BAD1) and AAD2 (BAD2) respectively.

Of the substrates analysed AAD2 (BAD2) showed greatest affinity towards GABald with a K_m value of 9μM and a high $kcat/K_m$ of 68 $M^{-1}s^{-1}$ (Table 4.1), suggesting GABald is an effective substrate for AAD2 (BAD2) and that accumulation and spontaneous cyclisation

of GABald to form Δ^1-pyrroline due to a non-functional AAD2 (BAD2) enzyme is a likely cause of 2AP accumulation in rice. I propose the biochemical pathway of 2AP production in rice (Figure 4.6) starts with proline being catabolised via putrescine into GABald, a substrate of AAD2 (BAD2). If AAD2 (BAD2) is present and functional it is able to convert the majority of GABald to γ-aminobutyric acid (GABA), but if AAD2 (BAD2) is absent or non-functional the majority of GABald is acetylated to form 2AP.

Putrescine is found in high levels in actively growing tissues where cells are rapidly dividing (Kakkar *et al*., 2000) and is catabolised to GABald by diamine oxidase (DAO) during the processes of lignification and cell wall stiffening (Sebela *et al*., 2001), i.e. after the majority of cell division has taken place. Formation of GABald is therefore likely to occur in young tissue that has stopped actively dividing and is undergoing cell wall stiffening. Tips of rice leaves contain higher levels of 2AP than the base of the leaf does (data not shown) while young leaves are more aromatic than old ones (Lorieux *et al*., 1996), which correlates with the proposed pathway of 2AP formation via putrescine (Figure 4.6). Dissipation of 2AP in older tissue is most likely due to the volatile nature of the chemical (Yoshihashi *et al*., 2005). AAD1 (BAD1) has a low affinity to GABald with a K_m value of 498μM but a high kcat/K_m of 27 $M^{-1}s^{-1}$ (Table 4.1) suggesting that despite the large K_m value GABald may still be a substrate for AAD1 (BAD1) *invivo* and that the presence of AAD1 (BAD1) may diminish the pool of GABald available to form 2AP. The similarity of preferred substrates of AAD1 (BAD1) and AAD2 (BAD2) makes it unclear as to why a non-functional AAD2 (BAD2) has such a major impact on the fragrance phenotype of rice. Fitzgerald *et al.,* (2008) has shown AAD1 (BAD1) accounts

for at most 30% of total AAD (BAD) gene expression within the leaves of non-fragrant rice, although this is increased by exposure to salt stress.

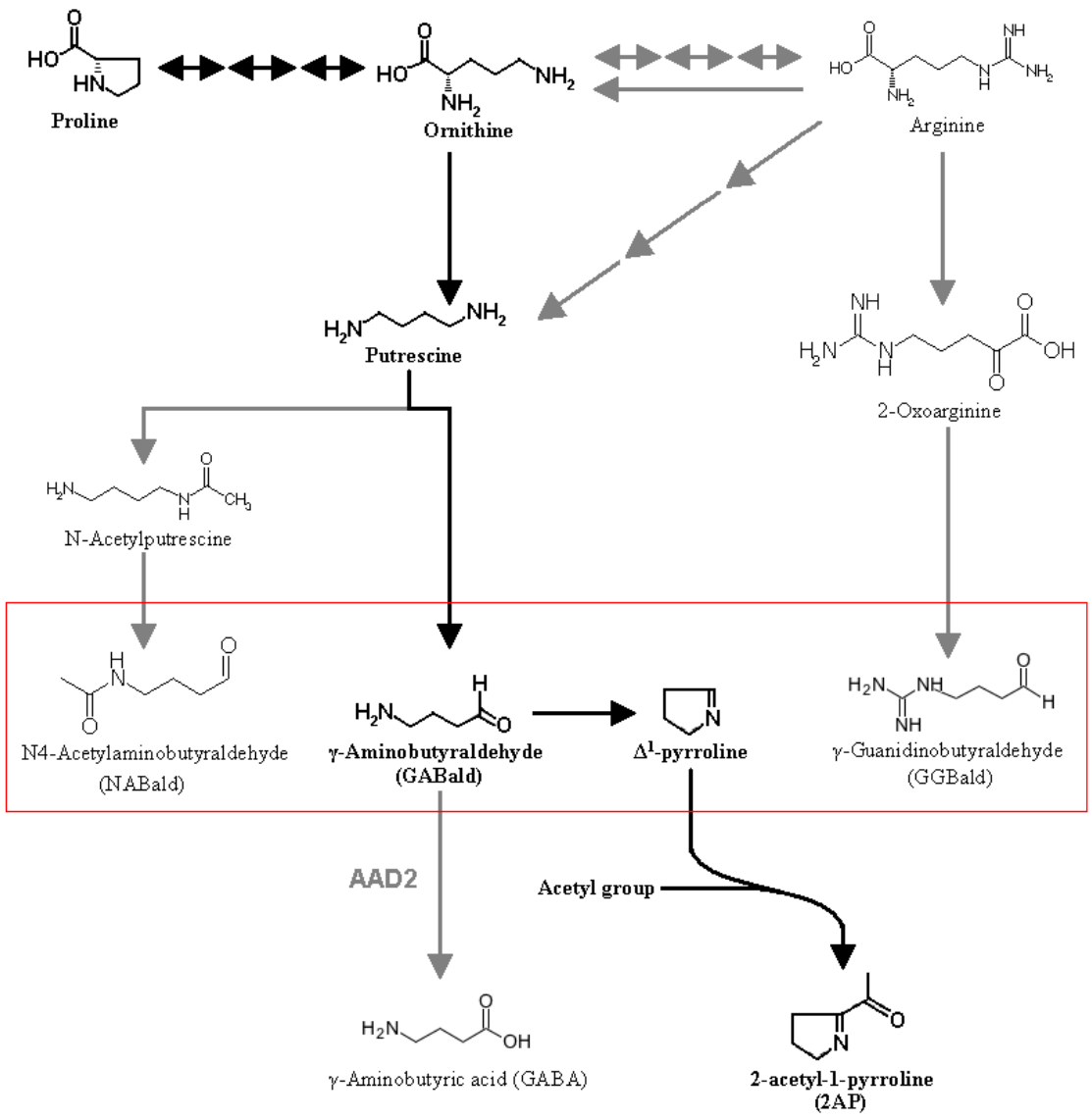

Figure 4.6 Pathway from proline to potential substrates (boxed) of AAD1 (BAD1) and AAD2 (BAD2) and to 2-acetyl-1-pyrroline via Δ^1-pyrroline when AAD2 (BAD2) is inactive (black) or to GABA when AAD2 (BAD2) is active.

It is therefore conceivable the fragrance phenotype of rice is altered by the action/inactivation of the AAD2 (BAD2) enzyme via a combination of its higher transcript levels, higher catalytic efficiency and higher substrate specificity towards GABald in comparison to that of AAD1 (BAD1). An absence of AAD2 (BAD2) is likely to cause an accumulation of GABald leading to higher 2AP production.

AAD1 (BAD1) may influence fragrance in rice, especially in saline conditions when AAD1 (BAD1) mRNA expression is higher and perhaps more importantly when levels of GABald are closer to that of the K_m value of AAD1 (BAD1). These two factors are likely to occur together as the levels of precursors of GABald, such as proline and polyamines, are known to increase in rice under salt stress. Lorieux *et al*. (1996) identified a minor locus for fragrance in rice located on chromosome four most likely placed between molecular markers RG214 and RG788. These are located very close to molecular marker RZ675, located at 23,974,544bp on chromosome four (as noted on NCBI). The gene encoding the AAD1 (BAD1) enzyme is located on chromosome four between 23,146,445 and 23,150,872bp (as noted on NCBI). This fragrance locus was only identifiable by Lorieux *et al*. (1996) when the AAD2 (BAD2) locus on chromosome eight was accounted for, it had less influence on 2AP accumulation in the varieties studied than AAD2 (BAD2). With their similar activities and with AAD1 (BAD1) being associated with a potential minor fragrance allele, it seems likely that both AAD1 (BAD1) and AAD2 (BAD2) have an influence on 2AP accumulation in rice. Mutations that lead to a non-functional AAD1 (BAD1) or a reduction in expression levels of AAD1 (BAD1) may cause an increase in 2AP accumulation similar to that observed by a non-functional

AAD2 (BAD2) and could explain the minor fragrance allele identified by Lorieux *et al*. (1996).

Does fragrance affect plant performance?

The identification of a perturbation of GABA synthesis via GABald being key to 2AP accumulation in rice leads to the question does this perturbation lead to a decrease in plant performance? Although there are reports of decreased yield in some fragrant varieties (Berner and Hoff, 1986; Singh *et al*., 2000; Aggarwal *et al*., 2002; Bhattacharjee *et al*., 2002; Nagaraju *et al*., 2002; Sriboonchitta and Wiboonpongse, 2005; Garg *et al*., 2006) which could be due to any number of genetic loci or the environment in which these varieties are grown (Yoshihashi *et al*., 2004), there does not appear to be significant performance problems with fragrant varieties. This may be because plants synthesise GABA via multiple pathways. The predominant pathway to GABA formation in plants is via direct decarboxylation of glutamate (Narayan and Nair, 1990; Snedden *et al*., 1995) and an alternative pathway of GABA synthesis in plants is from either arginine or ornithine decarboxylases via putrescine (Narayan and Nair, 1990; Kakkar *et al*., 2000).

Betaine aldehyde accumulation

Both AADs (BADs) showed lower affinity to bet-ald than they did to GABald, AAD2 (BAD2) showed higher affinity for bet-ald than AAD1 (BAD1) which had a K_m value of 63μM and a kcat/K_m value of 2 $M^{-1}s^{-1}$, similar K_m values have been reported, 68μM (Incharoensakdi *et al*., 2000) and 73.9μM (Oishi and Ebina, 2005), for BAD to bet-ald in betaine accumulating spinach. Oishi and Ebina (2005) reported a K_m value towards bet-

ald of 291μM for ZBD1 (BAD) from Zoysia tenuifolia another betaine accumulating species. ZBD1 from Zoysia tenuifolia is more similar to rice AAD1 (BAD1) than to rice AAD2 (BAD2). This may explain the poor substrate specificity of ZBD1 for bet-ald as this work shows that rice AAD2 (BAD2) is a more effective enzyme for the conversion of bet-ald to glycine betaine. Rice is a glycine betaine non-accumulator, reportedly because its genome encodes a choline monooxygenase (CMO) that is probably non-functional due to aberrant stop codons (NCBI accession number P0545E05.33 on chromosome 6) (Shirasawa *et al*., 2006). Although to date there have been no reports in the scientific literature of a CMO being isolated or characterised from any plant in the Poaceae family (Jagendorf and Takabe, 2001; Oishi and Ebina, 2005), the NCBI database reports the isolation of an apparently complete CMO cDNA from rice (AJ578494) (Niu *et al*., 2007). If this putative rice CMO is expressed and fully functional then a new theory as to why rice does not accumulate glycine betaine is called for. Perhaps it is expressed at low levels, alternatively the apparent localisation of AAD (BAD) in the peroxisome and of CMO, which lacks a peroxisomal target sequence, in the cytoplasm may separate the pool of the toxic intermediate bet-ald from AAD (BAD). This study has shown that AAD2 (BAD2) has higher affinity to bet-ald than AAD1 (BAD1) does, suggesting efforts to engineer glycine betaine accumulation in fragrant rice needs to replace the function of AAD2 (BAD2) without reducing the pool of its preferred substrate GABald. This could be achieved by the use of the codA gene which produces an enzyme that directly converts choline to glycine betaine, and probably has very low affinity for GABald. Targeting of this enzyme to the chloroplast would maximise salt tolerance and isolate the action of this enzyme from that of AAD2 (BAD2) and therefore

from the pool of GABald, there by maintaining fragrance in fragrant rice while increasing salt tolerance. This has been achieved in basmati rice (Mohanty *et al*., 2002), although there was no mention of the effect on the fragrance phenotype.

CHAPTER 5: General Conclusions

Fragrance in Basmati and Jasmine style rice is a recessive trait which results principally from the presence of elevated levels of the compound 2-acetyl-1-pyrroline (2AP) in the aerial parts of the plant. A deletion in the gene encoding AAD2 (BAD2) on chromosome 8 which disables the AAD2 (BAD2) enzyme is the most likely cause of fragrance.

Evidence for the gene that encodes AAD2 (BAD2) and *fgr* being the same gene comes from many lines of enquiry, including positional, genetic, biochemical and physiological: (i) the gene that encodes AAD2 (BAD2) is in the exact chromosomal location suggested by genetic mapping; (ii) the loss of function is consistent with a recessive trait; (iii) a gene of amino acid metabolism downstream of proline is suggested by metabolic evidence and AAD (BAD) is a credible biochemical candidate in this pathway; and (iv) elevated fragrance in response to stress has been reported, as would be predicted from this biochemistry. The complete association of the deletion in the gene that encodes AAD2 (BAD2) with fragrance in a wide range of unrelated germplasm is especially convincing evidence.

Knowledge of the most likely genetic cause of fragrance has allowed the development a perfect assay for fragrance in rice. A single tube allele specific PCR which allows determination of the genotypic status of an individual rice plant, either homozygous fragrant, homozygous non-fragrant or heterozygous non-fragrant, has practical utility for rice breeders worldwide. The assay is a simple robust method for screening rice to

determine its fragrance status across a wide range of rice varieties and within segregating populations using DNA isolated from rice following simple, inexpensive and rapid extraction protocols. The PCR products can be analysed easily and inexpensively on agarose gel or alternatively using more sophisticated high throughput equipment, making the assay a very versatile tool.

Understanding the major genetic cause of fragrance has assisted in elucidating the biochemical pathway that leads to accumulation of 2AP in rice.

This work shows that two enzymes previously annotated as BADs due to sequence homology, actually have a greater affinity towards aminoaldehydes and as such should be reclassified AADs. This work in this chapter has demonstrated for the first time the biochemical function of an enzyme (AAD2) produced from a gene that was, in the previous two chapters, linked to the fragrance phenotype in rice. The accumulation of 2AP in rice is explained by the absence of AAD2 (BAD2) activity leading to an increased level of its preferred substrate, GABald/Δ^1-pyrroline, the immediate precursor of 2AP.

However, there is much to be learned about the production of 2AP in rice as well as other organisms such as Pandanus or bacteria. For example the biochemical pathway leading to acetylation of GABald/Δ^1-pyrroline for the final step in 2AP production is uncertain and it is unknown whether this step is enzymatically or chemically driven. Further research may lead to the identification of enzymes and genetic loci responsible for this final step, which in turn may then lead to further enhancement of rice fragrance through selective

breeding or genetic engineering of rice cultivars. Alternatively, selection for up-regulated genes upstream of 2AP formation may lead to enhanced fragrance in rice cultivars.

AAD1 (BAD1) may also contribute to the accumulation of 2AP in rice. Assuming AAD1 (BAD1) is involved in 2AP accumulation it is possible down-regulation of the gene encoding AAD1 (BAD1) may lead to increased 2AP accumulation. Knockout mutants of the gene encoding AAD1 (BAD1) in existing fragrant cultivars or in cultivars crossed to existing fragrant cultivars could lead to super fragrant rice varieties, perhaps with a 2AP level similar to that of Pandanus leaf, ten times more than that of existing fragrant rice cultivars.

Genetic complementation experiments and further analysis of the biochemical reactions catalysed by AAD2 (BAD2) and AAD1 (BAD1) may provide further insights into the basis of fragrance in rice and other organisms. Mutation breeding may generate a whole range of new fragrant foods, such as potatoes and corn with disrupted AAD2 (BAD2) or AAD1 (BAD1) activity, that have increased levels of 2AP.

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