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Analysis of reporter proteins *GUS* and *DsRed* driven under the control of CaMV35S promoter in syncytia induced by beet cyst nematode *Heterodera schachtii* in Arabidopsis roots

Muhammad Amjad Ali^{1,2*}, Amjad Abbas¹

Abstract

Background: Cyst nematodes induce specialized feeding structures called syncytia in the plant roots. The expression of CaMV promoter in syncytia has remained topic of debate. The objective of this research was to study the activity of CaMV promoter by using reporter proteins like *GUS* and *DsRed* under the control of CaMV35S promoter in syncytia induced by *H. schachtii* in Arabidopsis roots.

Methods: pMAA-Red and pPZP3425 plasmids were used to study expression of *GUS* and *DsRed* in syncytia. The plants were grown in 2% Knop medium under sterile conditions in growth chambers at 25°C in long day conditions. *GUS* activity in syncytia was studied through staining of syncytia using X-gluc solution. *Ds-Red* fluorescence in syncytia was detected by using an inverse microscope equipped with UV filter.

Results: The expression analysis of *DsRed* protein driven by CaMV promoter demonstrated that this promoter is active in syncytia at all the time points. All the syncytia showed *DsRed* expression at 5 dpi. At 7 dpi, 10 dpi and 15 dpi over 90%, 80% and 50% of the syncytia showed *DsRed* fluorescence respectively. There was very high fluorescence in the syncytia as compared to the uninfected root segments due to high expression. CaMV::*GUS* lines showed *GUS* expression in 80% of 5dpi syncytia. However, unlike expression of *DsRed*, the number of *GUS* stained syncytia decreased quickly to around 50% at 7 dpi and to about 5% in the 15 dpi syncytia.

Conclusions: The results conclude that CaMV promoter is more active in younger syncytia as compared to older syncytia but can be used for expression in syncytia. Moreover, *DsRed* protein could be used as better reporter for evaluation of gene expression in syncytia as compared to *GUS*.



Introduction

Plant parasitic nematodes have a detrimental effect on agricultural productivity either by a direct damage to crops or serving as vectors for plant invading viruses, thereby resulting in over \$150 billion crop losses worldwide annually [1]. The cyst and root-knot nematodes within the family *Heteroderidae* are the most important plant parasitic species and are obligate endoparasites of plant roots. Cyst nematodes with their destructive migration activity travel intracellularly through the root to reach the elongation zone, where they select a cell at the periphery of the vascular bundle to become the initial syncytial cell. This cell expands through partial dissolution of cell walls of up to 200 times of the surrounding cells into a feeding site known as a syncytium [2,3]. Due to incorporation of many surrounding cells the syncytium becomes multinucleate and substantial hypertrophy of the syncytial elements results in enlargement of the feeding site (Figure 1A). This syncytium becomes the only nutrient source for the nematodes as they develop through subsequent sedentary life stages [4,5]. The common features of a syncytium include high metabolic activity, disintegration of the central vacuole, reduced number of secondary vacuoles, and increased numbers of organelles and wall ingrowths into xylem vessels [6,7]. The females of cyst nematode are fertilized by vermiform adult males. The male nematodes induce comparatively smaller syncytia than females and are covered in a cuticular casing (Figure 1B). The males die after fertilization of several females while the female dies after the completion of egg development, leaving the eggs contained within its enlarged body [8] (Figure 1A). The female mostly detaches from the root surface and hardens to form a protective cyst covering hundreds of eggs inside. The cyst with eggs can remain viable for many years in the soil before infective J2s hatch in favorable conditions to start a new life cycle [9].

The cauliflower mosaic virus 35S (CaMV35S) promoter has been extensively used for the constitutive expression of transgenes in many plant species [10,11]. A combination of CaMV35S promoter with omega element from Tobacco Mosaic Virus (TMV) used as translational enhancer resulted in enhanced expression of the gene of interest delivered with this promoter [12]. Holtorf *et al.* [13] compared different constitutive promoters (CaMV35S, CaMV35S+omega, *UBQ1* and

BTH6) and one inducible promoter (soybean heat-shock promoter *Gmhsp17.3*) and found that CaMV35S+omega is a very strong promoter for constitutive expression in all organs of *Arabidopsis thaliana*.

The CaMV35S promoter has been used to express a variety of reporter proteins such as *GUS* [14], *Luc* [15], *GFP* [16] or *DsRed* [17]. The *DsRed* protein was initially isolated from a sea anemone, *Discosoma striata* and is responsible for the red colour around the oral disk of a coral of the genus *Discosoma* [17]. The *DsRed* protein has a molecular weight of 28 kDa and a maximum emission wavelength of 583 nm and it emits a bright red fluorescent light [18]. Jach *et al.* [19] first used *DsRed* delivered by the CaMV35S promoter as a reporter for plant gene expression in tobacco and concluded that it is well suited, easy-to detect reporter protein for plant molecular biology instead of *GUS*, *Luc* or *GFP*. Very recently, we have used *DsRed* successfully as a fluorescent selectable marker for the selection of transgenic lines [20].

The objective of this study was to trace out the expression of CaMV35S promoter which contains two time 35S (CaMV2x35S) region plus omega element in the end of the CaMV35S sequence (Figure 2) by using *DsRed* and *GUS*. This study was carried out because of the debate about the activity or expression of CaMV35S promoter in the cyst nematode induced syncytia. For example, Urwin *et al.* [21] reported that CaMV35S is active in giant cells induced by root-knot nematode (*M. incognita*) but it was highly downregulated in syncytia induced by cyst nematodes (*H. schachtii*). Liu *et al.* [22] showed that a chemoreception-disruptive peptide delivered by CaMV35S considerably inhibits acetylcholinesterase in *Globodera pallida* induced syncytia in potato. Most of the reports have shown that this promoter is downregulated in cyst nematode induced syncytia [23,24] while Urwin *et al.* [25] demonstrated that CaMV35S is active in syncytia up to 10 dpi (days post inoculation). However, Goverse *et al.* [26] used green fluorescent protein (*GFP*) to monitor the activity of two constitutive promoters, CaMV35S and *TR2'*, in developing syncytia induced by *Globodera rostochiensis* in potato roots and found that CaMV35S is upregulated in syncytia until 13dpi. Similarly, CaMV35S has been used to suppress nematode development by overexpressing nematode repellent as well as nematode specific genes involved in nematode

establishment on the plants [27,28]. But there is still a debate about the activity of CaMV35S promoter in the cyst nematode induced syncytia. We have constructed CaMV35S promoter::reporter fusion lines to find out the expression of this promoter using two reporter proteins *GUS* and *DsRed*.

Methods

Construction of plasmid vectors CaMV2x35S + omega::GUS/DsRed fusions

For testing the expression of CaMV promoter in syncytia, promoter::reporter fusion lines were developed using two reporters, GUS (β -glucuronidase) [14] and DsRed [17]. For the promoter::GUS construct we used the vector pMAA-Red which had a CaMV2x35S+omega::GUS cassette. From the pMAA-Red construct, first three lines with maximum expression of GUS from the previous study were used for GUS staining in syncytia at different time points [14]. For the construction of CaMV::DsRed, pPZP3425 [29] was used by replacing GUS with the DsRed sequence as given by Ali *et al.* [20].

Plant transformation and selection of transgenic lines

The plasmid constructs containing CaMV::GUS (pMAA-Red) and CaMV::DsRed (pPZP3425) fusions were introduced into *A. tumefaciens* GV3101 for transformation of *Arabidopsis* plants by the floral dip method [30]. In case of pMAA-Red, the fluorescent transformed seeds were selected under an inverse microscope (Axiovert 200M; Zeiss, Hallerbergmoos, Germany) equipped with a DsRed fluorescence filter and put on soil to grow the next generation. Homozygous lines were selected based on visual observation as described by Ali *et al.* [20].

In case of pPZP3425 containing CaMV::DsRed cassette, the transgenic lines were selected on MS medium containing 3% sucrose (Murashige and Skoog, 1962) supplemented with 50 mM kanamycin as described by Szakasits *et al.* [29]. In this case three transgenic lines were selected for further studies.

Plant cultivation

Arabidopsis thaliana (L.) ecotype Columbia plants were grown on soil in growth chambers at 25°C in long day conditions (16 h light / 8 hour dark). For growth in

sterile conditions seeds were surface sterilized for 7 min in 10% (w/v) sodium hypochlorite and subsequently washed three times with sterile water. Seeds were placed in Petri dishes (9 cm) on a modified Knop medium with 2% sucrose [31] and grown for 12 days for nematode infection.

Nematode infection

The cysts of the sugar beet cyst nematode *Heterodera schachtii* were harvested from *in vitro* stock cultures propagated on mustard (*Sinapis alba* cv. Albatros) roots growing on 0.2 concentrated Knop medium supplemented with 2% sucrose [31]. The cysts were soaked in 3 mM ZnCl₂ as stimulus for hatching of J2 larvae under sterile conditions. The J2 larvae were then washed three times in sterile water and resuspended in 0.5% (w/v) Gelrite (Duchefa, Haarlem, The Netherlands) before inoculation. Roots of twelve-day-old *Arabidopsis* seedlings were inoculated under sterile conditions with about 50-60 juveniles per plant.

Histochemical detection of GUS activity

Histochemical detection of GUS activity was performed by staining [14] using X-gluc (Biomol, Hamburg, Germany) in 0.1 M sodium phosphate buffer pH 7.0 containing 0.1% Triton-X 100, 0.5 mM K₃[Fe(CN)₆], 0.5 mM K₄[Fe(CN)₆] and 10 mM Na₂EDTA. For GUS staining of syncytia, the infected roots of CaMV::GUS plants were incubated with X-gluc for 6 hours at 37°C. Staining was performed at 5, 7, 10, and 15 dpi (days post inoculation). Stained syncytia and uninfected roots were counted per line from three promoter::GUS lines and photographed under an inverse microscope (Axiovert 200M; Zeiss, Hallerbergmoos, Germany) having an integrated camera (AxioCam MRC5; Zeiss). The GUS expression in the root cap border cells was also photographed in the same way.

Detection of DsRed fluorescence in syncytia

Three lines of the CaMV::DsRed construct were grown on Knop medium and infected with nematodes as mentioned before. The number of syncytia having DsRed expression were counted and imaged at 5, 7, 10 and 15 dpi using an inverse microscope (Axiovert 200M; Zeiss, Hallerbergmoos, Germany) having a DsRed filter and an integrated camera (AxioCam MRC5; Zeiss). The DsRed expression in the root cap border cells was also photographed in the same way.

Results

Expression of reporter genes driven by the CaMV2x35S + omega promoter in the syncytia

The CaMV promoter that was used here contained the omega element as described in [13] and a doubled enhancer [32]. The sequence of CaMV promoter with two copies of 35S region along with omega element is shown in Figure 2. In order to test the activity of CaMV promoter in syncytia, we constructed promoter fusion lines with beta-glucuronidase (*GUS*) and *DsRed* fluorescent protein. For construction of CaMV.2x35S + omega::*DsRed* we used pZP3425 [29] in which *GUS* was replaced by *DsRed*. The transgenic lines used for the study were having the highest fluorescence in the seeds. For CaMV.2x35S+omega::*GUS* we used the pMAA-Red vector itself [20].

We determined the expression of *DsRed* and *GUS* driven by the CaMV promoter at different time points after infection. The lines, CaMV.2x35S + omega::*DsRed* L1, L2 and L3 contained 80, 101 and 82 syncytia in total, respectively and all of them showed *DsRed* fluorescence at 5 dpi (Table 1). The percentage decrease of total number of syncytia in fluorescent syncytia at different time points is given in Figure 3A. At 7 dpi, over 90% of the total syncytia were found fluorescent. Similarly, at 10 dpi and 15 dpi on an average from three lines over 80% and 50% of the syncytia showed *DsRed* fluorescence respectively (Figure 3A). Representative syncytia with *DsRed* fluorescence were also photographed at different time points (5, 7, 10 and 15 dpi) and are shown in Figure 4A. It is quite obvious from the figure that there was very high fluorescence in the syncytia as compared to the uninfected root segments. However, at 15 dpi, the expression level of *DsRed* also slightly went down showing decreased activity of the promoter with the passage of time.

For the CaMV.2x35S + omega::*GUS* construct we used the first 3 transgenic lines harboring the pMAA-Red vector having the highest expression in Ali *et al.* [20]. The pMAA-Red seedlings were grown on MS medium for 14 days and stained with X-gluc solution (see Materials and Methods) at 37°C for 6 hours. The seedlings had very high staining, showing that the CaMV.2x35S + omega promoter was highly and constitutively active in the seedlings under the control of this promoter (data not shown). After nematode infection, the transgenic lines CaMV.2x35S +

omega::*GUS* L1, L2 and L3 contained 58, 73 and 95 syncytia in total, respectively and over 80% of them showed *GUS* staining in syncytia after five days of infection (Table 1 and Figure 3B).

Different lines	Total #	5d		7d		10d		15d	
		+	-	+	-	+	-	+	-
+/- expression of reporter									
Promoter:: <i>DsRed</i> L1	80	80	0	75	5	71	9	43	37
Promoter:: <i>DsRed</i> L2	101	99	2	96	5	80	21	45	56
Promoter:: <i>DsRed</i> L3	82	78	4	73	9	67	15	53	29
Promoter:: <i>GUS</i> L1	58	50	8	37	21	19	39	3	55
Promoter:: <i>GUS</i> L2	73	62	11	31	42	22	51	2	71
Promoter:: <i>GUS</i> L3	95	81	6	55	40	39	56	13	82

Table 1: Number of syncytia showing the expression of reporter proteins under control of the CaMV2x35Somega promoter

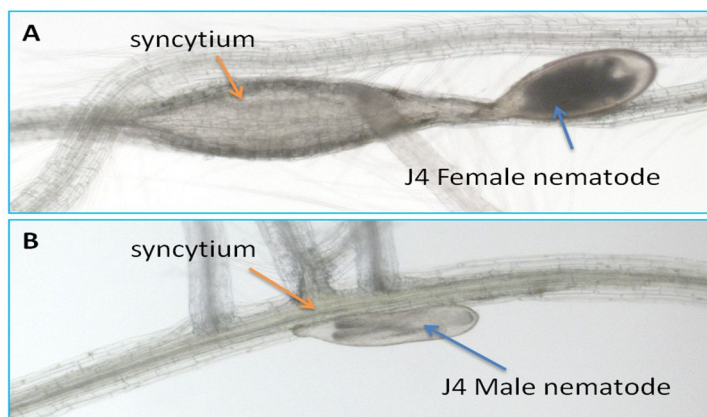


Figure 1: A: J4 male nematode and associated syncytia, B: J4 female nematode its associated syncytia (Reproduced from Ali *et al.* [36].

However, unlike expression of *DsRed*, the number of *GUS* stained syncytia decreased quickly to around 50% at 7 dpi and to about 5% in the 15 dpi syncytia (Figure 3B). Representative syncytia with *GUS* staining at different time points (3, 5, 7, 10 and 15 dpi) were photographed and are shown in Figure 4B. The syncytia at 3 and 5 dpi showed strong staining while the *GUS* staining in case of 7, 10 and 15 dpi was more confined towards the region of nematode attachment with the root. Nevertheless, at 15 dpi, the *GUS* staining was very low and only found at the point of nematode attachment, indicating that the activity of the CaMV.2x35S + omega promoter decreases with the passage of time in older syncytia.

Expression of *GUS/DsRed* driven by CaMV.2x35S + omega promoter in root cap border cells

EcoRI
Polylinker with unique sites
HindIII

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GTTACTAGATCTCTGAATTCACGTGGTACCGCGGCCGCAGATCTAAGCTTGCATGCCTGCAGGTTCGATCCCCCT
CAGAAGACCAGAGGGCTATTGAGACTTTTCAACAAAGGGTAATATCGGGAAACCTCCTCGGATTCCATTGCCAG
CTATCTGTCACTTTCATCGAAAAGGACAGTAGAAAAGGAAGGTGGCTCCTACAAATGCCATCATTGCGATAAAGGAA
AGGCTATCGTTCAAGATGCCTCTACCGACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAACATCGTGGAAA
AAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATGGCCGCTCTAGCATGGAGTCAAAGATTC
AAATAGAGGACCTAACAGAACTCGCCGTAAAGACTGGCGAACAGTTTCATACAGAGTCTCTTACGACTCAATGACA
AGAAGAAAATCTTCGTCAACATGGTGGAGCACACACATTTGTCTACTCCAAAAATATCAAAGATACAGCTCTCAG
AAGACCAAAGGGCAATTGAGACTTTTCAACAAAGGGTAATATCCGGAAACCTCCTCGGATTCCGATTGCCCAGCTA
TCTGTCACTTTATTGTGAAGATAGTGGAAAAGGAAGGTGGCTCCTACAAATGCCATCATTGCGATAAAGGAAAAG
CCATCGTTGAAGATGCCTCTGCCGACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAGCATCGTGGAAAAAG
AAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCACTGACGTAAGGGATGACGCACAAT
CCCCTATCCTTCGCAAGACCCTTCTCTATATAAGGAAGTTTCATTTCAATTTGGAGAGGACAGGCTTCTTGAGAT
CCTTCAACAATTACCAACAACAACAACAACAACAACAACAATTACAATTACTATTTACAATTACAATGGTCCGTC
    
```

Omega element from TMV
NcoI

Figure 2: The sequence of the promoter has two identical copies of 35S region, the base pairs (bp) with gray colour and the omega element is shown as underlined light green colour. The polylinker have unique restriction sites before the CaMV.2x35S+omega sequence, which are shown in the vector pMAA-Red [20]. The 915bp sequence of this promoter starting from 6501 bp (GCATGCCTGC) and ending at 7415 bp (TTACAATTACACC) can also be found on Genbank with the accession number JX025642. This promoter contains only one TATA box in the sequence.

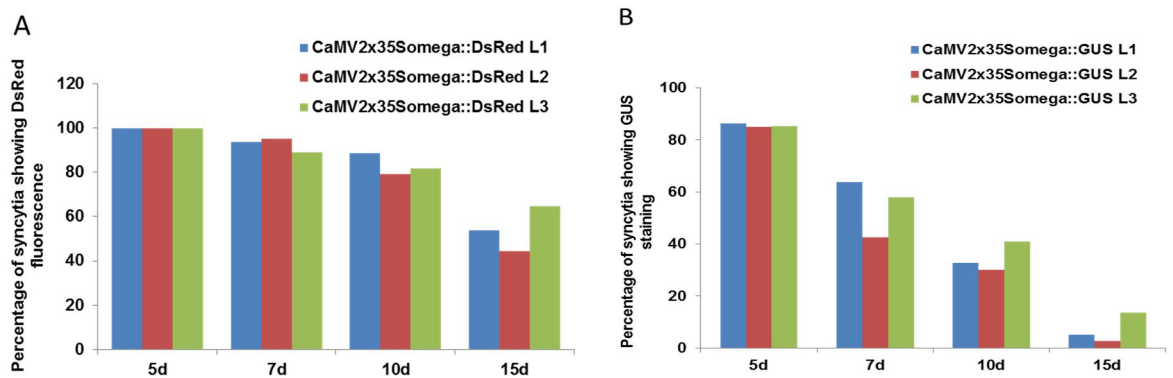


Figure 3: Percentage decrease in the number of syncytia expressing DsRed (A) and GUS (B) on y-axis when driven by CaMV35S promoter during different days on x-axis.

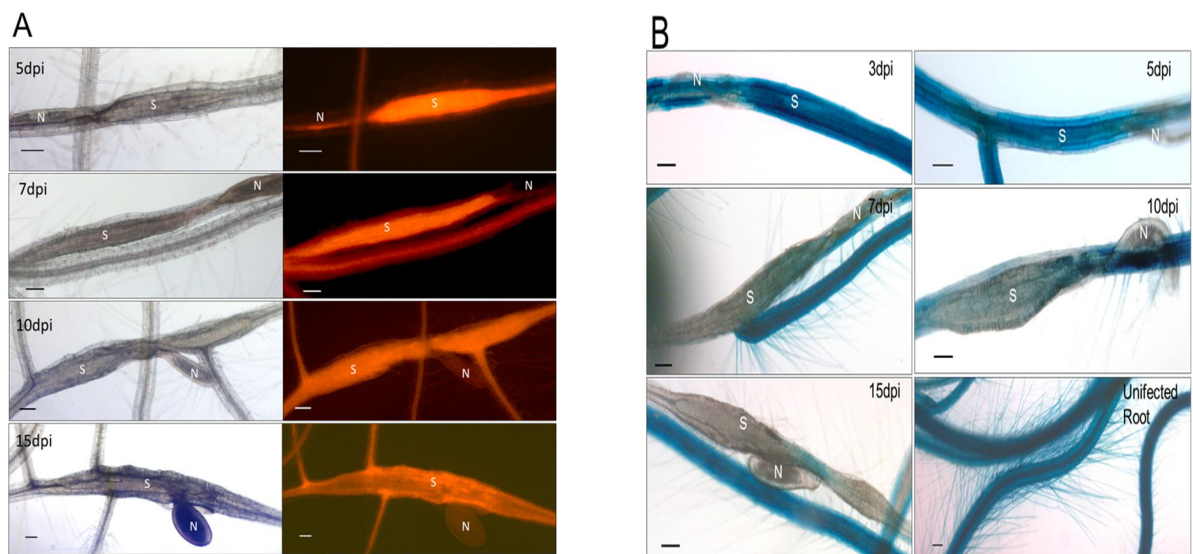


Figure 4: Expression of DsRed (A) and GUS (B) delivered by CaMV2x35Somega promoter in 3, 5, 7, 10 and 15dpi.

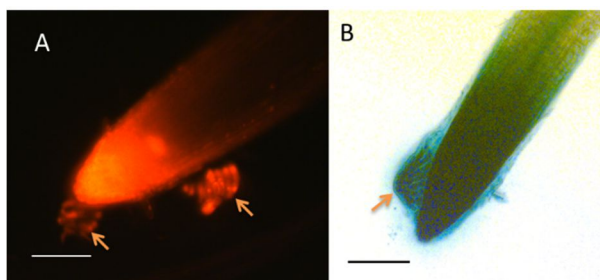


Figure 5: the reporter proteins *DsRed* (A) and *GUS* (B) are expressed in border cells when driven by 35S promoter. Bar = 100 μ m.

As this promoter was constitutively active throughout the plant tissues, the expression of both the reporter genes was found in the root cap border cells of the root tips. The CaMV.2x35S + omega:: *DsRed* as well as the *GUS* cassettes showed high expression in the root cap border cells as shown in Figure 5A and B respectively. All the transgenic lines showed the same expression of both the reporters in the root cap border cells, however, only the representative photos are shown here.

Discussion

Our results showed that the CaMV.2x35S+omega promoter was active at all the time points in case of *DsRed* with decreased expression only at 15 dpi. However, in case of *GUS*, the promoter was active in syncytia at 3, 5 and 7 dpi and was slightly downregulated at 10 dpi and almost inactive at 15 dpi. The molecular weight of *GUS* gene (*Uida*) based on the amino acid mentioned by Ali *et al.* [20] in Genbank is just over 68 KiloDaltons (kDa) which is higher than that of *DsRed* protein is around 25 kDa but this is not the molecular weight difference that the promoter delivered the *DsRed* (smaller protein) more efficiently into the syncytia as compared to *GUS*. What could be the reason for the better expression of *DsRed* in older syncytia as compared to *GUS*? *DsRed* is a fluorescent protein which does not need any cofactor or substrate which is needed for *GUS* staining (i.e. X-gluc) [14]. The substrate thus has to get into the syncytia to be available for the *GUS* enzyme to interact. Old syncytia have very strong outer cell walls which could make it difficult for the *GUS* substrate to penetrate into the old syncytia. Similar observations were reported by Goverse *et al.* [26] who expressed green fluorescent protein (GFP) in transgenic potato (*Solanum tuberosum*) plants under the control of the CaMV35S promoter. By using confocal laser scanning microscopy (CLSM), they investigated promoter activity in syncytia during potato cyst

nematode (*Globodera rostochiensis*) infection and observed GFP expression up to 13 dpi [26].

The CaMV.2x35S+omega promoter was used to overexpress some defense related genes like *RAP2.6* and *WRKY33* in syncytia during our studies and we found elevated expression of these genes in syncytia which resulted in enhanced nematode resistance in transgenic lines [20,33,34]. This suggested that this promoter could be used to express the gene of interest constitutively in plants, including syncytia. The CaMV promoter has been used for proteins to increase resistance against nematodes. For example, Liu *et al.* [22] successfully used the CaMV35S promoter for expressing synthetic chemodisruptive peptides *in planta* which disrupted the establishment of cyst nematode *Globodera pallida* in potato. Similarly, the CaMV35S promoter was used to overexpress different proteinase inhibitors which resulted in lower numbers of nematode with impaired development on several host plants including potato, rice and banana (Reviewed by [27]). This promoter has also been used to control the expression of RNAi or double stranded RNA of nematode parasitism genes to induce nematode resistance mediated by RNA interference [35]. However, Urwin *et al.* [21] indicated that CaMV35S is active in giant cells induced by the root-knot nematode *Meloidogyne incognita* but was highly downregulated in syncytia induced by cyst nematodes (*H. schachtii*). It has been shown in many studies that CaMV35S promoter is downregulated in cyst nematode induced syncytia [23,24] while Urwin *et al.* [25] concluded that CaMV35S is active in syncytia up to 10 dpi.

The results conclude that CaMV.2x35S+omega promoter is induced in younger syncytia and is downregulated in older syncytia with the passage of time but can be used for expression of transgenes in syncytia. Moreover, *DsRed* could be used as a better reporter for evaluation of gene expression in syncytia compared with *GUS*.

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