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The effect of adenosine monophosphate deaminase overexpression on the accumulation of umami related metabolites in tomatoes

Bee Lynn Chew^{1&2}, Ian D. Fisk³, Rupert Fray², Gregory A Tucker⁴, Zsuzsanna Bodi², Alison Ferguson², Wei Xia⁴ and Graham B. Seymour^{2*}.

¹School of Biological Sciences, Universiti Sains Malaysia, 11800 Penang, Malaysia,

² Division of Plant and Crop Sciences, School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough, Leicestershire LE12 5RD, United Kingdom,

³ Division of Food Sciences, School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough, Leicestershire LE12 5RD, United Kingdom.

⁴ Division of Nutritional Sciences, School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough, Leicestershire LE12 5RD, United Kingdom.

* Corresponding author E-mail: Graham.Seymour@nottingham.ac.uk;
Telephone number: 00 44 115 951 6323

21 **Abstract**

22 Taste is perceived as one of a combination of five sensations, sweet, sour, bitter, salty and umami. The umami taste
23 is best known as a savoury sensation and plays a central role in food flavour, palatability and eating satisfaction.
24 Umami flavour can be imparted by the presence of glutamate and is greatly enhanced by the addition of
25 ribonucleotides such as inosine monophosphate (IMP) and guanosine monophosphate (GMP). The production of
26 IMP is regulated by the enzyme adenosine monophosphate (AMP) deaminase which functions to convert AMP into
27 IMP. We have generated transgenic tomato (*Solanum lycopersicum*) lines over expressing AMP deaminase under
28 the control of a fruit specific promoter. The transgenic lines showed substantially enhanced levels of AMP
29 deaminase expression in comparison to the wild type control. Elevated AMP deaminase levels resulted in reduced
30 accumulation of glutamate and increased levels of the umami nucleotide GMP. AMP concentrations were
31 unchanged. The effects on the levels of glutamate and GMP were unexpected and are discussed in relation to the
32 metabolite flux within this pathway.

33

34 **Keywords:** umami, transgenic tomato, glutamate, inosine monophosphate and guanosine monophosphate

35

36 **Key message**

37 This study highlights the changes in umami related nucleotide and glutamate levels when the AMP deaminase gene
38 was elevated in transgenic tomato.

39

40 **Author contribution statement**

41 Bee Lynn Chew (BLC) planned the experiments and wrote the manuscript assisted by Graham Seymour. BLC
42 performed the cloning, tomato transformation, Q-PCR analysis and High Performance Liquid Chromatography
43 (HPLC-UV). Ian Fisk and Wei Xia assisted in the HPLC-UV analysis and data interpretation. Rupert Fray advised
44 on the cloning methods of the AMP deaminase gene. Zsuzsanna Bodi assisted in the Southern blot analysis and
45 interpreted the gel images. Alison Ferguson performed the glutamate enzyme assay. Gregory A Tucker helped in
46 interpreting the data.

47

48 **Acknowledgements**

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50 Malaysia (1001/PBIOLOGI/811258) for funding the project and other related expenses. Some of the methods
51 utilised were developed under BBSRC BB/F017014/1.

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58 **Introduction**

59 Palatability greatly affects preference, acceptance and the consumption of foods and is regulated to a large extent by
60 taste properties. There are five major taste modalities, sweet, salty, sour, bitter and the savoury taste, umami
61 (Yamaguchi, 1991). Umami functions to signal the presence of amino acid rich foods which are essential for human
62 nutrition and also promotes healthy growth and development. Yamaguchi (1991) and Ninomiya (1998)
63 demonstrated that the umami taste sensation was greatly influenced by the presence of the amino acid glutamate and
64 was potentiated by the presence of specific nucleotide monophosphates.

65
66 Ripe tomato has been reported to contain high levels of free glutamic acid of up to a level of 246 mg /100g fresh
67 weight, and this is one of the highest levels observed in any vegetable or fruit (Ninomiya, 1998). The process of
68 tomato ripening increases the concentration of the amino acids glutamate and aspartate, along with organic acids and
69 sugars (Inaba et al. 1980; Petro-Turza, 1986). The mechanisms regulating of glutamate levels in ripening tomato
70 have been investigated using a range of different transgenic experiments. Glutamate can be generated from 2-
71 oxoglutarate by the enzyme NADH-dependent glutamate dehydrogenase (GDH) and GDH shows a rise in activity
72 during ripening (Sorrequieta et al. 2010). Tomato plants over expressing an *Aspergillus nidulans* GDH showed twice
73 the levels of glutamate in the fruits compared to the non-transgenic controls (Kisaka and Kida, 2003). Similar results
74 were obtained when over expressing a native tomato GDH gene (Kisaka et al., 2007). In contrast, using artificial
75 microRNA technology, to silence tomato GDH genes resulted in a dramatic decrease in total GDH activity and
76 glutamate (Ferraro et al, 2015). These results indicate that GDH is involved in the synthesis of glutamate during
77 ripening in tomato. Other approaches to alter glutamate metabolism in tomato have involved inhibition of glutamate
78 metabolising enzymes such as glutamate decarboxylase (GAD) (Kisaka et al., 2006). Transcriptional control of
79 glutamate accumulation in tomato appears to be linked to master regulators of ripening such as the tomato
80 *FRUITFULL* homologs *TDR4/FUL1* and *FUL2* (Bemer et al, 2012).

81
82 Ribonucleotides, which are precursors of nucleic acids and which have other important cellular functions, have been
83 reported to synergistically enhance the umami effect of glutamate. Yamaguchi (1991) showed inosine
84 monophosphate (IMP) could lower the detection threshold of monosodium glutamate (MSG) by a factor of 100 and
85 guanosine monophosphate (GMP), which is present in abundance in shiitake mushrooms, also intensifies the umami
86 taste hence making it a desirable ingredient in Asian cooking worldwide (Nakajima et al. 1961).

87
88 In tomato, researchers have observed the accumulation of adenosine monophosphate (AMP), but not other
89 ribonucleotides and this is consistent with tight regulation of nucleotides within the cell and where accumulation of
90 IMP or GMP does not occur under normal conditions (Yamaguchi and Ninomiya 2000). We wanted to determine if
91 the levels of IMP or GMP could be altered in tomato with the potential, therefore, to enhance the umami taste
92 sensation already provided by the elevated levels of glutamate in the fruits. One approach to enhancing IMP levels
93 would be to increase the levels of IMP by up-regulation of a fruit expressed AMP deaminase gene. This gene
94 encodes an enzyme that converts AMP to IMP. Studies in *Arabidopsis thaliana* showed that the *EMBRYONIC*

95 *FACTOR 1 (FAC1)* gene encodes an AMP deaminase. In the tomato the most closely related sequence by homology
96 based on amino acid similarity was Solyc09g014770.2 (The Tomato Genome Consortium, 2012).

97

98 In this study, we investigated whether increased expression of an AMP deaminase gene in tomato in a fruit specific
99 manner, could produce elevated levels of IMP or GMP, with the aim of enhancing the potential umami taste active
100 components of the fruits.

101 **Materials and methods**

102 Plant material

103

104 Tomato (*Solanum lycopersicum*) seeds, cv. Micro Tom were grown in a growth room set at 22°C during the day and
105 18°C at night with a 16 hour photoperiod and average photosynthetically active irradiance levels of 164 $\mu\text{mol m}^{-2}$
106 s^{-1} . The plants were grown in 10 cm pots in Levington M3 compost and NPK enriched fertilizer (Hortifeed 15-07-
107 30, Lincoln, UK) applied at 1g / L with every watering after 3 weeks from sowing. The Micro Tom seeds were
108 obtained from Professor A.K.Thompson (Cranfield University, UK). Tomato fruits were harvested at various stages
109 of development and ripening, where mature green (MG) was taken as 40 days after anthesis, breaker was the first
110 visible colour change, then breaker+4 days (B4) and breaker+7 days (B7).

111

112 RNA and cDNA analysis

113

114 RNA was extracted from pericarp tissue using the RNeasy Plant Mini Kit by Qiagen (Germany). The synthesis of
115 the first strand of cDNA from the tomato fruit mRNA was performed using Super Script III Reverse Transcriptase
116 (Invitrogen, UK). The primer sequences to amplify the tomato AMP deaminase gene were designed to
117 Solyc09g014770.2. They were forward primer 5'TGAGCAAACAAAATGGATGC 3'and reverse primer
118 5'TCCCCTCAACATCCAAATTC 3'. The PCR reactions generated a single clean band and the sequence of the
119 amplicon was identical to Solyc09g014770 (Tomato Genome cDNA ITAG release 2.40, <https://solgenomics.net/>).
120 The AMP deaminase PCR product was cloned into the pDONR 221 (Invitrogen, UK) vector and then into the plant
121 Gateway transformation vectors pEN-L4-PG-R1 and pK7m24GW,3 which were supplied by the University of
122 Ghent (<https://gateway.psb.ugent.be>). The pEN-L4-PG-R1 contains the tomato polygalacturonase (PG) promoter to
123 drive the expression of the AMP deaminase gene in a fruit-related manner. These vectors are described in detail in
124 (Fernandez et al 2009). Plasmid preps were performed on the colonies obtained using GeneJET Plasmid Miniprep
125 Kit (ThermoScientific, USA) and insertion of the gene of interest was confirmed through sequencing.
126 Agrobacterium transformation of Micro Tom cotyledons followed the protocol of Sun et al. (2006) with transformed
127 plantlets maintained in culture in the presence of 100 $\mu\text{g} / \text{mL}$ kanamycin. PCR and sequencing were used to
128 confirmed the presence of the transgene.

129

130 Southern blot analysis

131

132 Total genomic DNA from the transgenic tomato fruit pericarp (ripening stage of breaker+7) was extracted using
133 DNeasy Plant Mini Kit (Qiagen, Germany). The DNA was digested with restriction enzyme *SpeI*, separated by 0.7%
134 agarose gel electrophoresis, and transferred onto a nitrocellulose membrane. Southern hybridization was performed
135 using standard protocols (Sambrook, Fritsch & Maniatis 1989), gel blots were hybridised to ^{32}P -labelled probes
136 (dCTP, [α - ^{32}P]- 3000Ci/mmol Perkin Elmer) generated using a random priming labelling kit (Rediprime II DNA
137 Labeling System, Amersham). The primer sequences for the probe were as follows:

138 Forward -AMPD-F: 5'- GGA ACC GTA GCC GGA GTA ATG -3'

139 Reverse -AMPD-R: 5'- TCT TTC ACC CAA GGA GCA AC -3'

140

141 Quantitative polymerase chain reaction (q-PCR) analysis

142

143 Expression analysis by q-PCR was undertaken using RNA from three transgenic lines (P4, P44 and P55). This was
144 performed on the LightCycler 480 Real-Time PCR Detection System (Roche, Switzerland) using LightCycler
145 Taqman Master, with reference to manufacturer's guidelines. Primer 3' software was used to aid the design of
146 primers and probes. The elongation factor gene (*Le-ELF-a*) obtained from Gene Bank (accession number: X14449)
147 was used as the control for normalizing the qPCR values across the samples. Control and target gene primers were
148 as follows:

149

150 Forward primer: 5'- TTG CAC TTG CAA CTG GAG AC-3'

151 Reverse primer: 5'- TTA TCG CGG TTC TTC TGC TT- 3'

152 Taqman probe: 5'- TTG GCC ATC GTC ACA TCC GG- 3'

153 Le-ELF forward primer: 5'-ACC TTT GCT GAA TAC CCT CCA TTG-3'

154 Le-ELF reverse primer: 5'-CAC AGT TCA CTT CCC CTT CTT CTG-3'

155

156 High Performance Liquid Chromatography (HPLC-UV) analysis for the quantification of nucleotides.

157

158 Fruits from homozygous transgenic lines P4, P44 and P55 at both (B4 and B7) ripening stages were analysed. There
159 were four biological replicates from each line (one fruit from each of four individual plants of each of the lines). The
160 tomato fruit with seeds intact were macerated with distilled water using an equivalent volume of water / weight of
161 tissue. Then the mixture was centrifuged at 10 000 x g. Clear serum samples of 200 µL were obtained from each
162 sample and subjected to the analysis. Serum samples were diluted 10 times and were subjected to HPLC-UV
163 analysis. Tomato nucleotide samples were then separated using a 5 µm Biphenyl 100A 250*4.6 mm column
164 (Kinetex, Phenomenex, Macclesfield, UK) at a constant flow rate of 1 mL/min, with isocratic mobile phase (10 mM
165 NH₄Ac: Methanol 99:1. GMP, IMP and AMP nucleotides were detected at 260 nm at retention times of 3.0, 3.2 and
166 4.3 min respectively. The concentration of nucleotides was quantified using external authentic standards.

167

168 Glutamate enzyme assay

169

170 Measurement of L-glutamic acid (L-glutamate) was performed using a two-step enzyme assay following
171 manufacturer's instructions for a microplate assay procedure (Megazyme, Wicklow, Ireland). Tomato serum from
172 the Micro Tom tomato fruits at B4 and B7 were extracted using the method previously defined for nucleotide
173 analysis. The samples were diluted 10 fold and 10 µL was used directly in the assay. L-glutamate was quantified by
174 reference to a standard curve and authentic standards.

175 **Results**

176

177 Introduction of the AMP deaminase gene into tomato plants

178

179 We prepared a binary vector harboring the tomato AMP deaminase gene (Solyc09g014770), under the control of the
180 PG promoter. Homozygous lines were identified by Southern blotting (Supplemental Fig.1). Three independent
181 transgenic lines with a single copy of the transgene were selected (P4, P44 and P55) for cultivation and further
182 analysis. The homozygous lines selected from T₁ progeny were used in the experiments on transgene expression,
183 nucleotide and glutamate quantification.

184

185 There were no obvious observable morphological differences, in terms of plant development and fruit production,
186 between the transgenic lines, in comparison to the azygous wild type control plants.

187

188 Transgene expression, nucleotide and glutamate quantification

189

190 Fruits at the developmental stages of mature green (MG), breaker+4 (B4) and breaker+7 (B7) were harvested from
191 homozygous lines, RNA extracted and samples were subjected to expression analysis using qPCR. AMP deaminase
192 gene expression was apparent at all stages of ripening (Fig. 1) and showed maximum expression at B4, for all lines
193 including the control. All transgenic lines had significantly ($P<0.05$) higher expression in comparison to the azygous
194 line at all stages of ripening. Line P55 had significantly ($P<0.05$) higher transgene expression at all stages of
195 ripening than any of the other transgenic lines or the control, and this was highest at B4.

196

197 LC-UV analysis of the tomato fruit serum, of ripening stages B4 and B7 identified the presence of nucleotides,
198 particularly adenylate (AMP) and guanylate (GMP) in all samples. Inosine monophosphate (IMP) was not detected
199 in our samples. AMP concentrations were significantly higher in the B7 ripening stage in comparison to B4 for all of
200 the lines including the control. There were no significant differences ($P>0.05$) in the levels of AMP between the
201 transgenic lines (P4, P44 and P55) and the azygous control (Fig. 2). The GMP concentration was significantly
202 higher in the B7 ripening stage in comparison to B4 for all of the lines including the control, furthermore the
203 transgenic tomato line P55 contained significantly greater ($p<0.05$) concentrations of GMP at B4 and B7, when
204 compared to the wild type control (Fig. 3). This transgenic line also showed the most substantial elevation of *AMP*
205 *deaminase* expression at both B4 and B7. The AMP levels for all of the samples were at least 5 fold higher than the
206 GMP levels in the tomato fruits. Glutamate analysis of the tomato serum samples indicated a significant ($P<0.05$)
207 reduction in glutamate concentration in P55 for both B4 and B7 and in P44 for B4 (Fig. 4).

208

209 **Discussion**

210 Previous studies have revealed that certain ribonucleotides, when present at low levels with glutamate,
211 synergistically enhance the umami flavour of foods; these nucleotides include IMP and GMP. However,

212 ribonucleotides levels are normally tightly controlled to ensure normal metabolism, whilst avoiding accumulation
213 which can lead to undesirable perturbations of the nucleotide pool. We investigated whether nucleotide levels could
214 be enhanced in tomato by up-regulation of AMP deaminase.

215
216 In Arabidopsis, the *AMP deaminase* gene *FAC1* was shown to be essential for the development of zygote to embryo
217 (Xu et al. 2005). AMP deaminase plays an essential role in plants through the maintenance of the purine nucleotide
218 cycle and altered enzyme activity can result in changes in the concentration of adenine and guanine nucleotide pools
219 leading to growth suppression and plant death (Dancer et al. 1997; Sabina et al. 2007).

220
221 We over expressed AMP deaminase on a fruit specific promoter construct. The fruits were found to grow and
222 develop normally without any evident morphological differences in comparison to the control. The transgenic lines
223 showed enhanced AMP deaminase expression. However, glutamate levels decreased as *AMP deaminase* expression
224 was elevated in comparison to the control. These results were somewhat unexpected as we would predict that
225 elevation of the AMP deaminase enzyme expression would lead to higher deamination activity, thereby converting
226 AMP into IMP, as has previously been reported by Yoneda et al. (2004).

227
228 IMP formation involves the conversion of the precursor ribose 5-phosphate into 5-phosphoribosyl-1-pyrophosphate
229 (PPRP). IMP is then converted to either adenylylate (AMP) via adenylosuccinate or oxidised to form guanylate
230 (GMP). Whilst it is not a closed system, excess AMP in the nucleotide pool can be converted to IMP through a
231 process catalysed by the enzyme AMP deaminase, also known as monoadenylylate deaminase. This enzyme converts
232 adenosine monophosphate (AMP) to inosine monophosphate (IMP) via a hydrolytic deamination reaction with the
233 release of ammonia. In the current model of this pathway IMP is the branch point precursor for the production of
234 AMP and GMP (Figure 5). AMP is synthesized by amino group substitution from IMP to form adenylysuccinate,
235 requiring a GTP energy donor and aspartate, the remaining reaction leading to the formation of AMP releases
236 fumarate (Stryer, 1998). Excess AMP has been shown to be channelled to a degradation pathway involving AMP
237 deaminase for the recycling of AMP back to IMP. GMP is also produced from IMP, although this is via an oxidation
238 reaction involving NAD⁺ as the hydrogen acceptor and xanthylate as the intermediate (Stryer, 1998).

239
240 We observed an increase in the levels of AMP from B4 to B7 but AMP remained at equivalent concentrations
241 between the transgenic lines and the control. Under normal conditions, AMP is regulated alongside ADP and ATP
242 as part of the energy balance within the cell. These processes may have a much bigger impact on the AMP levels
243 within the cell than AMP deaminase activity. Additional AMP or IMP may be channelled for the generation of
244 additional GMP instead, as indicated in our results (Fig. 3). This would operate to maintain AMP homeostasis and to
245 preserve energy (Zrenner et al. 2006). In our work, exceptionally high levels of *AMP deaminase* expression were
246 sufficient to enhance GMP in the nucleotide pool and IMP did not accumulate to high levels.

247

248 We measured the levels of glutamate in fruit tissues and somewhat surprisingly we found that glutamate
249 concentrations were lower in P44 at B4 and P55 at B4 and B7 in comparison to the azygous control. In pathways for
250 the biosynthesis of nucleotides, glutamate was expected to accumulate in the lines with higher levels of GMP due to
251 the conversion of IMP to GMP (Fig. 5). One explanation for these observations could be due to higher levels of
252 GMP / glutamate affecting other biochemical pathways. Glutamate is required for γ -aminobutyric acid (GABA)
253 biosynthesis through the GABA shunt pathway (Bouché and Fromm 2004). Glutamate decarboxylase (GAD)
254 catalyses the decarboxylation of glutamate to GABA and a short term tracer study showed that excess glutamate
255 may lead to the increase GAD activity and a higher rate of conversion of glutamate to GABA (Scott-Taggart et al.
256 1999). In a study on a GABA-rich tomato cultivar, 'DG03-9', GAD activity was prolonged into the ripening stages
257 and GABA transaminase (synthesis glutamate from GABA) activity was reduced (Akihiro et al. 2008). Therefore an
258 explanation for the reduced accumulation of glutamate in transgenic fruit is that elevated GMP levels result in
259 increased GAD activity during ripening, leading to conversion of glutamate to GABA.

260

261

262 **Conclusion**

263 In summary, three transgenic tomato lines (P4, P44, P55) with modified levels of AMP deaminase expressions
264 (P55>P44>P4) were generated. The line with the highest level of AMP deaminase expression had the highest
265 concentration of GMP and also surprisingly showed a reduced glutamate concentration.

266

267 **List of figures**

268

269 **Fig. 1: Quantitative PCR analysis of the AMP deaminase transgene expression for control and transgenic**
270 **lines P4, P44, P55 at three stages of ripening (MG=mature green, B4=breaker+4, B7=breaker+7). Error bars**
271 **are the SEM of nine independent fruits. Mean value of relative gene expression followed by same letter of the**
272 **alphabet at each stage of development were not significantly different (Duncan Multiple Range Test, $P \leq$**
273 **0.05).**

274

275 **Fig. 2: Adenlyate (AMP) quantification of transgenic fruits (P4, P44 and P55) at breaker +4 and breaker +7.**
276 **Error bars are the SEM of four biological replicates. Mean value of AMP concentration followed by same**
277 **letter of the alphabet for transgenic lines and wild type at each ripening stage were not significantly different**
278 **($P < 0.05$).**

279

280 **Fig. 3: Guanylate (GMP) quantification of transgenic fruits (P4, P44 and P55) at B4 (A) and B7 (B). Error**
281 **bars are the SEM of four biological replicates. Mean value of GMP concentration with significant differences**
282 **to the WT for each ripening stage was marked with * for $P < 0.05$**

283

284 **Fig. 4: Glutamate concentration (g/L) of transgenic of transgenic fruits (P4, P44 and P55) at B4 (A) and B7**
285 **(B). Error bars are the SEM of four biological replicates. Mean value of glutamate concentration with**
286 **significant differences to the WT for each ripening stage were marked with * for P<0.05**

287
288 **Fig. 5: Schematic illustrating the biosynthesis on adenylate (AMP) and guanylate (GMP) from inosinate**
289 **(IMP)**

290
291 **Supplementary Fig. 1:** Southern blot analysis of T1 progeny (1-8) from three independent T0 lines (P4, P44 and
292 P55) of transgenic Micro Tom DNA. Wild type (WT) and transgene bands in each line are shown. Homozygous
293 lines indicated by red boxes.

294
295 **Conflict of Interest Statement:**
296 The authors declare that they have no conflict of interest.

297
298
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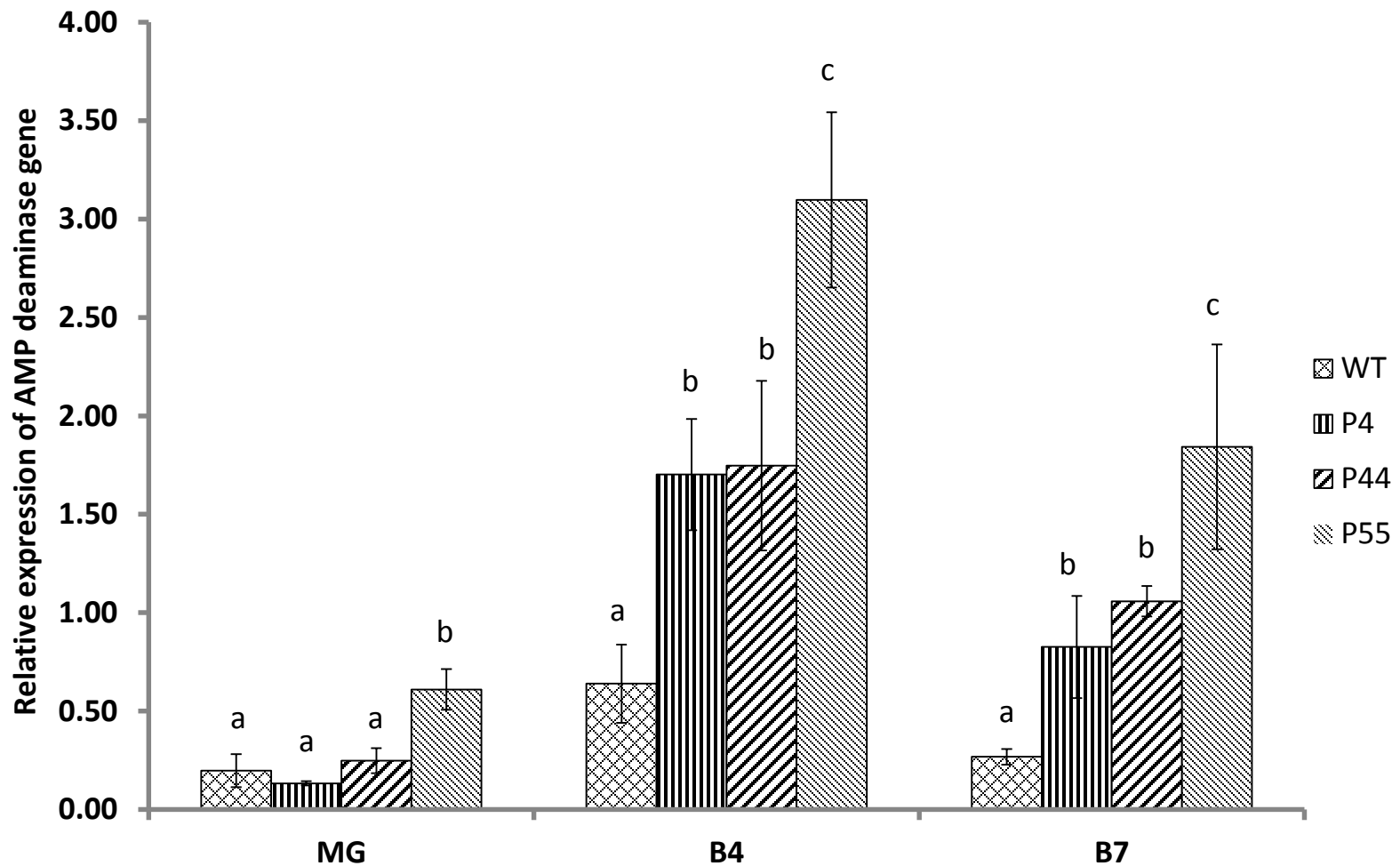


Fig. 1: Quantitative PCR analysis of the AMP deaminase transgene expression for control and transgenic lines P4, P44, P55 at three stages of ripening (MG=mature green, B4=breaker+4, B7=breaker+7). Error bars are the SEM of nine independent fruits. Mean value of relative gene expression followed by same letter of the alphabet at each stage of development were not significantly different (Duncan Multiple Range Test, $P \leq 0.05$).

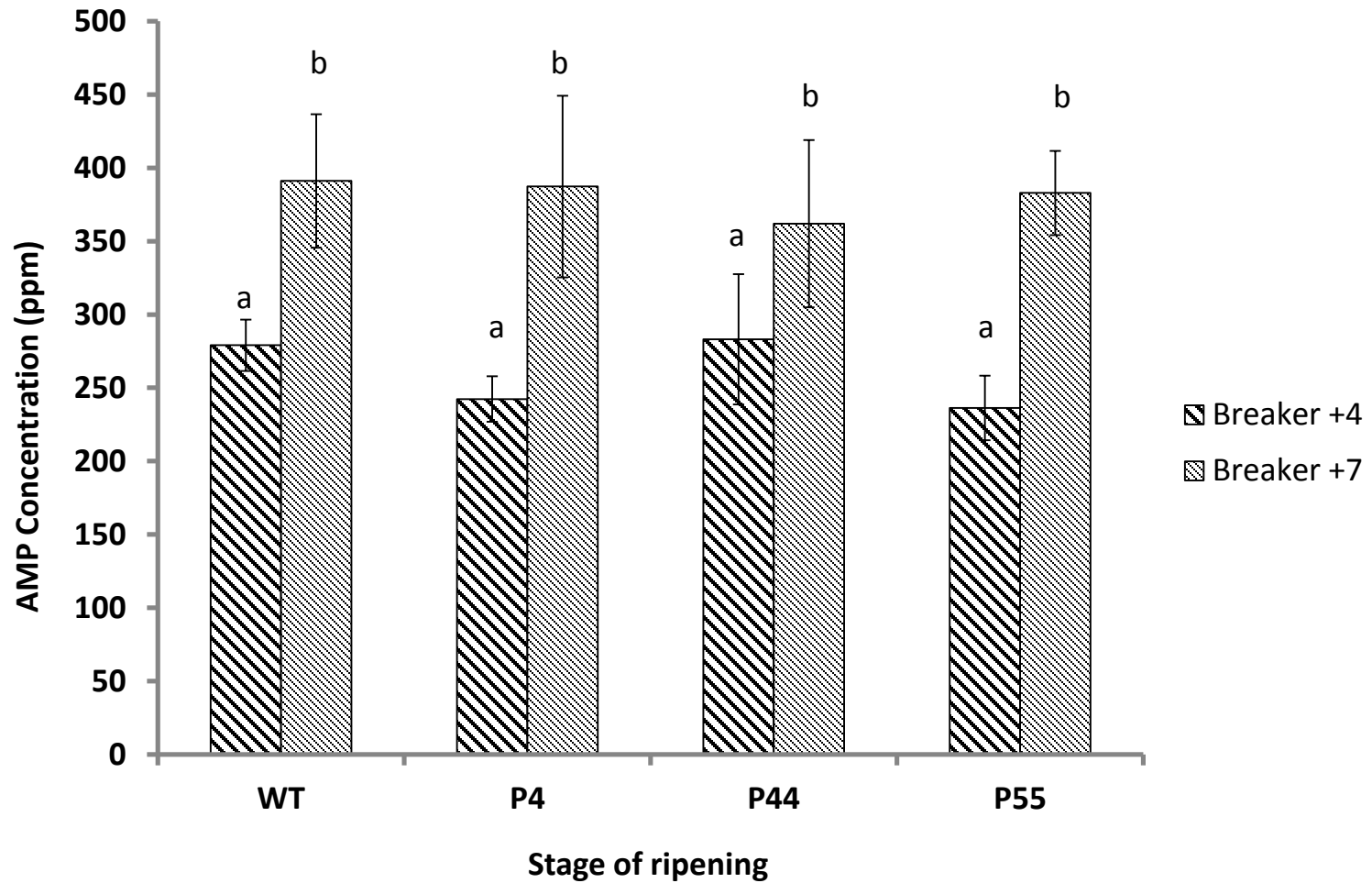


Fig. 2: Adenlyate (AMP) quantification of transgenic fruits (P4, P44 and P55) at breaker +4 and breaker +7. Error bars are the SEM of four biological replicates. Mean value of AMP concentration followed by same letter of the alphabet for transgenic lines and wild type at each ripening stage were not significantly different ($P < 0.05$).

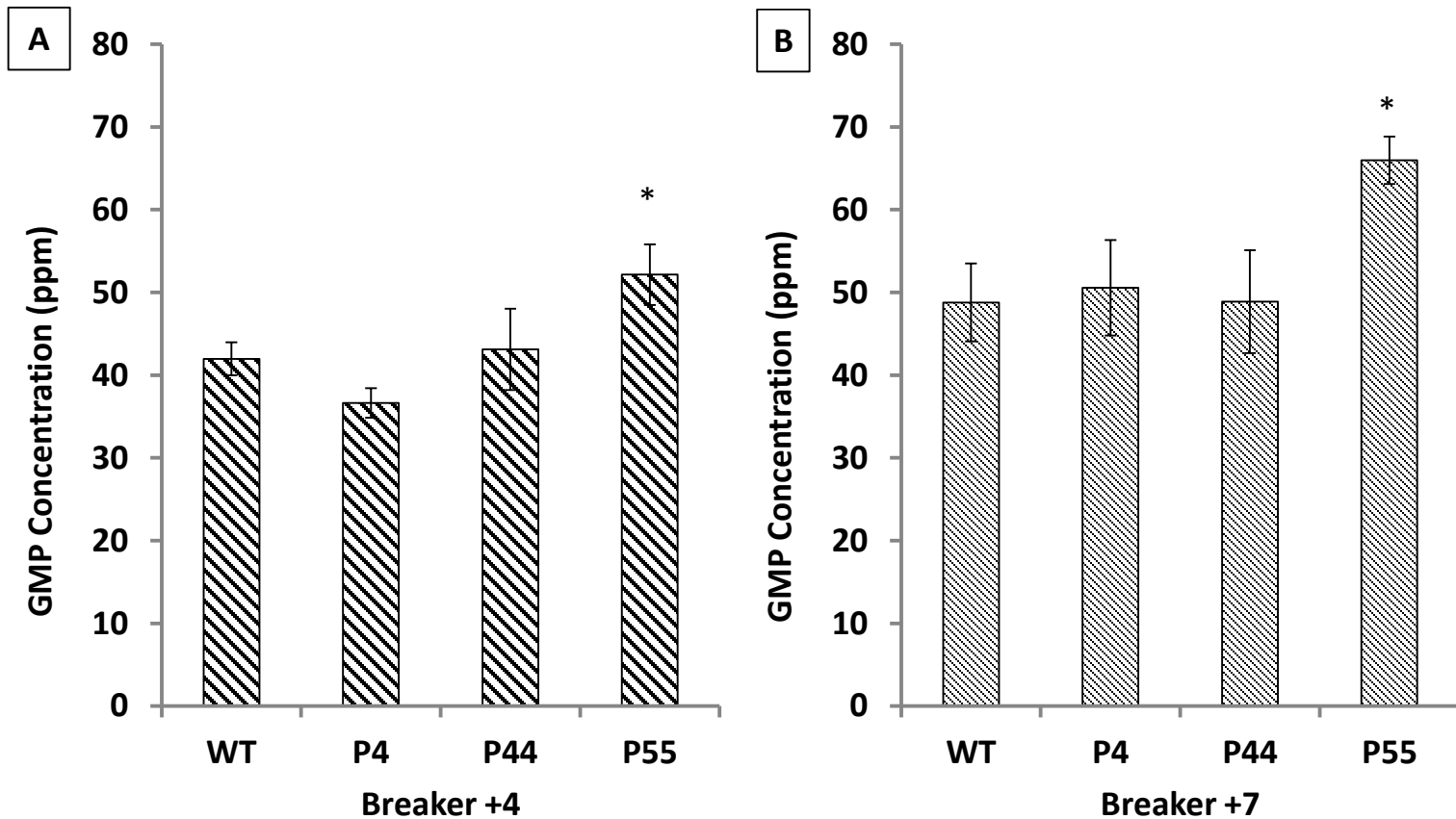


Fig. 3: Guanylate (GMP) quantification of transgenic fruits (P4, P44 and P55) at B4 (A) and B7 (B). Error bars are the SEM of four biological replicates. Mean value of GMP concentration with significant differences to the WT for each ripening stage was marked with * for $P < 0.05$

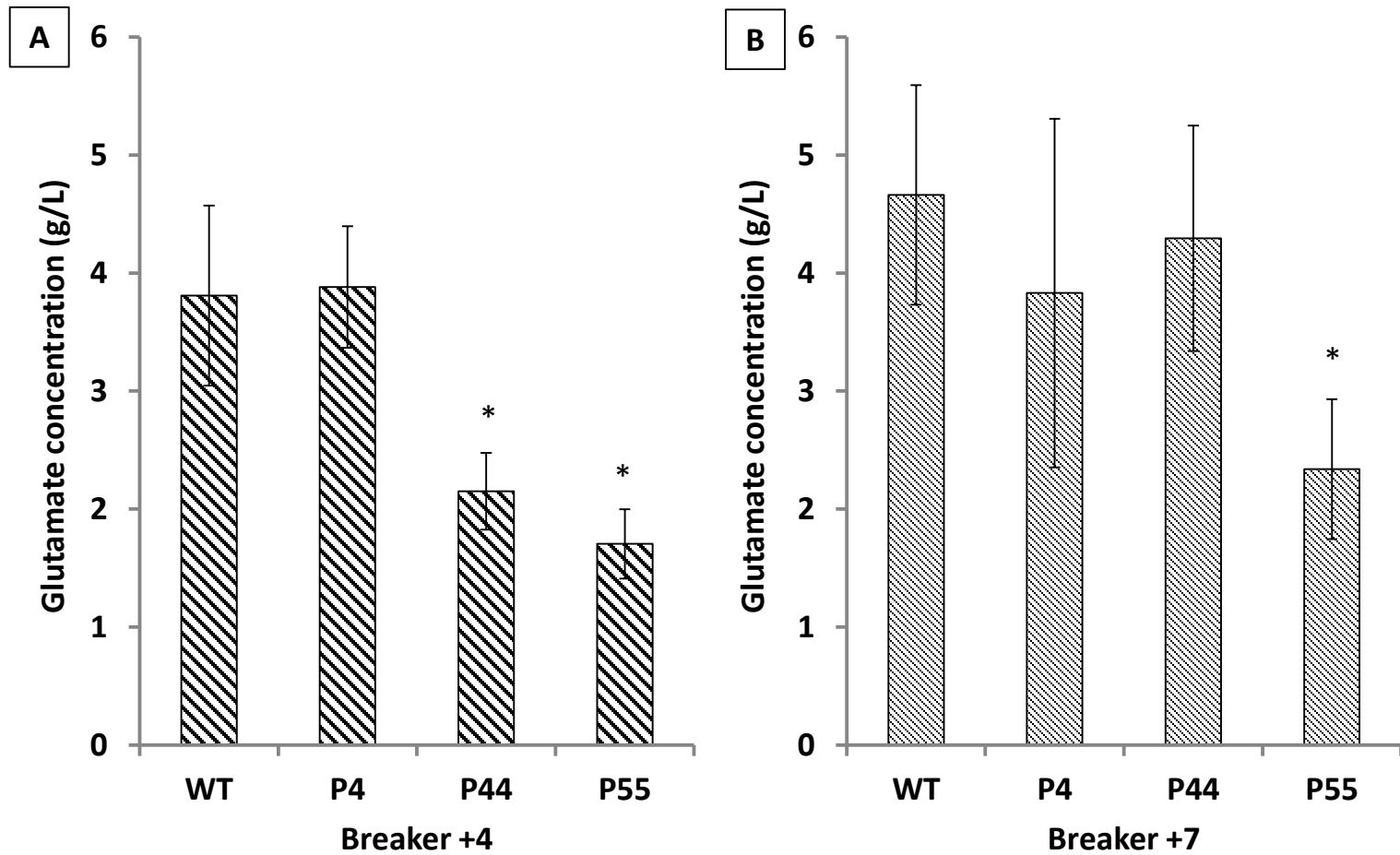


Fig. 4: Glutamate concentration (g/L) of transgenic of transgenic fruits (P4, P44 and P55) at B4 (A) and B7 (B). Error bars are the SEM of four biological replicates. Mean value of glutamate concentration with significant differences to the WT for each ripening stage were marked with * for $P < 0.05$

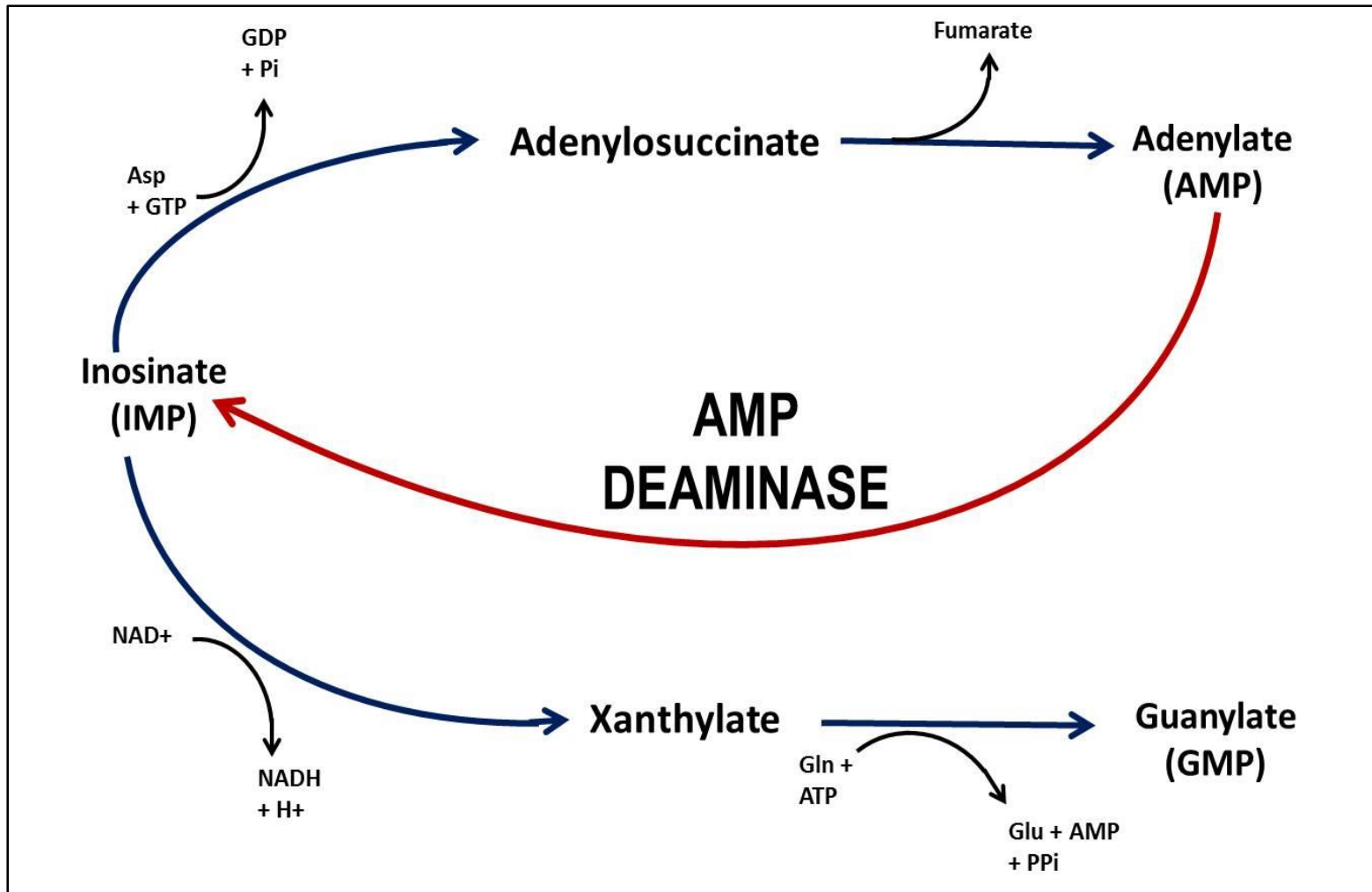
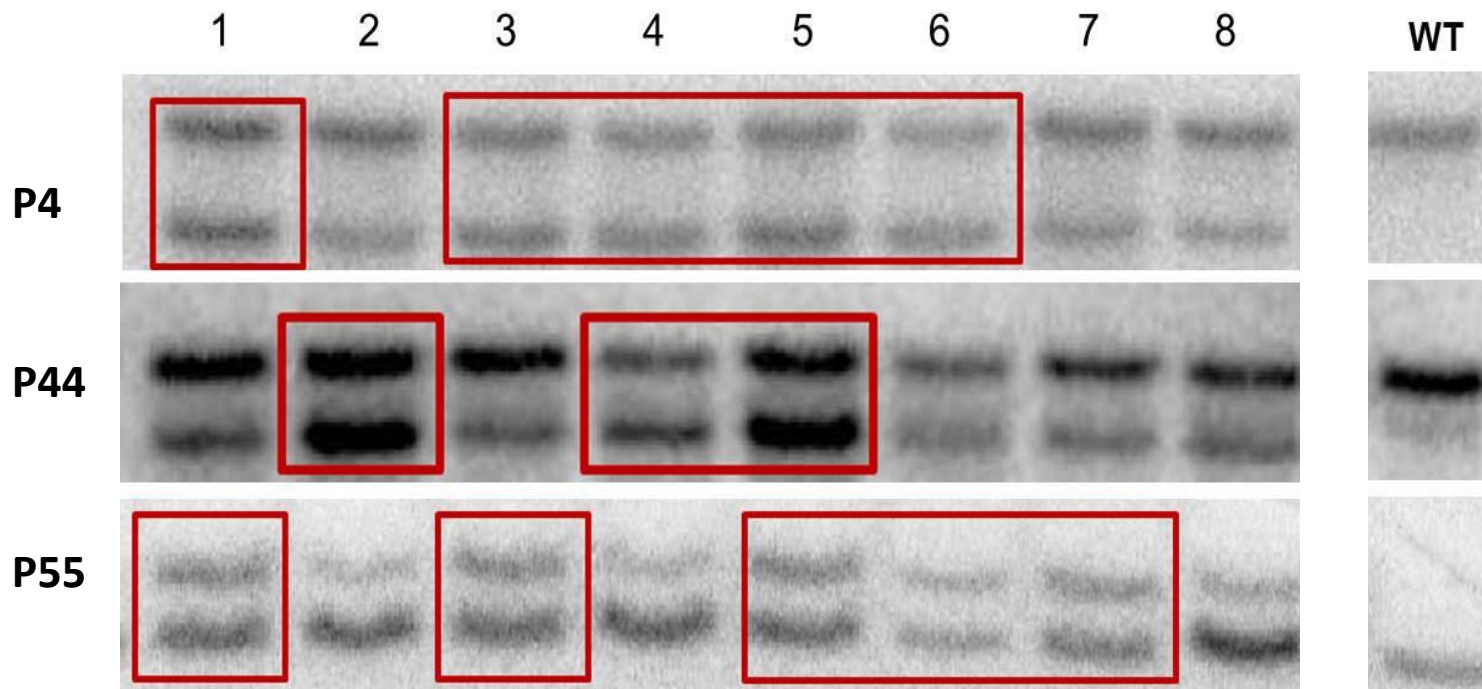


Fig. 5: Schematic illustrating the biosynthesis of adenylate (AMP) and guanylate (GMP) from inosinate (IMP)



Supplementary Fig. 1: Southern blot analysis of T1 progeny (1-8) from three independent T0 lines (P4, P44 and P55) of transgenic Micro Tom DNA. Wild type (WT) and transgene bands in each line are shown. Homozygous lines indicated by red boxes were selected for subsequent analysis.