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Identification and Cloning of Transcripts from Triterpenoid-Induced Neuronal Outgrowth in Neuro-2a Cells from Mus musculus

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Identification and cloning of transcripts from triterpenoid-induced neuronal outgrowth in Neuro-2a cells from *Mus musculus*

by

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Abstract

Neurodegenerative diseases severely reduce quality of life and are often responsible for the death of those suffering from them. In addition to the societal toll these diseases inflict on populations, the health care costs are estimated to be hundreds of billions of dollars annually for the U.S. alone. Unfortunately, direct pharmacological treatment for some of the most common of these diseases, such as Alzheimer's, remains elusive. Traditional medicine in China and India have recommended a variety of plants for preventing and treating these diseases. For example, *Centella asiatica* (L.) Urban, also knows as Gotu Kola, is believed to treat and prevent many common ailments and diseases, including Alzheimer's disease and other dementia-related diseases. *C. asiatica* has been shown to contain phytochemicals, such as asiatic acid (AA) and madecassic acid (MA), which are believed to be responsible for the physiological response by humans to *C. asiatica* extracts. The present research examined the transcriptomes of mouse Neuro-2a cells treated with AA, MA, and ethanol (vehicle) to discover gene transcripts that were highly expressed (10X or greater) by both AA and MA treatments compared to ethanol alone. This resulted in a core set of 23 transcripts, which was used to determine a subset of transcripts for cloning into a mammalian overexpression plasmid (pcDNA-DEST40). High level expression of the five transcripts (*Mpp3-204*, *Pak1-206*, *Tardbp-204*, *Usf1-210*, and *Zc3h15-204*) following AA and MA treatments was verified using qPCR. The suite of plasmids containing these transcripts will be used in subsequent experiments with neurons in culture to explore whether one of these proteins or a combination of them is sufficient for significantly increased neurite outgrowth and length, as was demonstrated with direct AA and MA treatments.

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1. Introduction

Over the past two decades, remarkable progress has been made in elucidating the causes of neurodegenerative diseases. The term degenerative diseases of the nervous system is an umbrella description that includes several conditions that primarily affect nerve cells in the brain (Brown et al., 2005). Neurons are the nervous system's cornerstone, including the brain and spinal cord. Normally, neurons cannot reproduce or replace themselves when they are damaged or killed. Examples of degenerative diseases of the nervous system include Parkinson's, Alzheimer's, and Huntington's. Neurodegenerative disease (N.D.) is a common and growing cause of mortality and morbidity worldwide, particularly in the elderly (Erkkinen et al., 2018).

N.D. is distinguished by the gradual loss of selectively susceptible populations of neurons, as opposed to select static neuronal loss caused by metabolic or toxic disorders (Dugger et al., 2017). Alzheimer's disease (A.D.) is one of the major neurodegenerative disorders and the most common type of dementia. A.D. was first recognized more than 100 years ago, but only in the past 30 years has there been a significant increase in the study of its symptoms, causes, risk factors, and treatments. It is defined by a gradual loss in cognitive, functional, and behavioral alterations that often begins with memory degradation that affect people aged 65 or older. People with A.D. develop a gradually worsening ability to remember new information over time. The most common symptoms include memory loss and cognitive decline (Alzheimer’s Association, 2013), which currently produces dementia in 5.8 million U.S. citizens, and this number will likely increase to 13.5 million by 2050 (Alzheimer's Association, 2019).

A.D. dementia is projected to have a devastating impact on global populations by 2050, with 131 million affected (Cummings et al., 2020). Research and technological advances have improved our quality of life and prolonged longevity. Unfortunately, a greater lifespan is accompanied by an increase in the prevalence of age-related disorders, such as Alzheimer's. These diseases cost the U.S. economy billions of dollars each year in direct health care costs and lost opportunities; it is estimated that $321 billion was required for Alzheimer's and related diseases in 2022, which included out-of-pocket spending estimated at $81 billion (2022 Alzheimer's Disease Facts and Figures). In addition to the financial costs, there is an immense emotional burden on patients and their caregivers. As the number of elderly citizens increases, these costs will also increase. The costs of A.D. are accelerating, rising from one trillion globally in 2018 to a projected $2 trillion in 2030 (Cummings et al., 2020). Despite extensive research efforts to find a cure for Alzheimer's disease, we still have no long-term solution. Effective therapeutic and preventative treatments are urgently needed to combat the devastating cognitive decline observed in patients with A.D. However, many potential remedies and medications for neurodegenerative diseases have been derived from traditional medicine (Cooper et al., 2017).

In China, traditional Chinese medicine (TCM) has theories and a wealth of valuable experience in preventing and treating Alzheimer's disease for over thousands of years (Liu et al., 2014). Recently, herbal medicines used to treat Alzheimer's disease in China have been founded on TCM or contemporary pharmacological theories; this approach has resonated regarding the etiology and pathophysiology of Alzheimer's disease. Evidence shows that TCM therapy may provide complementary cognitive

advantages in treating Alzheimer's. Furthermore, Chinese herbs may be helpful when considering multiple target regulations, particularly when compared to single-target antagonists. Many potent pharmacological substances derived from Chinese herbal remedies have been found to treat various disorders, including diabetes, microbial infections, allergies, inflammation, and cancer. Many Chinese herbs have been suggested to enhance human health by stimulating blood circulation and providing extra energy. Chinese herbs are a fantastic source of medications for testing that may be useful to people living with Alzheimer's (Wu et al., 2011). Following TCM, prescriptions composed of a complicated range of many different herbs are utilized to treat A.D. clinically are being utilized in Alzheimer's disease as a new pathway to memory and cognitive function enhancement (Liu et al., 2014).

In Ayurvedic**,** traditional Indian medicine has also contributed to the expanding list of useful substances. The findings supported ancient therapies for nervous system illnesses, particularly memory-related ailments like dementia. Attempts have been undertaken to conduct experimental studies on Ayurvedic medicine to understand better its impact on geriatric disorders, such as Alzheimer's disease (Orhan et al., 2012). For example, *Centella asiatica* (L.) Urban has been utilized as an Ayurvedic brain tonic remedy for centuries. *C. asiatica*, a potential phytopharmaceutical, demonstrates total neuroprotection by reducing oxidative stress, blocking enzymes, and decreasing the production of amyloid plaques in A.D. (Nishteswar et al., 2014).

Centella asiatica, also known as Indian Pennywort, is a member of the plant family Apiaceae. *C. asiatica* (Figure 1) is a perennial creeper that is slightly aromatic and a valuable medicinal herb in both the Old and New Worlds. Found in tropical and

subtropical regions of India at an altitude of 600 meters, the plant is native to Southeast Asia, Sri Lanka, India, parts of China, western South Sea islands, Madagascar, South Africa, and can be found in southeastern U.S.A., Mexico, Venezuela, Colombia, and eastern South America.

Figure 1. *Centella asiatica* plant (Gray et al., 2018)

C. asiatica is one of the most important herbs for treating skin problems, healing wounds, and revitalizing nerves and brain cells, and is thus known as a "brain food" in India. Centella's use in food and beverages has grown over the years, owing to its health benefits, such as a source of antioxidants, anti-inflammatory properties, wound healing, memory-enhancing properties, and many others (Seevaratnam et al., 2012). In the European Pharmacopeia, Commission E of the German Ministry of Health, and World Health Organization (WHO), the plant is described for the effects of wound healing and memory enhancement (Orhan et al., 2012).

Scientific studies have shown the existence of various biochemical components through chemical content analysis of plants associated with traditional medicines (Seevaratnam et al., 2012; Udari, 2018). The primary chemical compounds of medical interest in *C. asiatica* are triterpenes and their derivative molecules, such as asiatic acid

(AA), asiaticoside, madecassic acid (MA), madecosside (Figure 2), thankuniside, brahmoside, brahminoside, and brahmic acid. Asiaticoside and madecassoside are found primarily in the leaves and to a lesser extent in the roots (Chandrika et al., 2015). Due to these physiologically important components related to humans, Centella is thought to have an essential role in medical and nutraceutical applications.

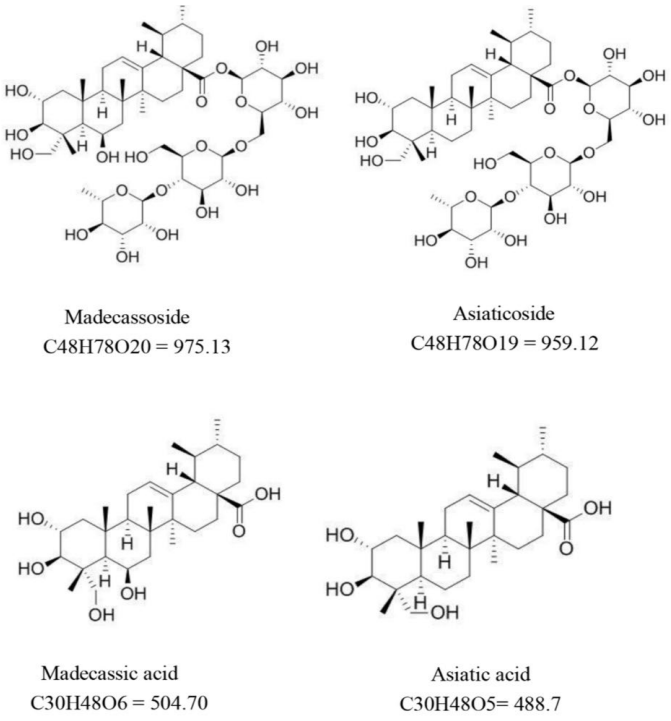

Figure 2. Molecular structures of triterpenoids in CA extracts (Sun et al., 2020).

Studies in cell culture and animal models have supported the beneficial effects of *C. asiatica* (CA) on the nervous system. Studies have indicated that the combination of asiatic acid and madecassic acid stimulates neuronal differentiation and neurite elongation in PC12 and SH-SY5Y cells, respectively (Jiang et al., 2016; Soumyanath et al., 2005). *In vivo*, *C. asiatica* extracts enhanced neuronal dendritic properties in the hippocampus and amygdala (Rao et al., 2006; Rao et al., 2009). In addition, *C. asiatica* treatment during the postnatal development stage improved neuronal

morphology and promoted higher brain function in mice (Rao et al., 2005). Furthermore, a *C. asiatica* water extract increased the expression of antioxidant and mitochondrial genes in mice while improving cognitive function (Gray et al., 2018).

Few human studies have examined the effects of CA in a placebo-controlled setting; however, CA treatment for 12 weeks in mentally disabled children was shown to improve general mental ability and behavioral problems (Rao et al., 1977). Furthermore, it was demonstrated that CA has anxiolytic activity in humans; a single 12 g oral administration of CA significantly reduced acoustic startle response in healthy subjects compared with the placebo group (Bradwejn et al., 2000). Nevertheless, more research on *C. asiatica*'s clinical activities is needed to investigate its potential in treating disease.

Previous research has demonstrated the effects of two phytochemicals found in *C. asiatica*, asiatic acid (AA) and madecassic acid (MA), on cultured mouse Neuro2a (N2a) cells. For example, in a dose-dependent manner, AA and MA led to increased neurite extension and combined length (Nathan and Tucker, 2019). A follow-up study examined the effects of 5 μM of AA or MA on N2a cells in culture, where both AA and MA led to increased neurite extension and combined length of neurons (Fatimah, 2020). That study also found that AA and MA led to similar genes being expressed at the highest levels (threshold of 10X or greater than ethanol vehicle). The present study aimed to expand upon these data by further investigating the transcriptome data to identify key genes that are expressed in common between AA- and MA-induced changes to N2a cells, and then prepare these genes for overexpression in these same cells. Future studies can then determine if overexpression of these genes alone or in combination are sufficient to produce significant increases in neuronal outgrowth or length of neurons.

2. Goals and Hypotheses

1. Identify highly expressed genes from mouse Neuro-2a cells after exposure to asiatic acid (AA) and madecassic acid (MA) using transcriptome data.

Hypothesis: There will be a set of highly expressed genes of interest in common between neurons exposed to AA and MA.

2. Verify transcriptome expression data of candidate genes using real-time quantitative PCR.

Hypothesis: The transcriptome data of genes of interest will correspond to qPCR data of these same genes.

3. Clone candidate genes into mammalian expression plasmids for future overexpression studies in cultured neurons.

Hypothesis: Candidate genes can be effectively cloned into mammalian expression plasmids.

3. Materials and Methods

3.1 Transcriptome Analysis

The mouse neuronal cell line Neuro-2a (American Type Culture Collection; CCL-131) was the source of the RNA used for transcriptome sequencing (Alqam, 2020). As previously described in that study, these cells were exposed to 5 μM of asiatic acid, 5 μM of madecassic acid, or vehicle (ethanol) before the RNA was harvested using TRIzol (Invitrogen). The RNA was then prepared with RNA-Seq technology (Illumina) followed by sequencing with a HiSeq 4000 instrument (Illumina). There were a total of 354 million reads across 12 samples (average of 29.5 million reads per sample). The RNA-Seq data were processed using the ArrayStar program in Lasergene 15.2 (DNASTAR) using *Mus.musculus.GRCm38.cdna.all.fasta* as the reference file (uswest.ensembl.org) and *gencode.vM20.annotation.gff3* as the annotation file (gencodegenes.org; Frankish et al., 2018). The reads per kilobase million (RPKM) values were subjected to ANOVA F-tests with false discovery rate (Benjamini Hochberg) correction applied. Transcript P values at or below 0.05 (4861 transcripts) were considered significant. Data from ArrayStar were exported into Microsoft Excel for further processing.

3.2 PCR Amplification

The RNA samples used for transcriptome analysis (described above) were treated with the TURBO DNA-free Kit (Invitrogen) to remove DNA, followed by cDNA generation with 300 ng of RNA using the Superscript III First-Strand Synthesis system (Invitrogen) according to the manufacturers' instructions. The resulting cDNA was used as template for amplification of the transcripts of interest (*Mpp3-204*, *Pak1-206*, *Tardbp-204*, *Usf1-210*, and *Zc3h15-204*). The PCR primers used for each gene were designed by using the

first 21 nucleotides for the forward primer, and the reverse complement of the last 21 nucleotides for the reverse primers. The leader sequences for Gateway technology (Invitrogen) 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3' and 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTC-3' were added to the forward and reverse primers, respectively. To allow for future cloning into the mammalian expression vector pcDNA-DEST40 (Invitrogen) that is designed for C-terminal protein fusion (V5-6xHis tag), the native stop codons were converted to sequences coding for either glycine (*Pak1-206* and *Zc3h15-204*) or leucine (*Usf1-210*, *Tardbp-204*, *Mpp3-204*). The primers used to amplify each of the five transcripts are shown in Table 1.

Table 1. The primers used to amplify the five transcripts of interest in this study. The Gateway-specific forward (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCT-3') and reverse (5'-GGGGACCACTTTGTACAAGAAAGCTGGGTC-3') leader sequences are not shown in the table. The underlined sequences are stop codons that replaced the native stop codons TGA or TAA.

Transcript	Forward Primer (5' to 3')	Reverse Primer (5'-3')
Pak1-206	ATGGATGTAGCCACAGGGCAG	TCCGTGATTGTTCTTGGTTGC
Usf1-210	ATGAAGGGGCAGCAGAAAACA	TAAGTTGCTGTCATTCTTGAT
Tardbp-204	ATGTCTGAATATATTCGGGTA	TAACAGCACTACTTTCAATGA
Mpp3-204	ATGCCAGTACTGTCTGAAGAC	TAACCTCACCCAGCTGATGGG
Zc3h15-204	ATGTATCGCCATGCACTTCCT	TCCTCATTCTTCTAAGTCAAG

3.3 Amplicon Purification and Gateway Cloning

The amplified products described above were visualized in agarose gels and subsequently excised using a razor blade. The DNA was purified from the gel matrix using the PureLink Gel Extraction Kit (Invitrogen). The amplicons were then cloned into the donor vector pDONR-221 (Invitrogen) using Gateway BP Clonase II Enzyme mix (Invitrogen) according to the manufacturer's instructions. After *E. coli* (One Shot OmniMAX 2 $T1^R$) colony selection and confirmation of successful cloning into pDONR-221, the gene sequences were then transferred from the donor plasmids to the destination plasmid pcDNA-DEST40 using Gateway LR Clonase II Enzyme mix (Invitrogen) without any modifications to the provided protocol. Again, *E. coli* colonies were screened for destination plasmids containing the gene of interest. The plasmid constructs were isolated using PureLink Quick Plasmid Miniprep Kit (Invitrogen) or PureLink HiPure Plasmid Filter Maxiprep Kit (Invitrogen) for pDONR-221 and pcDNA-DEST40 constructs, respectively.

3.4 qPCR Analysis

For gene expression validation studies, qPCR primers (Table 2) were designed using the PrimerQuest Tool (Integrated DNA Technologies; IDT) with the reference transcript sequences (*Mus.musculus.GRCm38.cdna.all.fasta*; uswest.ensembl.org). A StepOne Real-Time PCR System (Applied Biosystems) was used to detect amplification using SYBR Green as the fluorescent agent. The thermocycler conditions were: 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The threshold cycle (Ct) values were generated by the Step One software (Applied Biosystems) and then transformed using the ΔΔCt method. Each reaction contained 10 µL of SYBR Green

PCR Master Mix (Applied Biosystems), 1 µL (1 ρmol) of each of the forward and reverse primers, 1 µL of cDNA template, and 7 µL of water. For the analyses, the housekeeping transcript was *GAPDH-213* (GenBank: GU214026.1).

Table 2. The forward and reverse primers used for qPCR analysis of the five genes of interest in this study and the housekeeping transcript (*GAPDH-213*), along with the amplicon lengths of the products.

Transcript	Forward Primer (5' to 3')	Reverse Primer (5'-3')	Amplicon (bp)
Pak1-206	CTGTATGGATGAAGGCCAGATAG	CTCTTGATGTCCCTGTGAATGA	99
Usf1-210	AACGGAGGGCTCAACATAAC	CATAGAGCAGTCTGGGATGATTT	98
Tardbp-204	TGGTCACTCGAAAGGGTTTG	CACACCATCGCCCATCTATC	98
Mpp3-204	CCAGACGAGATCAGCCAAAT	AGAGGGCACGCATGAATAC	118
Zc3h15-204	AGGTGGTGATGAGGTTGATG	CCACAGTGATACCTGTCTCTTC	95
GAPDH-213	AACAGCAACTCCCACTCTTC	CCTGTTGCTGTAGCCGTATT	111

3.5 Graphing Software

The qPCR data and heatmaps were generated using OriginPro 2022 (OriginLab Corporation), with the heatmaps using the "Heat Map with Dendrogram v2.0" app available through the OriginLab File Exchange. The clustering of columns and/or rows in the heatmaps used the group average clustering method with Euclidean distances. Plasmid maps were generated using the SeqBuilder Pro program in Lasergene 15.2 (DNASTAR).

4. Results and Discussion

4.1 Transcriptome Analysis

The transcriptome data collected previously (Alqam, 2020) was used to mine for key genes that showed high expression under both asiatic acid (AA) and madecassic acid (MA) treatments. To accomplish this, a heatmap of the 4861 mouse transcripts that showed significant differences between the treatments (ANOVA F-test) was generated (Figure 3). Despite the similarities of the highest expressing genes examined previously (Alqam, 2020), examining all of the significantly different transcripts clearly shows the three AA samples clustering together with overall patterns very different from the MA or ethanol (vehicle) treatments. Additionally, although the MA and ethanol treatments clustered by their replicates, their patterns were remarkably similar to each other. This analysis suggests that the AA treatment led to a more diverse suite of changes than the MA treatment.

Further analysis of the significantly different transcripts between the AA, MA, and ethanol treatments by Alqam (2020) showed 37 transcripts total between AA and MA treatments that were at least 10X higher than the ethanol control (Figure 4). Of these transcripts, 23 were common between the two phytochemical treatments. A heatmap of these 23 transcripts allowed for resolution of the expression patterns between the treatments (Figure 5). For the cloning aspect of the current research, 10 of the 23 transcripts were identified as potential targets due to (1) completeness of the reference transcript sequence, and (2) reasonable length for amplification (*i.e.* 200-1500 bp). Of the 10 transcripts, 5 were able to be amplified from cDNA by routine PCR and were therefore moved into the cloning pipeline. These transcripts were *Pak1-206*, *Tardbp-204*,

Usf1-210, *Mpp3-204*, and *Zc3h15-204*. A heatmap of these five transcripts was created to show expression differences between the treatments and replicates (Figure 6).

4.2 qPCR Analysis

Before the five transcripts (*Pak1-206*, *Tardbp-204*, *Usf1-210*, *Mpp3-204*, and *Zc3h15-204*) were processed further, qPCR was used to verify that these transcripts were expressed at a higher level with the AA and MA treatments compared to the ethanol vehicle. The five transcripts showed at least 2X greater levels compared to ethanol alone, with *Mpp3-204* under AA and MA treatments showing the highest levels of expression at approximately 7X those of ethanol (Figure 7). The qPCR expression level changes of these transcripts from the phytochemical treatments were much lower compared to the transcriptome data, yet were high enough to warrant further investigation. Discrepancies between qPCR and transcriptome data are not uncommon due to the gene-specific nature of qPCR compared to the discovery approach of transcriptome sequencing that is more likely to slightly misrepresent expression levels of particular genes or transcripts (Fassbinder-Orth, 2014).

4.3 Biochemical and Physiological Functions

The five genes of interest in this study code for proteins involved in a variety of cellular functions. For example, Pak1 is a p21 (RAC1) activated kinase has serine/threonine kinase activity. This protein is involved in nervous system development, neurotransmitter secretion, and observational learning (NCBI Gene Database, 2022a). The Pak family has been linked to neurite outgrowth, spine morphology, synaptic plasticity, and learning and memory (Pan et al., 2015). The Pak3 protein has been associated with mental retardation, possibly through involvement in neuronal networks (Boda et al., 2004). Tardbp-204 is a

TAR DNA binding protein is involved in RNA polymerase II binding activity and pre-mRNA intron binding activity, with expression in the central nervous system among other areas within a mouse. The human ortholog have been linked to Parkinson's disease, amyotrophic lateral sclerosis, and motor neuron disease (NCBI Gene Database, 2022b). For example, the human protein TARDBP-43 was linked to human subjects with familial Alzheimer disease and Down syndrome (Lippa et al., 2009).

Usf1 is an upstream transcription factor 1 protein that is involved in a variety of processes, including cell cycle regulation, immune response, and ultraviolet radiation response (NCBI Gene Database, 2022c). Despite the general nature of this transcription factor, polymorphisms of Usf1 were linked with Alzheimer's disease-associated lesions, with the authors speculating that Usf1 may be regulating key genes involved in lipid metabolism (Isotalo et al., 2012). Mpp3 is short for membrane protein palmitoylated 3 (MAGUK p55 subfamily member 3), and is thought to be involved with PDZ domain binding activity (NCBI Gene Database, 2022d). PDZ domains in proteins have been associated with signal transduction (Lee and Zheng, 2010), although a direct connection between Mpp3, PDZ, and neuron growth and development was not found in the scientific literature. The zinc finger CCCH-type containing 15 protein (Zc3h15) is also associated with signaling, such as the cytokine-mediated signaling pathway (NCBI Gene Database, 2022e). Recently, it was reported that overexpression of Zc3h15 in human glioblastoma cells was linked with poor survival of patients with glioblastoma (Hou et al., 2022). That study demonstrated that Zc3h15 expression led to an increase in EFGR protein, which is known to be highly expressed in glioblastoma cells.

4.4 Cloning into Expression Plasmids

The popular Gateway system of cloning was used to insert the transcripts of interest into the pDONR-221 donor plasmid and subsequently into pcDNA-DEST40 using BP and LR Clonase reactions, respectively (Figures 8-12). The pcDNA-DEST40 plasmid is designed specifically for high-level expression of genes of interest in mammalian cell lines using the cytomegalovirus (CMV) promoter. Other features of this plasmid include a gene coding for neomycin resistance in mammalian cell lines, as well as a V5-6x His tag that can be added to the C-terminals of genes of interest. In the present study, the stop codons of the five transcripts of interest were changed to codons specific for glycine or valine in order to allow the expressed proteins to include the V5-6x His tag. The V5 region of the protein can be detected with readily available antibodies, while the 6x His tag allows for facile purification of the expressed protein using nickel-based chromatography.

5. Conclusion

The present experiment further demonstrated that asiatic acid (AA) and madecassic acid (MA) treatments to Neuro-2a mouse cells in culture has a profound effect on the transcriptome, with AA causing much greater holistic expression shifts than MA. Nevertheless, AA and MA had a core set of 23 transcripts that were highly expressed (10X higher than ethanol vehicle treatment), suggesting that these transcripts play important roles in the increase of neurite extension and combined length observed previously with AA and MA treatments (Nathan and Tucker, 2019; Alqam, 2020). Five of the 23 core set of transcripts were amplified and cloned into a mammalian expression plasmid (pcDNA-DEST40) for future transfection experiments with cultured mouse

neuron cells. It is hypothesized that overexpression of one of these transcripts or a combination of transcripts will be sufficient to achieve significant changes to neuron extension and length that was observed with direct AA and MA treatment.

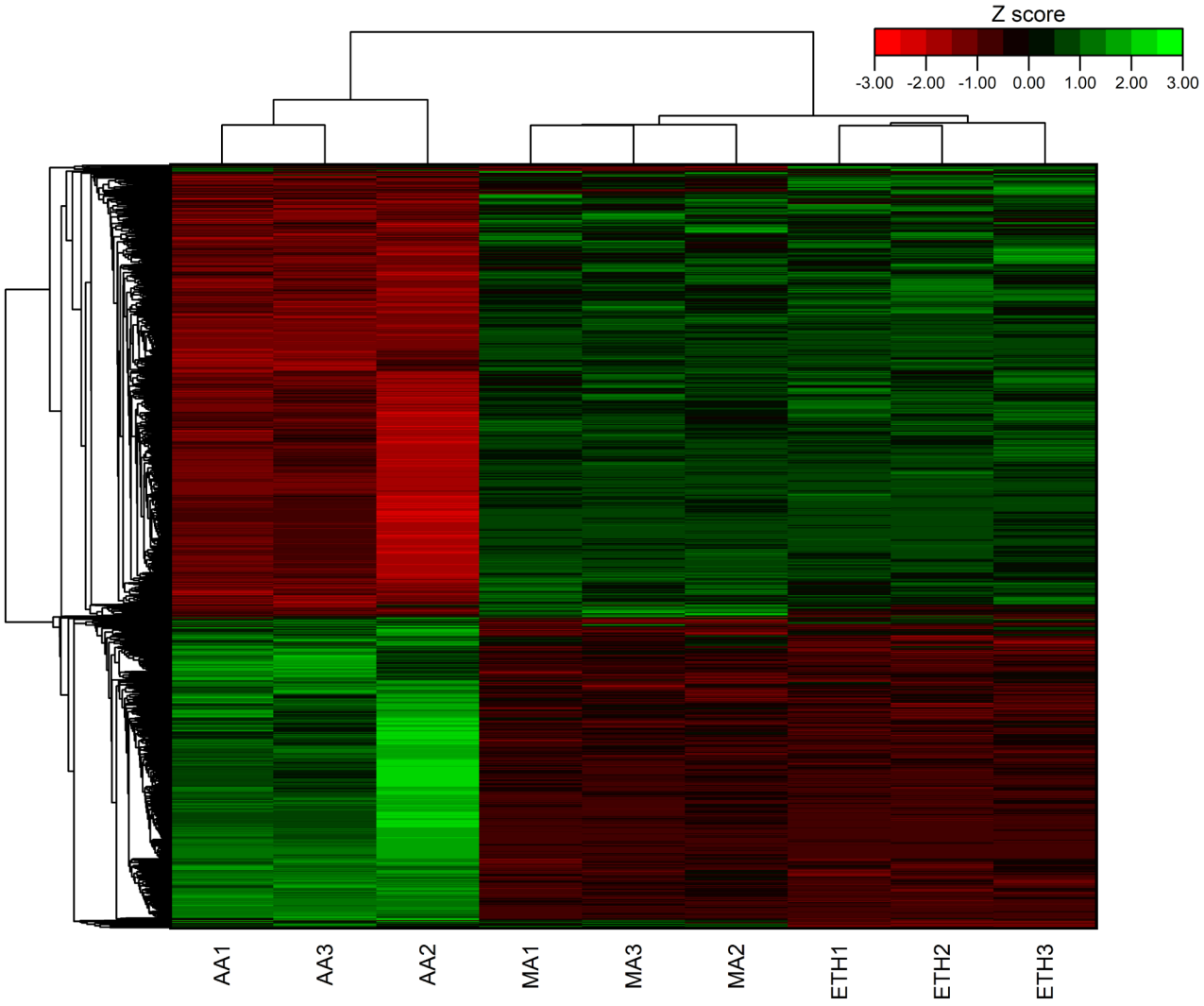

Figure 3. Heatmap of the 4861 mouse transcripts were significantly different (ANOVA F-test) with an average minimum of 1 RKPM for each sample. AA=asiatic acid treatment, MA=madecassic acid treatment, ETH=ethanol vehicle.

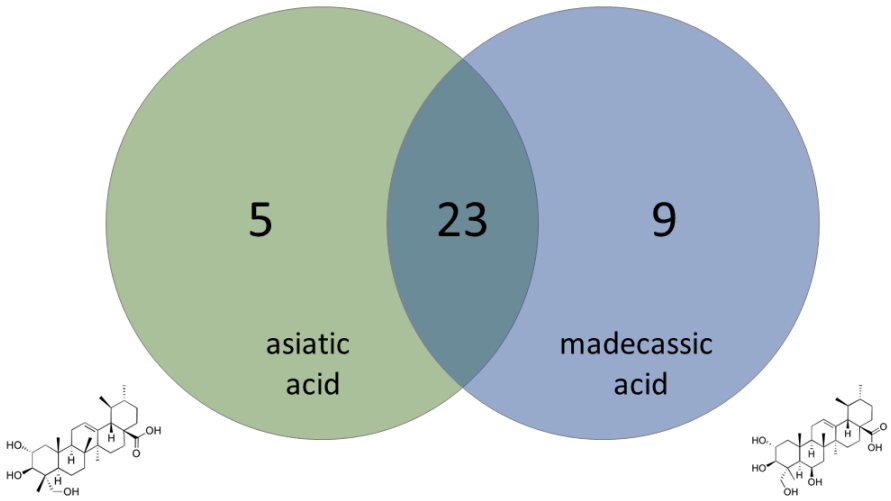

Figure 4. Venn diagram of transcripts with at least 10-fold higher average RPKM values in both asiatic acid (AA) and madecassic acid (MA) treatments compared to the ethanol vehicle (ETH). Transcripts were significantly different using an ANOVA F-test ($P \leq 0.05$). Image from Alqam (2020).

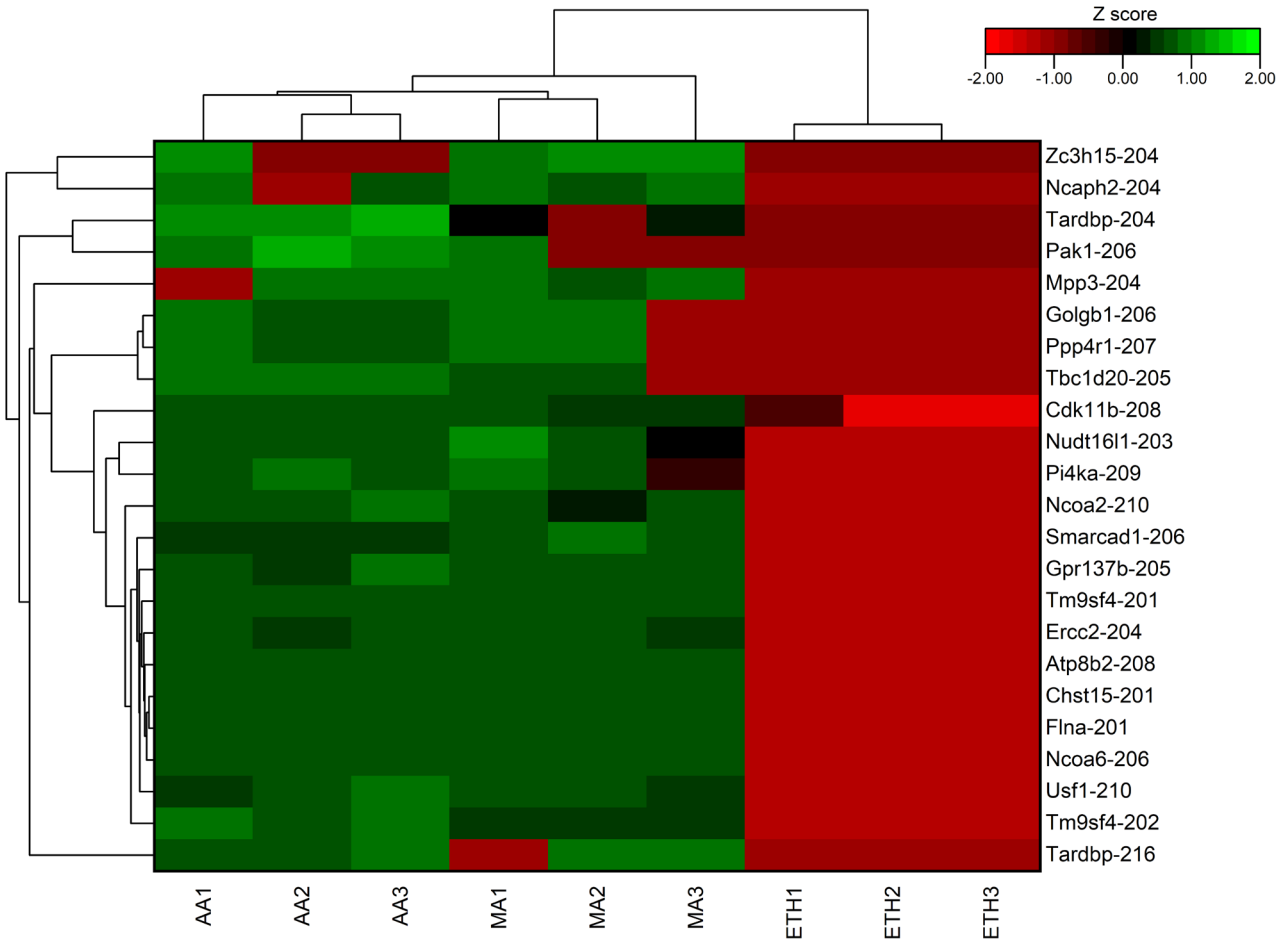

Figure 5. Heatmap of the 23 mouse transcripts that had at least 10-fold higher average RPKM values in both asiatic acid (AA) and madecassic acid (MA) treatments compared to the ethanol vehicle (ETH). Transcripts were significantly different using an ANOVA F-test ($P \leq 0.05$).

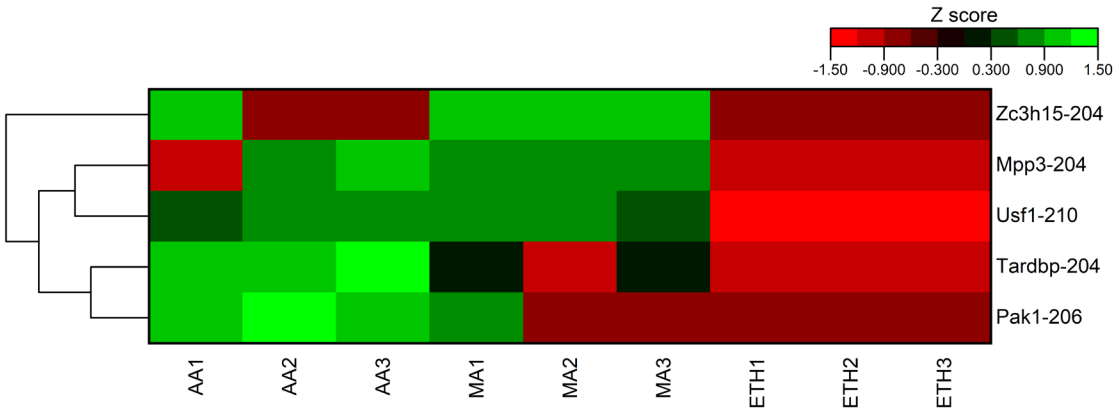

Figure 6. Heatmap of the 5 mouse transcripts used in the cloning experiments. AA=asiatic acid treatment, MA=madecassic acid treatment, ETH=ethanol vehicle. Transcripts were significantly different using an ANOVA F-test ($P \leq 0.05$).

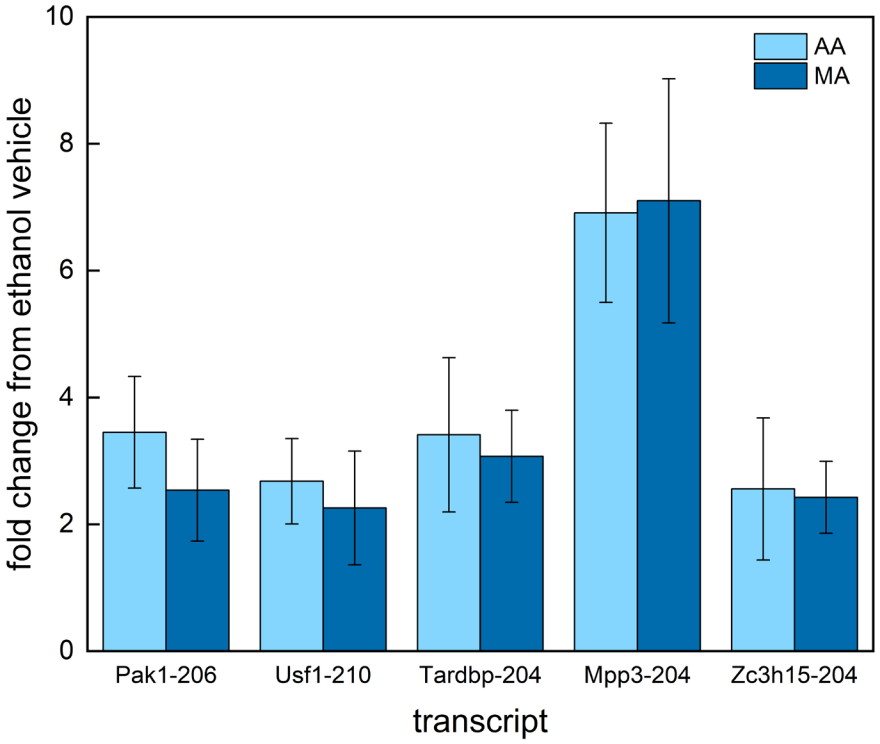

Figure 7. qPCR data for the 5 transcripts of interest that were expressed in mouse neurons exposed to asiatic acid (AA) or madecassic acid (MA). Values are mean with standard deviation of the fold change compared to ethanol vehicle.

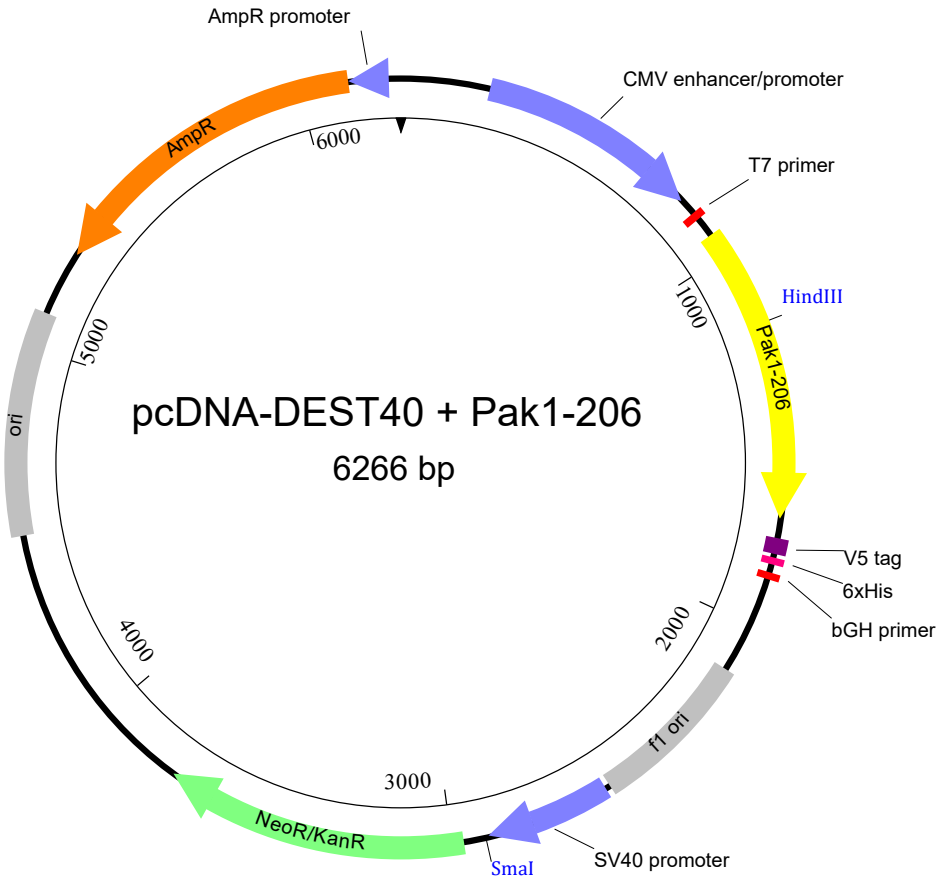

Figure 8. The mouse transcript Pak1-206 in the pcDNA-DEST40 plasmid. The transcript was cloned using Gateway technology from pDONR-221.

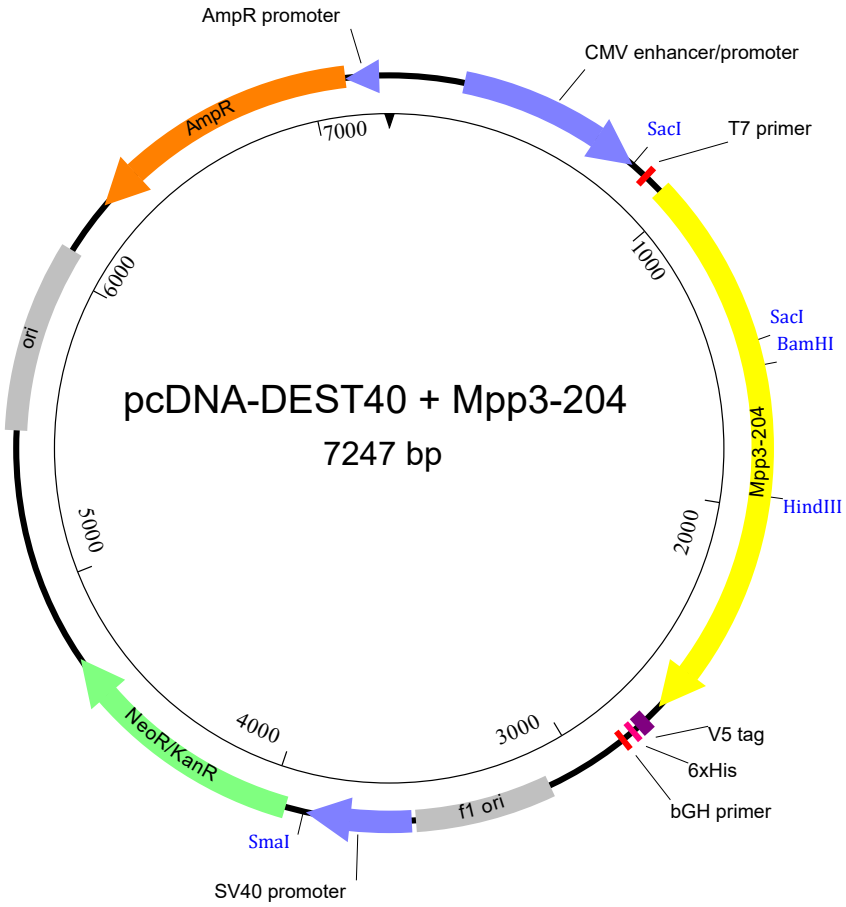

Figure 9. The mouse transcript Mpp3-204 in the pcDNA-DEST40 plasmid. The transcript was cloned using Gateway technology from pDONR-221.

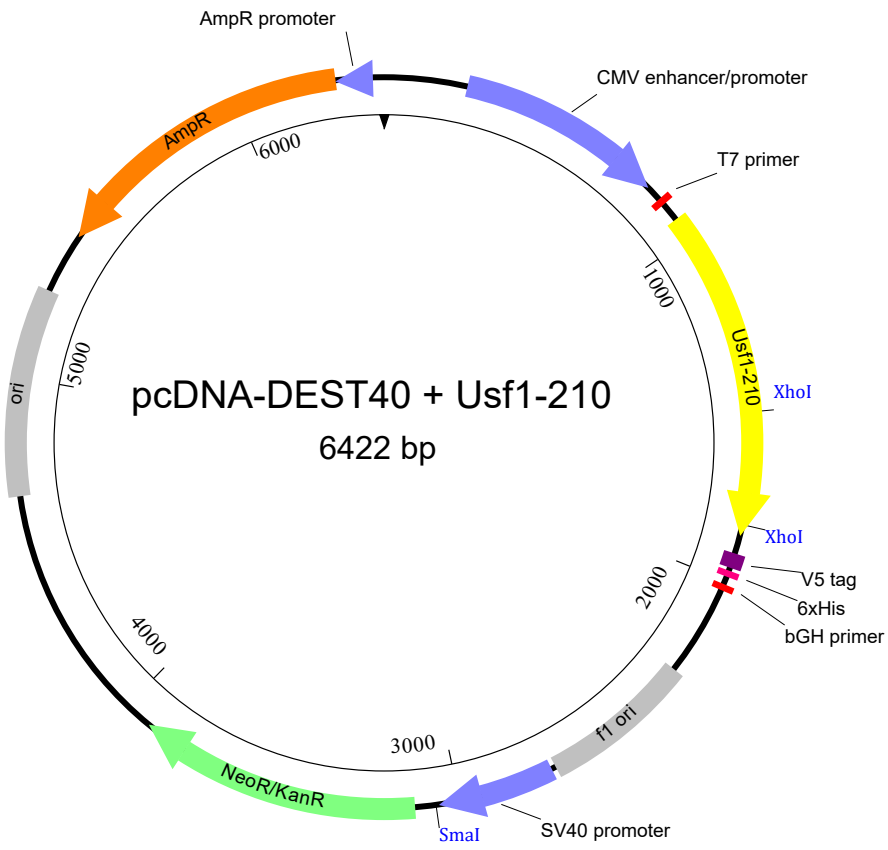

Figure 10. The mouse transcript Usf1-210 in the pcDNA-DEST40 plasmid. The transcript was cloned using Gateway technology from pDONR-221.

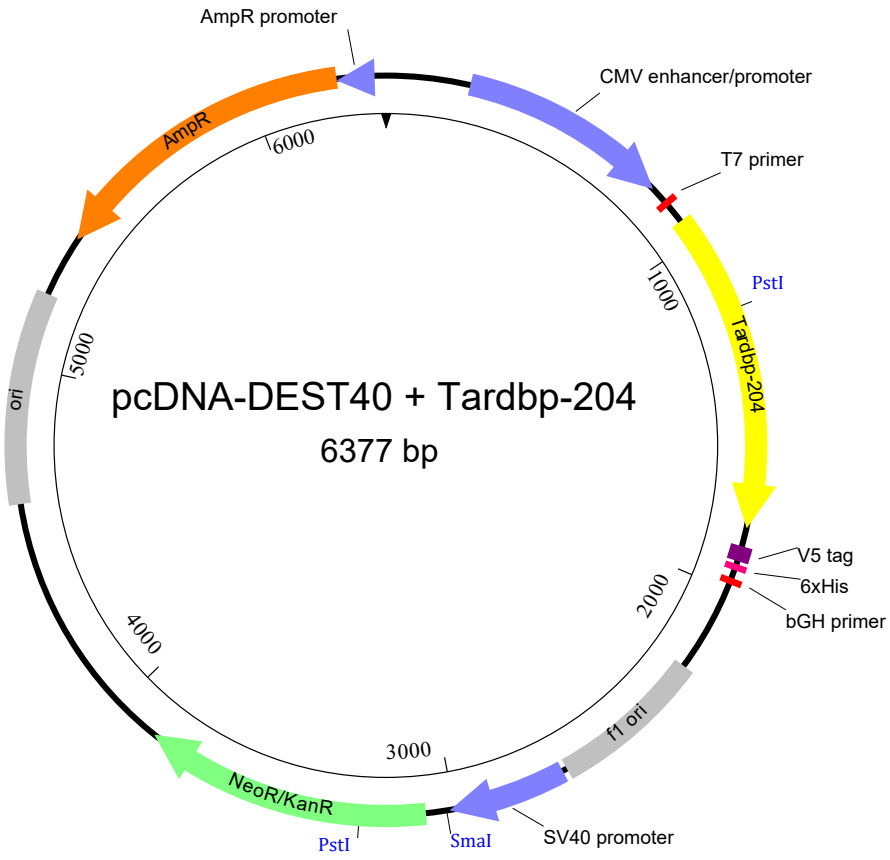

Figure 11. The mouse transcript Tardbp-204 in the pcDNA-DEST40 plasmid. The transcript was cloned using Gateway technology from pDONR-221.

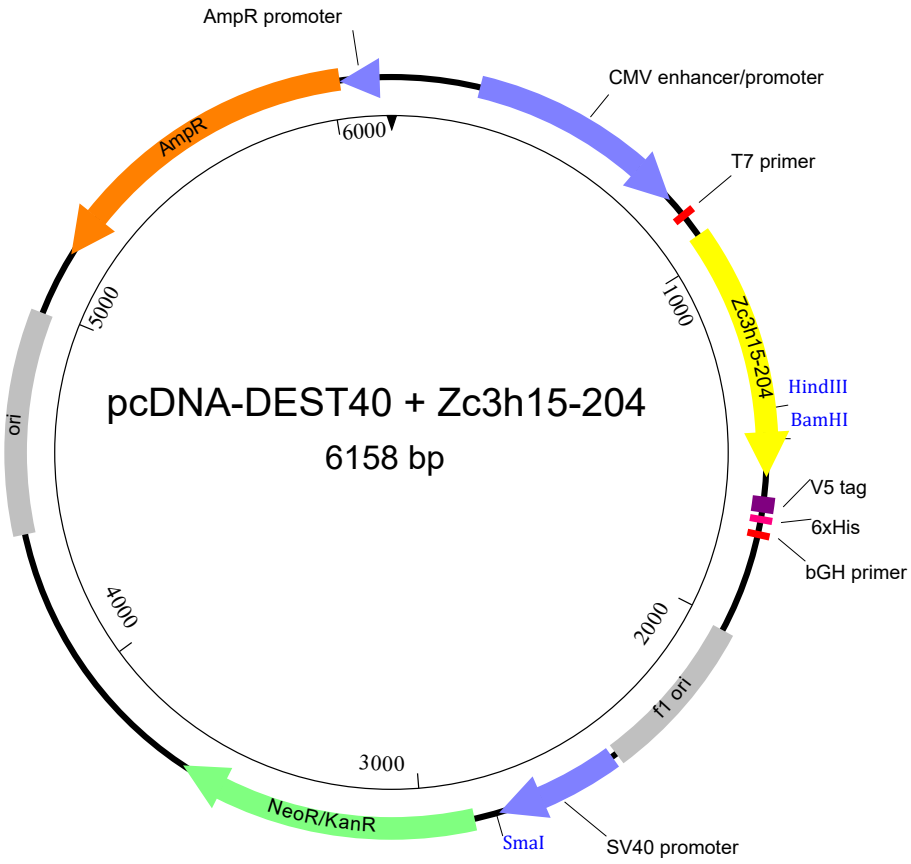

Figure 12. The mouse transcript Zc3h15-204 in the pcDNA-DEST40 plasmid. The transcript was cloned using Gateway technology from pDONR-221.

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