The CaMV 35S promoter is the most widely used promoter in plant biotechnology - despite being derived from a pathogenic virus. How and why did that happen? Here's…

A Short History of the CaMV 35S Promoter

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The Mosaic Disease (1921 – 1937)

The effects of the Cauliflower mosaic virus (CaMV) were first noted in **1921** in Chinese cabbage, where it caused mosaic-like necrotic lesions on leaf surfaces[1]. In the following years, the disease was regularly observed on Chinese cabbage, turnip or pot-herb mustard[2]. However, it was only in the **1930s**, that scientists invested more time and resources to investigate the disease[2]. This was spawned by severe yield losses on cabbage fields in the American Midwest, which strikingly were preceded by a heavy infestation of the field with aphids[2]. During this time, similar mosaic-like lesions were found on cauliflower in California, prompting investigations if these were caused by the same disease[3]. In a **1937** study, using infected cauliflower plants collected in California, C. M. Tompkins found that he could transmit the disease from the infected cauliflower plants to 51 different vegetable varieties, all belonging to the crucifer family (Cruciferae/Brassicaceae)[3]. This family includes, e.g., cauliflower, broccoli, cabbage, kale, turnip, kohlrabi or Chinese cabbage[3]. In the same study, he found that at least three different aphids can function as insect vectors, all three common inhabitants of cauliflower crop fields, thereby indicating that it is indeed a virus that causes the disease[3]. Although originally referred to simply as the 'mosaic disease', the virus was named Cauliflower mosaic virus, due to its described isolation from cauliflower[3].

The Cauliflower Mosaic Virus (1937 – 1978)

In the late **1940s**, research on the CaMV intensified once more, this time primarily in Europe, where it caused devastating losses in cauliflower and broccoli harvests across Great Britain[4]. As this was just after the end of World War II the impact was especially dramatic, as food was

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already a scarcity[4]. One of the first important findings in the following years was that CaMV is a non-circulative (and non-persistent) virus, meaning that it does not enter its aphid vector, but just 'sticks' to the insects stylet, and is thereby transported from an infected plant to a healthy one[5]. Interestingly, in 2007, researchers were able to pinpoint the exact position, at which the virus is perceived by the insect, an area roughly 5 µm long and less than 1 µm wide at the most distal tip of the aphid's stylet[6]. Through another important finding in the **1960s**, the CaMV was identified as the first plant virus containing double-stranded DNA[7]. This is of particular importance, because this feature is a pre-requisite for the viral DNA to be transcribed in plant cells[7]. Furthermore, this was the first indication that CaMV is a pararetrovirus (in contrast to the more commonly known single-strand RNA-containing retroviruses), even though this was only determined much later[8]. In **1980**, the whole genome (8024 double-stranded, circular base pairs (bp)) of the virus was annotated and found to contain six putative open reading frames[9,10]. At this point, scientists started to focus on deciphering the molecular details of plant infection by the virus. In the early 1980s it was discovered that the six coding regions are transcribed as only two mRNAs, the short, monocistronic 19S RNA, and the whole-genome covering 35S mRNA[11]. While the 19S RNA encodes a single protein, which was later found to be involved in gene silencing suppression in the host cell, the long 35S RNA serves as a template for whole genome replication, and is furthermore spliced into four individual mRNAs[12–14]. The 35S RNA also has two very curious features; (I) although serving also as a template for the genome, it is actually longer than the genome, as the 5' and 3' ends overlap by 200 nucleotides (nt); and (II) it has an unusually long 600 nt leader sequence[15]. This 600 nt leader was later found to be transcribed into '*massive amounts*' of 21 to 24 nt sense and antisense RNAs, which could function as 'decoys' during infection, to divert the host cell's silencing machinery from the actual coding 35S mRNAs[16]. However, the most important finding during that period was that the *19S* and *35S* reading frames were found to be highly expressed in infected plant cells, implying that the virus must have inserted its own double-stranded DNA into the plant cell, and that this inserted piece of viral DNA must contain all elements necessary to initiate transcription at high levels in host cells[11,15].

The Cauliflower Mosaic Virus meets Plant Biotechnology (1978 – 1985)

At this point it is important to note that in the late 1970s and early **1980s**, the field of plant molecular biology and genetics/genomics was still in its infancy[17]. *Arabidopsis thaliana* had just

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been accepted as a model system (see also 'A Short History of *Arabidopsis thaliana* (L.) Heynh. Columbia-0'[18])[17]. Direct transformation of a living plant with a transgene was not possible yet and very few individual genes had been cloned or studied at all. Furthermore, only a single promoter functional in plants had been fully described – the bacterial *octopine synthase* gene promoter, while a single promoter from plants, the pea *Ribulose-1,5-bisphosphate carboxylase* small subunit promoter, had been roughly located[17,19,20]. Accordingly, when it became clear that CaMV inserted its DNA into plant cells, and that this DNA was then expressed at high levels, plant biologists immediately recognized the potential use of CaMV as a cloning vector for plant transformation, and for expressing their genes of interest in the plant[11,21,22]. This resulted in two paths of research: First, researchers tried to insert a foreign gene into the genome of CaMV to determine whether this will get inserted and expressed in the host cell as well. Secondly, they attempted to identify the exact DNA sequences responsible for the strong expression of the CaMV genes in plant cells. Regarding the first path, researchers quickly progressed, and in the mid-eighties had successfully cloned bacterial and mammalian genes into the CaMV genome, and demonstrated that these genes were then transferred and expressed in plant cells[23–25]. However, they also realized that CaMV would only tolerate the insertion of short DNA fragments (~250 bp), and with the recent establishment of *Agrobacterium*-mediated plant transformation (see also 'A Short History of Plant Transformation'[26]), the research in CaMV-mediated plant transformation was subsequently abandoned in the early 1990s[27,28]. However, the second research path, the identification of the exact sequences that control gene expression in CaMV-infected cells, turned out to be a much bigger success.

The CaMV 35S Promoter (1985-2000)

Up until **1985** it was almost impossible to over- or misexpress a gene of interest *in planta* – nowadays an invaluable and indispensable tool to study the function of a specific gene. The identification of the CaMV 35S promoter would finally change this. In order to define the exact sequences controlling viral gene expression *in planta*, researchers first created several deletion variants of the roughly 1000 bp promoter region of the *35S* gene and fused these variants upstream of the *human growth hormone* (*hgh*) gene[29]. Notably, they used *Agrobacterium tumefaciens* to transform plant cells with their *35S::hgh* variants, not the CaMV itself[29]. They found that DNA sequences 46 bp upstream of the *35S* gene resulted in minimal expression, while a 343 bp fragment led to strong gene expression across all plant tissues tested[29]. The full 343 bp

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segment was therefore designated the 'CaMV 35S promoter', while the 46 bp segment was considered as the so-called 'minimal promoter'[29]. In follow-up studies, these 343 bp could then be further subdivided into several individual stretches, which would promote expression in different cell types or tissues, in either an additive or combinatorial fashion[30,31]. Based on these groundbreaking findings, numerous versions of the promoter emerged over the course of the following years; for example, simply placing two CaMV35S promoters in a tandem led to enhanced strength of the expression system[32]. Furthermore, the 46 bp minimal promoter also proved to be a highly useful tool: In the following years, short regulatory sequences within different gene promoters were identified that were bound by specific transcriptional activators[33–36]. By combining these activating elements with the minimal 35S promoter, scientists generated promoters that could promote gene expression in combination with the right activator[33–36]. This could be a plant hormone, such as auxin, therefore activating gene expression in a pattern reflecting the endogenous auxin concentration, or in response to external addition of the hormone[33]. Moreover, effectors could also be animal hormones such as estrogen and glucocorticoid, or ethanol, all of which are normally not present in the plant, therefore giving researchers complete control over when and where expression could be induced[35–37]. Finally, by combining the minimal 35S promoter with transcriptional binding sites for pathogen-responsive activators, researchers could engineer constructs that would confer enhanced resistance in the event of a pathogen attack[34].

This last point was not the main finding that made the CaMV 35S promoter so appealing to crop scientists, however. In **1986**, merely a year after the CaMV 35S promoter was correctly described, it was used to promote expression of the *5-enolpyruvylshikimate-3-phosphate synthase* (*EPSP*) gene in transgenic petunia[38]. The EPSP is an essential enzyme in the aromatic amino acid biosynthetic pathway[39]. And this enzyme is also the specific target for the herbicide glyphosate[40]. Accordingly, plants that overexpress the *EPSP* gene from the 35S promoter acquire an increased tolerance towards glyphosate treatment[38]. This successful engineering of the first transgenic herbicide-tolerant plants combined two major scientific breakthroughs of the early 1980s – the establishment of *Agrobacterium*-mediated plant transformation and the identification of the CaMV 35S promoter – and together these three milestones, all published within three years, meant a giant leap forward for both the plant science community and the developing field of plant biotechnology[29,38,41]. Over the course of the following 20 to 30 years, the 35S promoter became

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the most frequently used promoter in plant biotechnology, and almost every genetically modified crop plant that made it into our fields carries a version of this promoter[42].

The CaMV 35S Promoter as Target for Anti-GE Activists (1990-today)

The creation of genetically engineered crop plants not only gave a boost to plant science, it also activated the anti-GE (Genetic Engineering) movement. And in the 1990s, the 35S promoter became one of their main targets[43]. Interestingly though, there was no biosafety-incident or something comparable, that spawned a reasonable fear of the 35S promoter – it was a combination of insufficient outreach and bad public relations work from the scientific community, and the mere origin of the 35S promoter from a pathogenic virus[42]. There were, however, two incidents that clearly contributed to tarnishing the reputation of the 35S promoter in the public eye: the Petunia field trial in Germany, in **1990**, and the Pusztai affair in Great Britain, in 1998[44,45].

In the 1980s, researchers at the Max-Planck Institute for Plant Breeding Research (MPIPZ) in Cologne, Germany, were working on transposable elements in maize (so called 'jumping genes' or 'transposons')[46,47]. They found that white flowers from petunia could be converted into salmon red flowers, by introducing a maize transgene under control of the CaMV 35S promoter[46]. They then used these red flowering plants in a field trial to identify transposons in petunia, arguing that if enough plants are sowed out, the rare 'jump' of a transposon into the introduced maize transgene would be readily identified[48]. This insertion event should render the maize transgene inactive, thereby turning the red flower back to white – a clear visible sign that a transposon had been 'trapped'[48]. The chance for this to happen was estimated to be around 0.0001 %, and so the expectation was that only a few individual flowers in the population of over 30 000 plants would revert back to their white color[48]. The result, however, was a reversion rate of almost 60 %[48]. The researchers later discovered that this was mostly due to epigenetic gene silencing, the auto-inactivation of gene expression - a protective mechanisms of the plant cell if expression appears to get out of control[48,49]. The plant cell had simply turned off the CaMV 35S promoter because it was too strong, which also demonstrates why the CaMV had evolved its 19S protein as a silencing suppressor - something only discovered many years later in 2007[12,48]. While this was an exciting but surprise finding for the plant science community ('epigenetic gene silencing' was not well studied or understood at that time), it also became a public relations problem for the

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MPIPZ[44]. The petunia experiment was the first field trial with transgenic plants in Germany, a country that is notoriously critical and reserved when it comes to genetic engineering[44,50,51]. The trial therefore was accompanied by protests from anti-GE activists claiming that the scientists did not understand genetic engineering well enough to undertake such an experiment outside of the controlled environment of a green house without considerable risks[51]. The scientists, on the other side, were both interested in the scientific outcome of the experiment, but also to demonstrate to the public that they were able to control such an experiment[44,51]. Needless to say, the results did not go over well with the public, and the protesters felt reassured of their claim[44]. In the following two decades, there were some further field trials of GE-plants in Germany, but as of 2015, 75 % of Germans are still opposed to growing genetically modified crops and since 2013 no further field trials with GE crops were approved (www.bvl.bund.de/EN/)[52]. Interestingly, more than 25 years after the petunia trial, in 2017, red-colored commercial petunias were recalled from stores worldwide when it was discovered that they were actually transgenic – they carried the same maize transgene that was used at the MPIPZ in 1987[53,54]. This episode was widely publicized in Germany at the time, and certainly contributed to a majority of Germans still remaining critical when it comes to genetic engineering[52]. However, even though the 35S promoter was indeed indirectly responsible for this public relations debacle for the German plant science community, at the time, the 35S promoter was not yet singled out as a threat to the environment and human health, and the activists focused on genetic engineering as a whole instead. This would change with the Pusztai affair in **1998**[55].

In the wake of increasing consumer concerns over the safety of genetically modified food, renowned protein scientist Árpád Pusztai, who was in the midst of conducting the first major study on possible health effects of transgenic crops, was interviewed on British TV about his ongoing experiments[56]. He stated that the trials included rats that were fed genetically modified potatoes and that they seemed to be less healthy than rats that were fed the unmodified counterpart[56]. He also acknowledged that he could not tell what caused these effects, and that he actually had concerns about the experimental design and the controls included[56]. Accordingly, he stated that more testing was needed until any firm conclusions could be reached[56]. However, he then went on to state that, if given the choice to eat transgenic crops now he '*wouldn't eat it*', adding that he thought it was '*very, very unfair to use our fellow citizens as guinea pigs*'[56,57]. Not surprisingly, this last sentence resulted in a major pushback against genetic engineering from the

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public, which was further exacerbated by how the Rowett Research Institute (Pusztais employer) subsequently handled the situation[56,58]. Overwhelmed by the massive backlash the institute received from the public and media, the Rowett Institute panicked and shut down Pusztai's lab, collected all lab books, suspended him indefinitely, and, worst of all, forbade him from talking to the press[56,57]. The director also released a statement, in which he described Pusztai's data as 'a total muddle', and apologized for releasing 'misleading information', before an investigation into the case had even begun[58]. This reaction drew massive criticism from both, fellow scientists and the public[57]. The scientific community, who valued Pusztai as a highly reputable colleague, and certainly an authority in the field, were shocked by his harsh treatment, and noted that *'it is an unacceptable code of practice by the Rowett and its Director, Professor James, to set themselves up as arbiters or judges of the validity of the data which could have such a profound importance not only for scientists, but also for the public and its health'*[57]. For members of the public, similarly, the actions taken by the institute seemed as if the Institute was trying to silence a dissident member, and cover up his findings[57,58].

So what was all of this about? In his experiments, Pusztai fed transgenic potatoes expressing a snowdrop lectin (*Galanthus nivalis agglutinin* (*GNA*)) to rats, to test for any effects this would have on their health[59]. Plant lectins are sugar-binding proteins involved in cell immunity by detecting specific sugar chains on the surfaces of proteins, but also on viruses or bacteria[60]. GNA was shown to be toxic to some insects, among them several major crop pests[61]. So in this case, binding of GNA to the insect pest would kill the insect[61]. At the same time, GNA was shown not to be toxic to mammals, including rats (as demonstrated by Pusztai himself in 1990)[62]. Based on these findings the idea was developed to create transgenic crops expressing *GNA*, to enhance their tolerance to insect pests[60]. One of the first such crop plants was a potato expressing the *GNA* from the CaMV 35S promoter[63]. Pusztai's feeding trial was now intended to check for any health risks for mammals coming from such transgenic crop plants. In order to do this, he fed the rats either transgenic potatoes, non-transgenic potatoes, or non-transgenic potatoes that were laced with GNA[59]. And what he found was that several of the rats that were fed the transgenic potatoes, were less healthy than the rats that were fed the unmodified counterpart, or the unmodified but GNA-laced potatoes[59]. However, the observed effects were also highly variable from potato to potato, and most effects could not be traced back to the expressed GNA, as GNA-supplementation, even at high concentrations, did not result in the same effects on the rats as

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GNA transgene expression[57,59,64]. Therefore, Pusztai speculated that most of the effects were not due to the expressed GNA protein, but to the transgene itself, the position where it was inserted, the transformation procedure, or general alterations in the composition of the potato caused by the procedure to obtain the genetically modified plant[59,64]. Eventually, further analysis of Pusztai's data by external experts and an investigation by a commission set up by the Royal British Society all found that the Pusztai experimental setup was indeed clearly flawed, and that for this reason, no conclusions could be drawn from his findings[57,64–66]. They showed, for example, that the nutritional value varied widely between the different transgenic potatoes, as well as to their parental line, which already makes it impossible to distinguish if any effects were caused by the transgene, or simply were due to this variation[64]. Furthermore, as this diet is not suitable for rats, the animals were all protein-starved, affecting their general physiology, again making it impossible to trace any effects back to the transgene[64]. Also, the controls were flawed. The transgenic potatoes were created by transformation of cultured cells and regeneration of plants from these cells[63]. However, the wild type control plants had not gone through such a procedure[57,64,65]. This is especially important, as plants derived from tissue culture exhibit somaclonal variation, which can account for a wide spectrum of effects, most of all the observed differences in nutritional value between the different potatos[57,64,65,67]. And there are many more problems with the study as detailed in the various reports[57,64–67]. Thus, it is clear that this study did not provide any evidence for or against any effects caused by the transgene or the expressed protein. However, one thing almost everybody agreed on in the end, is that the Pusztai affair is a prime example for how *not* to handle potentially troubling findings, and how essential it is for scientists to stay in contact with, and explain their work to, the general public.

Now how does this relate to the CaMV 35S promoter? Several anti-GE groups and activists immediately picked up this story and, for some reason, highlighted the CaMV 35S promoter as the potential culprit of the observed health effects[43,55]. Greenpeace released a statement saying, '*For all we know they might have been caused by the virus used to transfer the alien DNA to the potatoes. This is the same virus used in Monsanto's Roundup Ready soy that is available in markets around the world*', clearly ignorant to the fact that the CaMV 35S promoter is not a virus, but a short stretch of DNA, and the active ingredient in Roundup Ready Soy is also not a virus, but the EPSP synthase[55]. In this case, it was solely the origin of the CaMV 35S sequence from a virus that brought it to the attention of these groups. The word 'virus' certainly has a

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negative connotation in most people's mind, and thus seemed to be a good way to activate as many people against GE as possible. And other activists published work along similar lines[43]. So overall, the Pusztai study was simply a badly planned and poorly executed work, which most likely would have been significantly improved during a peer-review process, if some of the results had not been prematurely broadcast publicly on TV. But the poor handling by the people involved, in combination with a scientific community that failed to sufficiently inform and educate the public about genetic engineering and genetically modified organisms in general for over a decade, allowed this to escalate into an affair that shifted public perception of genetic engineering and genetically modified crops from healthy criticism to outright rejection. Unfortunately, the damage done could not be rectified to this day. Furthermore, due to the continuing lobbying of anti-GE activists, the reputation of the CaMV 35S promoter was also severely tarnished by this event, and it has since become one of the buzzwords of the GE-movement. This is probably best illustrated by a 2009 paper from famed anti-GE activist and pseudo-scientist Mae-Wan Ho, who claimed that eating transgenic crops carrying the CaMV 35S promoter could promote HIV in humans[68]. This claim is 'backed up' in the paper by a truly amazing line of argumentation: "*In humans, P-TEFb is required by HIV-1 for its transcription and replication. The long terminal repeat of HIV-1 has minimal promoter activity in the absence of the viral Tat protein. The CaMV 35S promoter, on the other hand, is strongly active in plant cells in the absence of any viral protein. Thus, the presence of CaMV 35S promoter effectively facilitates the transcription of HIV and other viruses*"[68].

The CaMV 35S Promoter Today (2000-today)

To this day the CaMV 35S promoter remains the most commonly used promoter in plant science. Nonetheless, use of the CaMV 35S promoter is slowly decreasing due to several reasons. The number one reason being that today, in contrast to the 1980s, many alternatives to the CaMV 35S promoter are available to researchers. In academia, the *Arabidopsis UBIQUITIN10* promoter was identified in the mid-1990s as a strong promoter, active in all tissues of the plant body – indeed, the two major selling points of the CaMV 35S promoter – and was ready to replace it as a plant-derived promoter to use in plants[69,70]. By that time researchers had also discovered that the 35S promoter was actually not active in all tissues and cell types, but sometimes exhibited a 'patchy' pattern, something not seen for the UBQ1 or 10 promoters[71]. Furthermore, by the year **2000**, the *Arabidopsis* genome had been sequenced and annotated, uncovering all genes and their putative

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regulatory sequences[72]. Such sequences could now be easily cloned and enabled scientists to express their genes of interest under control of their respective native promoters, or very targeted in specific cells and tissues, and at physiological concentration levels. Gene silencing, which caused the problem in the Petunia field trial, prompted scientists to employ the 35S promoter in conjunction with the p19 silencing suppressor, further complicating the applicability of this promoter[73]. Finally, reports emerged that the 35S promoter could affect the expression not only of the downstream transgene, but also other genes in its vicinity, possibly via its enhancer regions, which further confounded the use of the 35S promoter[74–76]. Thus, while the CaMV 35S promoter is still used heavily in scientific studies, many now favor the use of endogenous promoters and/or the *UBIQUITIN10* promoter.

In agriculture, over 80 % of GE-crops in the field still carry a version of the CaMV 35S promoter, among those the most widely farmed varieties such as the Roundup Ready soybean, *Bt* corn and cotton, and the 'Sunset' papaya resistant to the papaya ringspot virus[77–80]. These crops have been found to be safe by all the major scientific institutions, and have been consumed by humans and livestock for decades now, without any negative health effects[81–86]. Since the generation, subsequent field-trials, safety tests and governmental approvals of a transgenic crop line are arduous, time-consuming and expensive, a switch to a different standard promoter will be a long-term project. The use of the CaMV 35S promoter is, however, limited because of multiple overlapping patents on it[87]. Among other things, this has led to enhanced use of similar, or related promoters, such as the figwort mosaic virus 34S promoter (FMV 34S)[87,88]. It was also found that in monocots, such as rice and corn, the CaMV 35S is not as active as it is in dicots, leading researchers to switch to, e.g., the later discovered *rice actin 1* or *maize Ubi-1* promoters[89–92]. So even though the switch is happening at a slower pace in applied agriculture than it is in academia, the variety of promoters used is steadily increasing also in this area.

Nonetheless, since its description in 1985 the CaMV 35S promoter has been the standard promoter used in all plant science and plant biotechnology, and has certainly propelled the research field forward like hardly any other discovery.

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