

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

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Abstract

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

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

Key message

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

Author contribution statement

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

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Introduction

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

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

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

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

Materials and methods

Plant material

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

RNA and cDNA analysis

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

Southern blot analysis

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

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

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

Quantitative polymerase chain reaction (q-PCR) analysis

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

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

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

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

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

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

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

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

Glutamate enzyme assay

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

Results

Introduction of the AMP deaminase gene into tomato plants

We prepared a binary vector harboring the tomato AMP deaminase gene (Soly09g014770), under the control of the PG promoter. Homozygous lines were identified by Southern blotting (Supplemental Fig.1). Three independent transgenic lines with a single copy of the transgene were selected (P4, P44 and P55) for cultivation and further analysis. The homozygous lines selected from T₁ progeny were used in the experiments on transgene expression, nucleotide and glutamate quantification.

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

Transgene expression, nucleotide and glutamate quantification

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

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

Discussion

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

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

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

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

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

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

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

Conclusion

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

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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 on 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.

Conflict of Interest Statement:

The authors declare that they have no conflict of interest.

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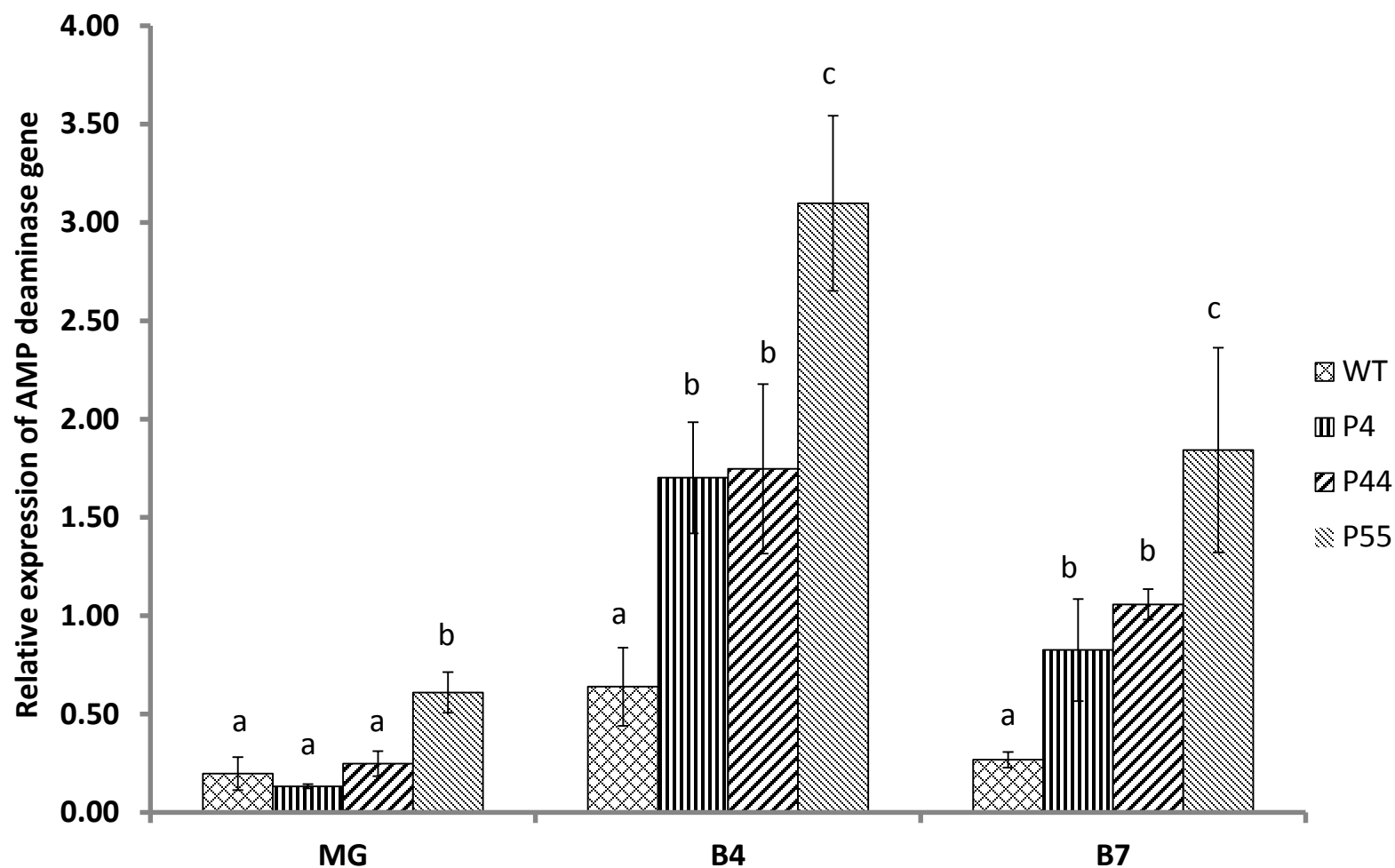


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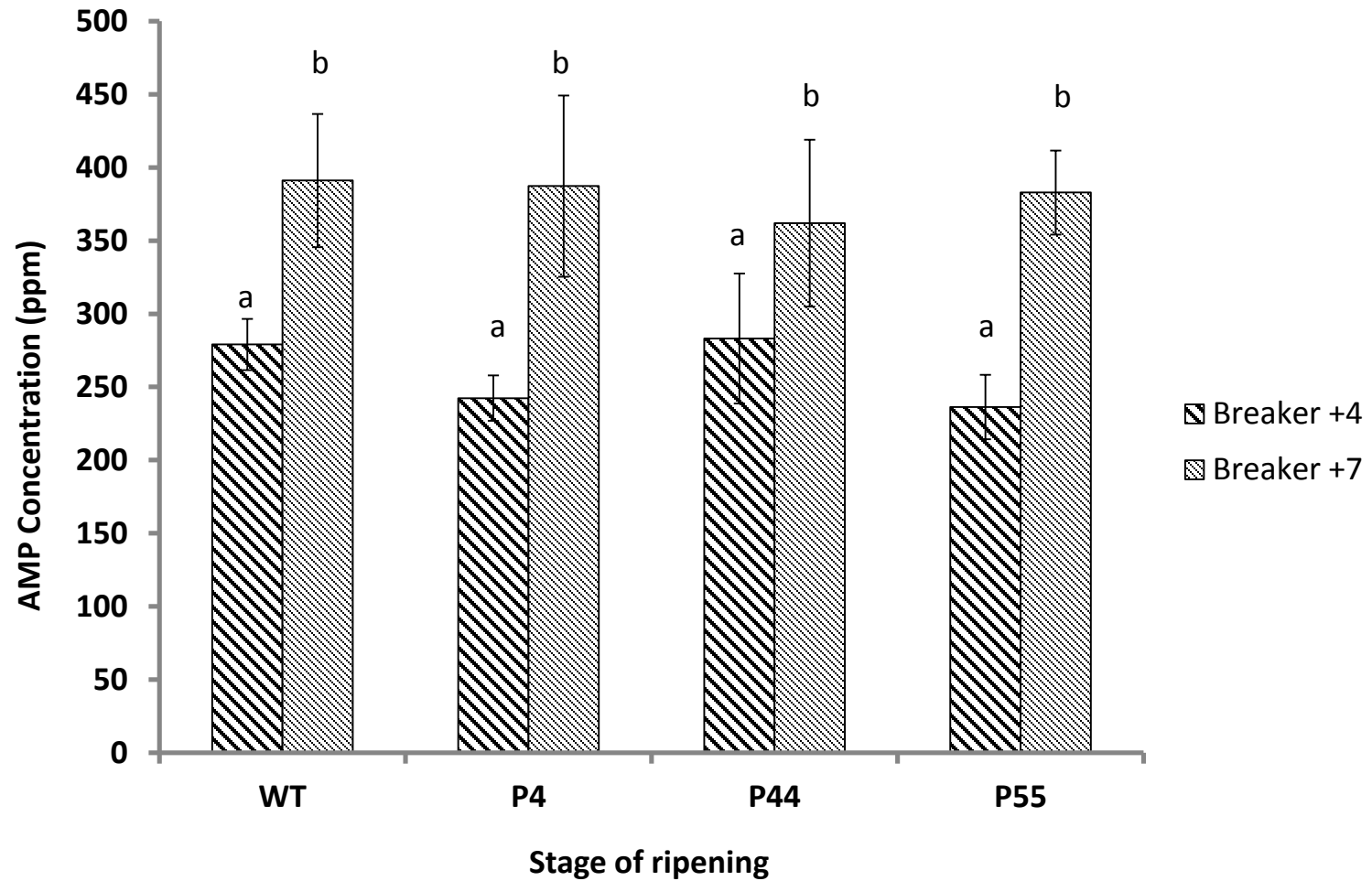


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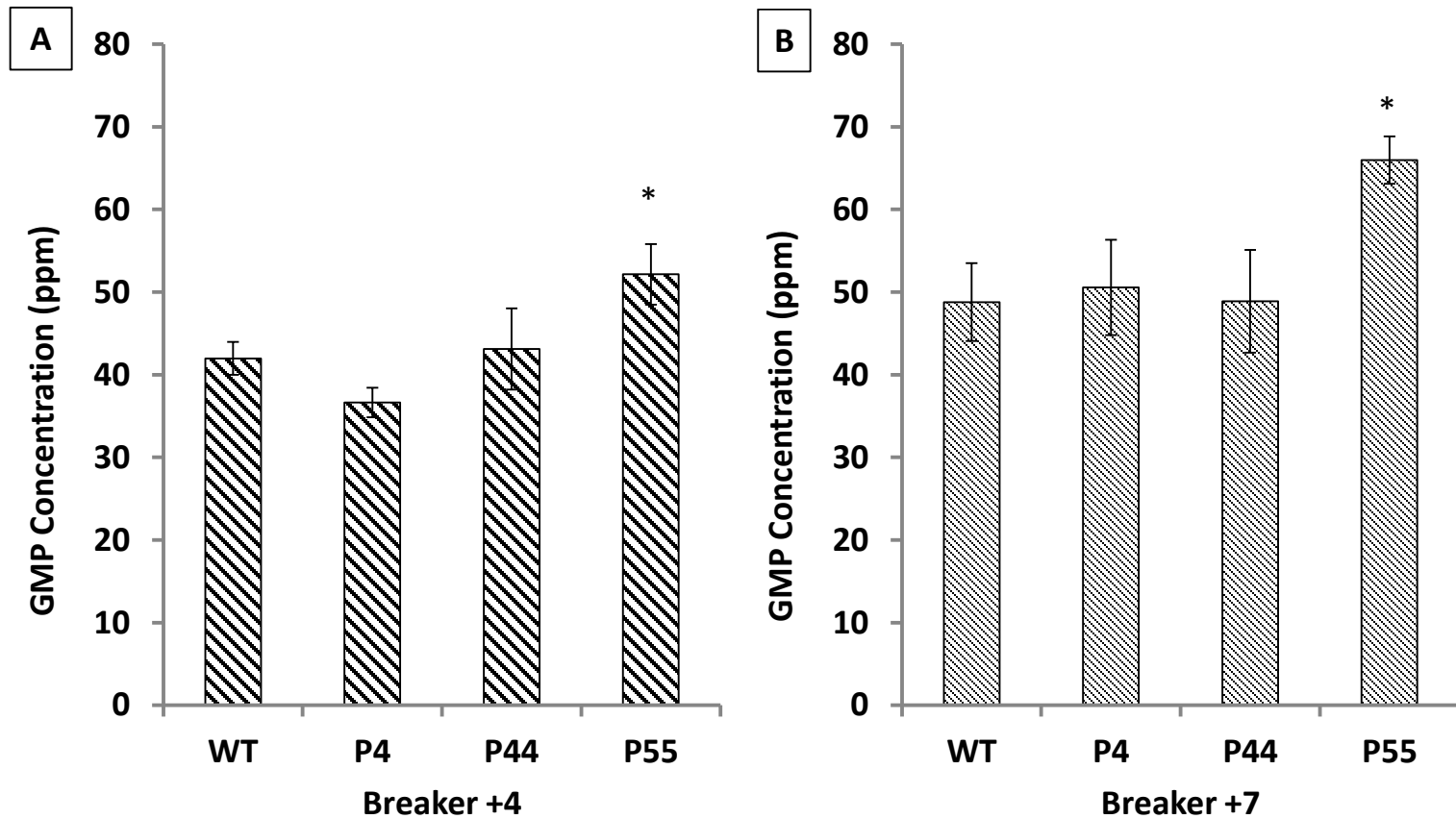


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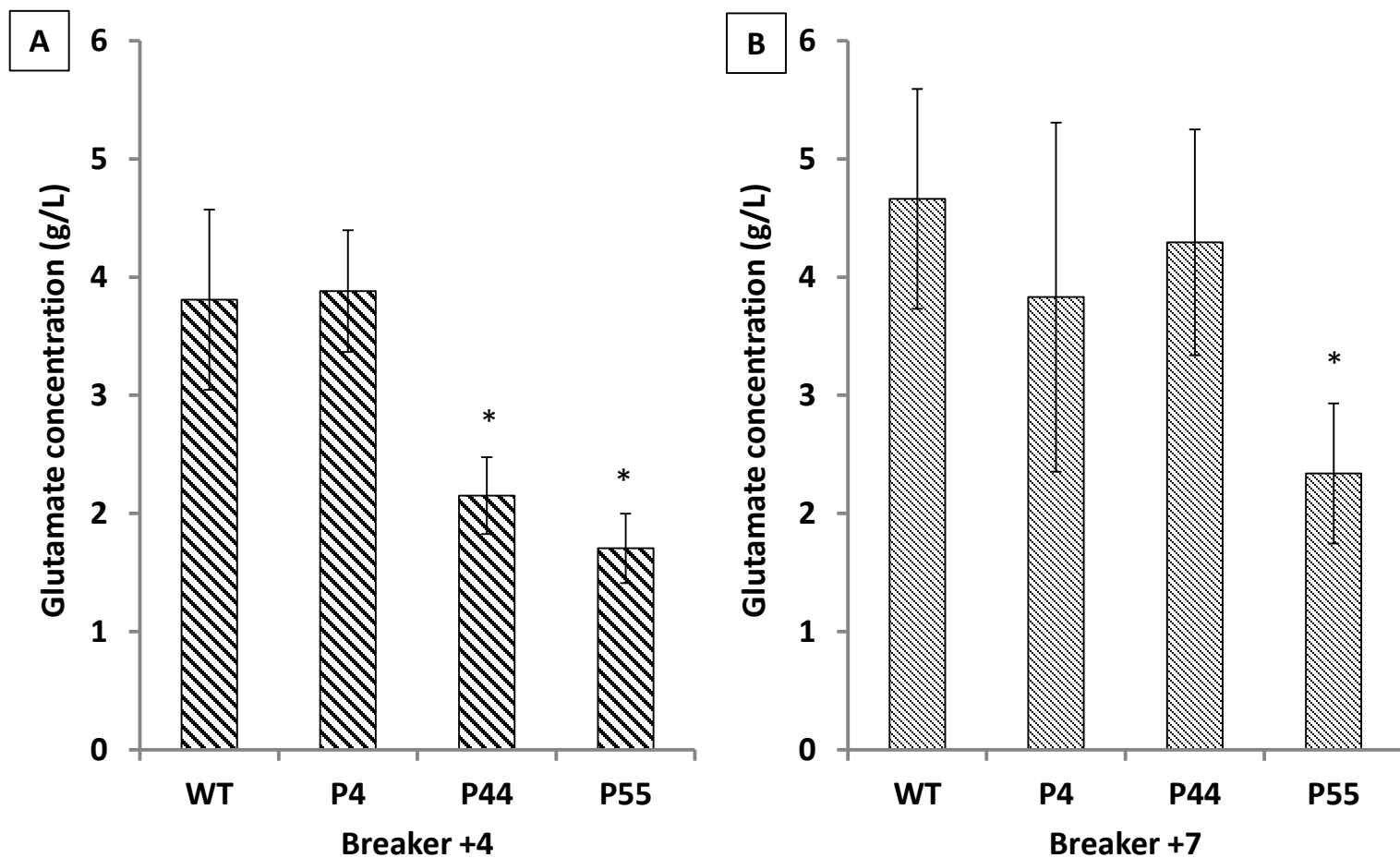


Fig. 4: Glutamate concentration (g/L) of transgenic of transgenic fruits (P4, P44 and P55) at B4 **(A)** and B7 **(B)**. Error bars are the SEM of four biological replicates. Mean value of glutamate concentration with significant differences to the WT for each ripening stage were marked with * for $P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

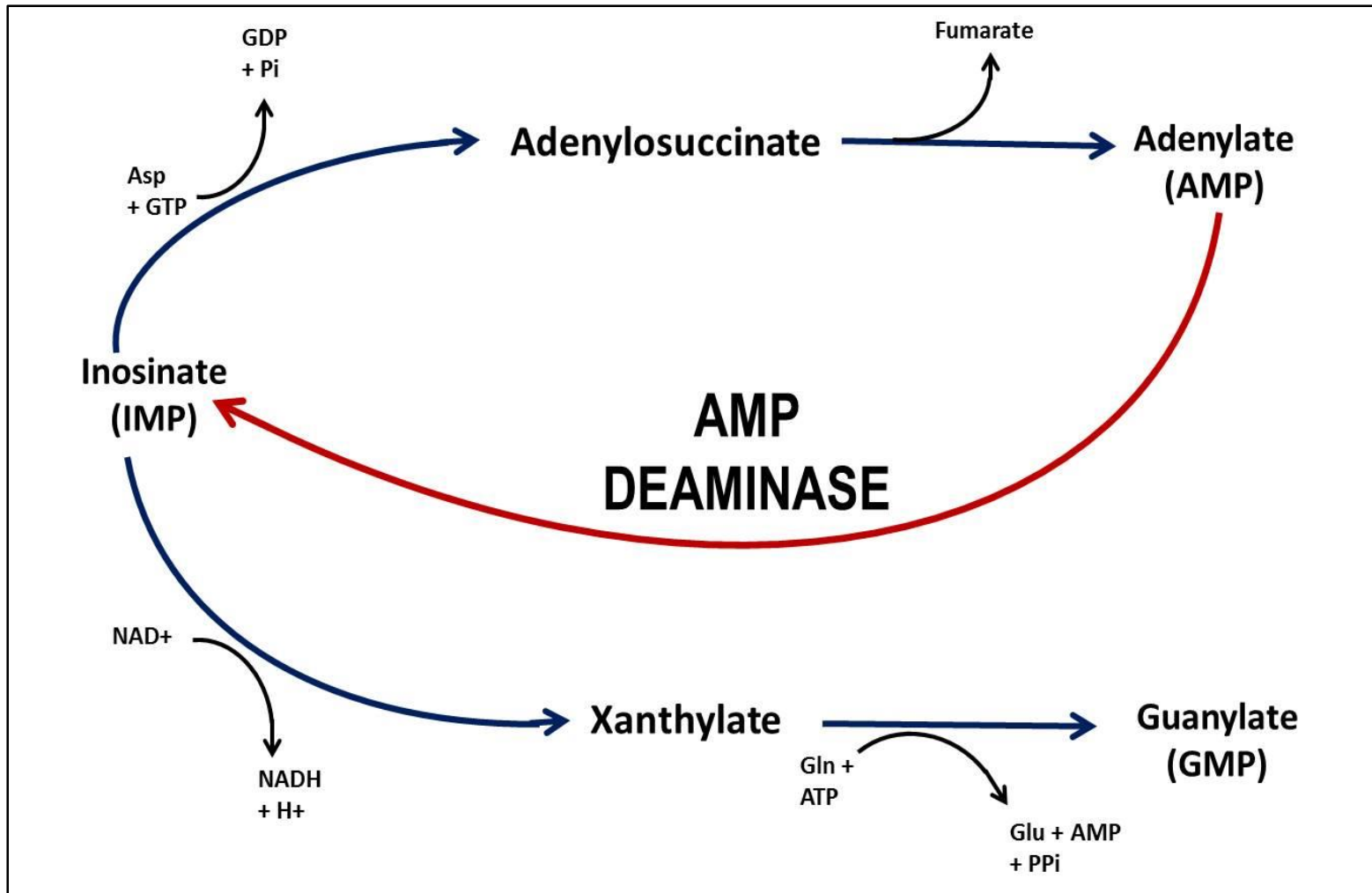
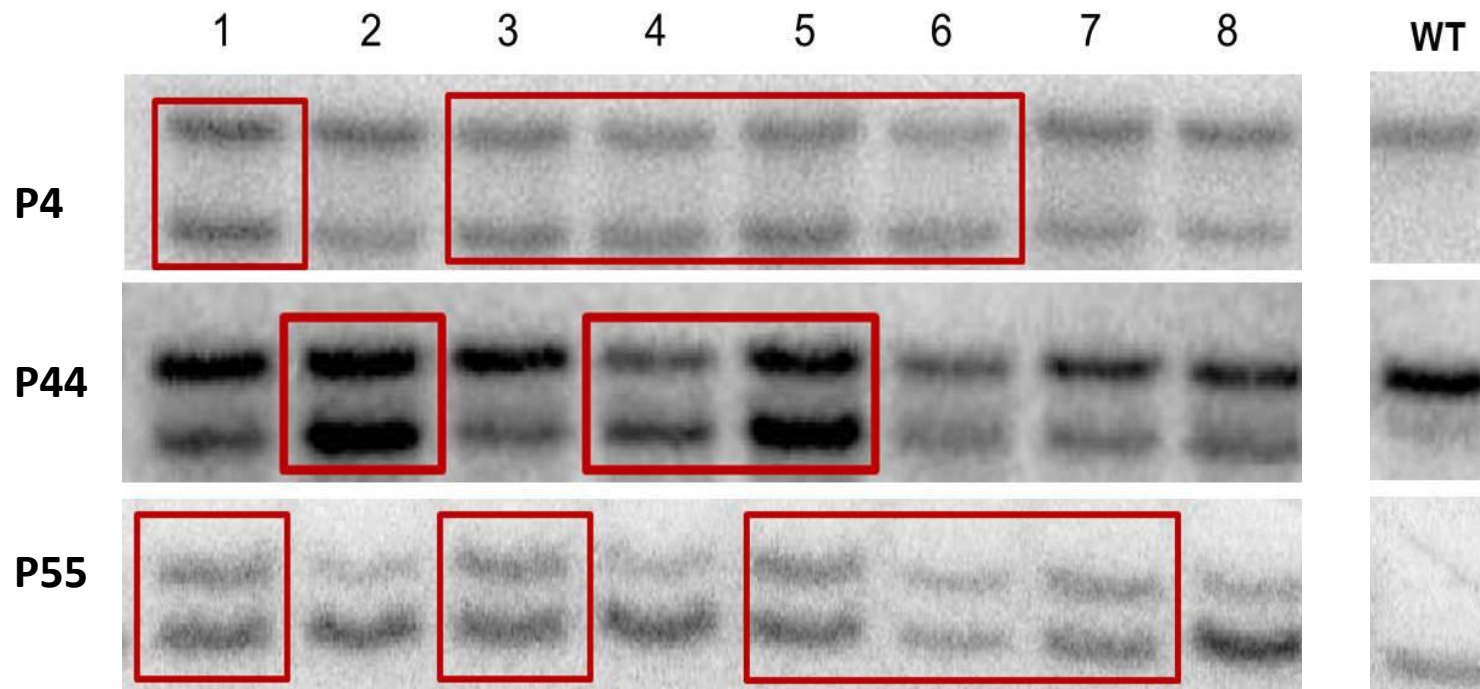


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