Enhancement or Attenuation of Disease by Deletion of Genes from Citrus Tristeza Virus

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Stem pitting is a common virus-induced disease of perennial woody plants induced by a range of different viruses. The phenotype results from sporadic areas of the stem in which normal xylem and phloem development is prevented during growth of stems. These alterations interfere with carbohydrate transport, resulting in reduced plant growth and yield. Citrus tristeza virus (CTV), a phloem-limited closterovirus, induces economically important stem-pitting diseases of citrus. CTV has three nonconserved genes (p33, p18, and p13) that are not related to genes of other viruses and that are not required for systemic infection of some species of citrus, which allowed us to examine the effect of deletions of these genes on symptom phenotypes. In the most susceptible experimental host, Citrus macrophylla, the full-length virus causes only very mild stem-pitting symptoms. Surprisingly, we found that certain deletion combinations (p33 and p18 and/or p13) induced greatly increased stem-pitting symptoms, while other combinations (p13 or p13 plus p18) resulted in reduced stem pitting. These results suggest that the stem-pitting phenotype, which is one of more economically important disease phenotypes, can result not from a specific sequence or protein but from a balance between the expression of different viral genes. Unexpectedly, using green fluorescent protein-tagged full-length virus and deletion mutants (CTV9Δp33 and CTV9Δp33Δp18Δp13), the virus was found at pitted areas in abnormal locations outside the normal ring of phloem. Thus, increased stem pitting was associated not only with a prevention of xylem production but also with a proliferation of cells that supported viral replication, suggesting that at random areas of stems the virus can elicit changes in cellular differentiation and development.

Viruses are obligate parasites with relatively small genetic coding capacity and must exploit a portion of host genes for invasion, multiplication, and spread. Although viruses of plants have been focused upon because of the diseases that they cause, the ultimate interaction when a virus evolves with a host is likely no disease. Yet, as viruses interact with plant hosts, they do sometimes cause disease. When disease occurs in a plant, it is often accidental due to the virus moving to a new host presented to it by agricultural practices. Disease symptoms usually occur on portions of the plant that develop and grow subsequent to viral infection. Rarely do symptoms occur in areas of the plant that are fully developed at the time of infection. Disease often results from interference with differentiation or development. Yet, when diseases do occur, they can cause severe damage to plants, and in agricultural crops, diseases cause economic losses, sometimes even preventing some crops from being grown.

Interference with differentiation or development results in numerous phenotypes. Lack of chloroplast development resulting in chlorosis is probably the most common virus-induced symptom. The resulting reduced photosynthesis causes reduced growth. Stem pitting is a common virus-induced phenotype of perennial woody plants that results from interference with stem growth. A range of different viruses distributed throughout the plant virus taxonomy induces stem pitting in a range of plant species, including numerous Prunus species, apples, vinifera grapevines, citrus, and avocado, usually resulting in a slow decline of growth and poor yields. In healthy and in normally developed areas of infected trees, the cambium, which is between the phloem and xylem, divides and differentiates in opposite horizontal directions, producing new xylem on the inward side and new phloem on the bark side, resulting in increased girth of the tree trunk and branches. Stem pits develop in areas where development is disrupted. The surrounding areas grow normally, leaving the disrupted areas as indented areas or pits. Although this disease phenotype is common in virus-infected perennial woody plants, there is little understanding of the processes that cause the stem pits.

Citrus tristeza virus (CTV), a phloem-limited virus, is a member of the genus Closterovirus of the family Closteroviridae (2, 14). The 19.3-kb single-stranded positive-sense genomic RNA of CTV is organized into 12 open reading frames (ORFs) (12, 15). ORFs 1a and 1b are directly translated from the genomic RNA as two overlapping polyproteins that encode two papain-like proteinases and methyltransferase-, helicase-, and RNA-dependent RNA polymerase-like domains (12). The 10 3’ genes are dispensable for replication at the single-cell level and are expressed through a nested set of 3’-terminal subgenomic (sg) RNAs (10, 20). CTV encodes a signature gene block, conserved among the members of Closteroviridae, comprising a 6-kDa hydrophobic protein; HSP70h, a homologue of the ubiquitous cellular heat shock protein; a 61-kDa protein; and minor coat protein (CPm) and major coat protein (CP). The last four proteins are involved in the formation of flexuous filamentous bipolar virions of 2,000 nm by 10 to 12 nm (21, 24). The proteins CP, p20, and p23 were shown to be involved in suppression of host RNA silencing in Nicotiana spp. (13). Additionally, the members of Closteroviridae encode 1 to 5 unique species-specific nonconserved genes with no sequence
identity with available sequences. CTV possesses three such genes (p33, p18, and p13), which are dispensable for systemic infection of certain citrus species (26). Recently, we reported that CTV was apparently able to extend its host range by acquiring these non-conserved genes (27). Acquisition of the p33 gene allowed systemic infection of sour orange and Eureka lemon trees, that of the p33 or the p18 ORF allowed infection of grapefruit trees, and that of the p33 or the p13 ORF allowed infection of calamondin trees (27).

The host range of CTV is limited to Citrus spp. and close relatives. Infections with almost all CTV isolates are symptomless in some citrus hosts; those isolates that do cause disease symptoms do so in only a small subset of their host range. Yet, some CTV isolates cause severe economic losses in citrus (2, 14). In the early 1900s, CTV destroyed entire citrus industries, particularly in South America. Currently, CTV continues to limit citrus production in much of the citrus-producing world. The virus causes a range of disease phenotypes in citrus, but the phenotype that is presently the most economically important is referred to as “stem pitting.” Trees with severe stem pitting grow poorly, lack vigor, and yield small, unmarketable fruit. Sasaki et al. (19) found that isolates causing severe stem pitting had a tissue tropism slightly different from the meristematic cells at the interface between phloem and xylem. Brlansky et al. (4) examined stem pitting induced by CTV by light and electron microscopy. In areas of the stem pits, the cambium appeared to be missing and the lack of new xylem formation resulted in a depression or pit in the surface of the stem as the normal areas continued increasing the girth of the stem. Proportionally to the density of pits, the function of the phloem and plant growth and vigor are reduced.

Certain isolates of CTV cause stem pitting in specific citrus varieties (9, 11). The remarkable feature of the CTV-stem pitting association is the high degree of specificity. For example, some isolates cause stem pitting in sweet orange but not in grapefruit. Others cause stem pitting in grapefruit but not in sweet orange. Others cause the phenotype in both, and others cause it in neither. This level of specificity continues throughout a range of other citrus species and even among different cultivars within a species, such that there are literally hundreds of phenotypes. Thus, among its whole host range, a particular isolate will cause stem pitting in only a small subset, perhaps only one species or even a subset of the species. Why is the induction of stem pitting so specific?

We created an infectious cDNA clone of CTV (20) that has allowed us to examine its interaction with its hosts (7, 26) and recently showed that the virus acquired three genes to extend its host range (27). The ability to systemically infect some young citrus trees with CTV mutants with deletions of combinations of the p33, p18, and p13 genes provided an opportunity to examine the effect of the lack of these genes on the development of symptoms caused by CTV. In the most susceptible experimental host, Citrus macrophylla, the full-length virus causes only very mild stem-pitting symptoms. Surprisingly, we found that certain deletion combinations induced greatly increased stem-pitting symptoms, while other combinations resulted in reduced stem pitting. These results suggest that the stem-pitting phenotype, which is one of the more economically important disease phenotypes, can result from a balance between the expression of different viral genes. The general expectation was that stem pitting induced by CTV would be due to specific viral sequences that could be used to identify isolates causing severe stem pitting. However, the changes in stem-pitting phenotypes shown here were due to removal of sequences instead of different sequences. Unexpectedly, at the pitted areas, the virus was found in locations outside the normal ring of phloem. Increased stem pitting was associated not only with a prevention of xylem production but also with a proliferation of cells that supported viral replication, suggesting that the virus can elicit changes in cellular differentiation at random areas of stems.

MATERIALS AND METHODS

Maintenance of CTV deletion mutants and inoculation of citrus plants. The following were produced from cDNA constructs previously described (20, 22, 23, 26): full-length cloned virus CTV9; deletion mutants CTV9Δp33, CTV9Δp18, CTV9Δp13, CTV9Δp33Δp18, CTV9Δp33Δp13, CTV9Δp18Δp13, and CTV9Δp33Δp18Δp13; green fluorescent protein (GFP)-tagged wild-type virus CTV9-GFPC3; and deletion mutants CTV9Δp33-GFPC3 and CTV9Δp33Δp18Δp13-GFPC3. The virus-infected plants were maintained in C. macrophylla under greenhouse conditions. Bark pieces or buds from infected plants were used to graft transmit the virus. A minimum of 4 to 5 plants were inoculated and maintained in a temperature-controlled greenhouse. The grafted plants infected by these viruses were pruned at 1 week after inoculation, which was followed by growth of a new flush. The infectivity of test plants was determined by analyzing extracts from young stems by double-antibody sandwich indirect enzyme-linked immunosorbent assay (DAS-1-ELISA) using CTV-specific antisera (8).

The main stems of plants infected with the full-length virus (CTV9) and deletion mutants at 3 months postinoculation (mpi) were examined with the bark removed, and pictures were taken under visible light.

Examination of GFP fluorescence in stems and stem pits. Cross sections of stems with bark tissue and vertical sections of wood with bark removed from plants infected with CTV9-GFPC3, CTV9Δp33-GFPC3, or CTV9Δp33Δp18Δp13-GFPC3 were examined for GFP fluorescence under a Zeiss Stemi SV11 UV fluorescence dissecting microscope (Carl Zeiss Jena GmbH, Jena, Germany) with an attached Olympus Q-color 5 camera (Olympus America, Inc., Center Valley, PA).

RESULTS

Deletions of the p33, p18, and/or p13 ORF greatly affect stem-pitting symptoms. Previously, we reported that deletions in the p33, p18, or p13 ORFs in all possible combinations in CTV (T36) (Fig. 1A) still allowed the virus to systemically infect the following hosts: C. macrophylla, Mexican lime (C. aurantifolia), sweet orange (C. sinensis), C. indica, C. hystrix, C. micrantha, Persian lime (C. latifolia), and citron (C. medica) plants (27). These results allowed the examination of the effect of deletion of genes on symptom production. Except on C. macrophylla and Mexican lime plants, the full-length virus (CTV9) and all deletion mutants induced mild or symptomless infections with little or no stem pitting. However, some of the mutants induced severe stem pitting in both C. macrophylla and Mexican lime plants. The phenotypes of the mutants in both plants were similar, but for brevity, here we report the results only for C. macrophylla.

Because the full-length virus was known to induce mild stem pits on the mature trunk and limbs at 2 to 3 mpi, we examined the stem-pitting symptom phenotype of the deletion mutants. CTV with a deletion of the p33 or p18 ORF induced more severe stem pitting with a substantial increase in the number and depth of pits (Fig. 1B). The mutant with the deletion of the p18 ORF induced stem pits slightly milder than those of the mutant with the p33 deletion; nonetheless, the stem pits elicited by both deletion mutants were severe compared to those elicited by the full-length virus (Fig. 1B). In contrast, CTV with a deletion of the p13 ORF

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A replication gene block

quintuple gene module

(A) CTV9

(B) CTV9Δp33

(C) CTV9Δp18

(D) CTV9Δp13

(E) CTV9Δp33Δp18

(F) CTV9Δp33Δp13

(G) CTV9Δp18Δp13

(H) CTV9Δp33Δp18Δp13

B

Healthy

CTV9

CTV9Δp13

CTV9Δp33

CTV9Δp13

CTV9Δp33Δp13

CTV9Δp13

CTV9Δp33Δp13

CTV9Δp13
induced no visible stem pits, and it induced even milder symptoms than the full-length virus (Fig. 1B). These data demonstrated that deletion of the p33 ORF (presence of the p18 and p13 ORFs) or the p18 ORF (presence of the p33 and p13 genes) increased the stem pitting by CTV, but the deletion of the p13 ORF (presence of the p33 and p18 ORFs) resulted in reduced stem-pitting symptoms.

We next examined the effects of double or triple gene deletions on induction of stem pits. The mutants with deletions of the p18 and/or the p13 ORF plus the p33 ORF induced greatly increased numbers and sizes of stem pits compared to those induced by mutants with the p33 deletion alone (Fig. 1B). In contrast, the mutant with deletions of both the p18 and p13 ORFs induced no visible stem pits, which was similar to the finding for the mutant with the single deletion of the p13 ORF. Both of these mutants induced reduced stem pitting compared to the full-length virus. Even though the mutant with the deletion of the p18 ORF induced severe stem pits, the mutant with deletions of the p13 ORF plus the p13 ORF induced no visible stem pits. The triple-gene-deletion mutant, CTV9Δp33Δp18Δp13, induced stem pits that were the most severe compared to those induced by the other deletion mutants (Fig. 1B).

**Association of virus-infected cells with stem pits.** We developed the full-length virus and deletion mutants CTV9Δp33 and CTV9Δp33Δp18Δp13 with a GFP marker in order to monitor virus movement and distribution in citrus trees (6, 26). The GFP ORF was constructed as an extra gene between the CPm and CP genes, and free GFP accumulates in the cytoplasm of cells as CTV replicates (6). The location of CTV replication is limited to phloem-associated cells, companion cells, and phloem parenchyma cells. Since CTV does not replicate in sieve elements or xylem cells, GFP does not accumulate in these cells (7). In cross sections of stems, a ring of meristematic cambium cells is located between the phloem to the outside and the xylem on the inside. Normally, the virus is limited to the ring of phloem-associated cells (6, 26). In rapidly growing citrus, the bark will slip, such that when the bark is pulled away from the tree at the loose cambium, the phloem is attached to the inside of the excised bark, while the xylem remains with the stem. In cross sections of CTV9-GFP-C3-infected mature stems, as expected, the fluorescence was restricted to the ring of phloem-associated cells (Fig. 2A).

Since CTV9Δp33 and CTV9Δp33Δp18Δp13 induced severe stem pitting compared to the relatively mild stem pitting induced by the full-length virus, we next examined how the stem pits were associated with the virus-infected cells. We examined the mature wood of trees infected with GFP-tagged full-length virus (CTV9) and deletion mutant CTV9Δp33 or CTV9Δp33Δp18Δp13 at 3 mpi. After the bark was removed, the new xylem on the outside of the woody stems was observed under a fluorescence microscope for the presence of GFP fluorescence associated with infected cells in the woody stems. As expected from previous observations (6, 7, 26), most of the fluorescence by GFP was restricted to the inside of the bark that was removed, except that a few fluorescent areas were associated with the few pitted areas induced by the full-length virus, CTV9 (Fig. 2B). In contrast, at 3 mpi the mature wood (bark removed) from trees infected with CTV9Δp33-GFP-C3 or CTV9Δp33Δp18Δp13-GFP-C3 had a large number of large fluorescent areas associated with the more confluent pitted areas (Fig. 2B). In those areas, some cells susceptible to CTV did not remain attached to the bark but instead remained in the pitted areas, with much more GFP fluorescence associated with the pits of the severely affected stems.

To further confirm the association of virus with stem pits, we examined the vertical sections of the stems with the bark removed from GFP-tagged full-length virus- and deletion-mutant-infected plants at 3 mpi (Fig. 2B). We found a large amount of fluorescent patches deep in the wood extending close to the central (pith) region of the wood in CTV9Δp33-GFP-C3- and CTV9Δp33Δp18Δp13-GFP-C3-infected plants (Fig. 2B). In contrast, a few tiny fluorescent patches were observed at the periphery of wood from GFP-tagged full-length virus-infected plants (Fig. 2B). Taken together, our data suggest that a large amount of virus is associated with stem pits in *C. macrophylla*.
FIG 2 GFP-tagged CTV deletion mutant-infected cells associated with pits in mature stems of C. macrophylla at 3 months postinoculation. (A) Cross sections of stems showing the presence of fluorescence from GFP-tagged viruses in the stems. Note that fluorescence was restricted to phloem-related tissue in CTV9-GFPC3-infected stems, whereas in deletion mutant-infected plants, a substantial amount of fluorescence was detected in areas normally composed of xylem cells. (B) CTV9-GFPC3-, CTV9Δp33-GFPC3-, and CTV9Δp33Δp18Δp13-GFPC3-infected C. macrophylla mature stems with the bark removed showing pitting symptoms under visible light (VIS) and UV light (UV) and stems split vertically shown under UV light (VS-UV).
DISCUSSION

Previously, we reported that CTV contained nonconserved genes (p33, p18, and p13) and that different combinations of these genes allow extension of the CTV host range (27). Yet, the virus can systemically infect most of its hosts with one, two, or three of the genes deleted. A few other viruses have been shown to tolerate deletion of a gene, but these mutants generally cause attenuated symptoms in their hosts (e.g., see references 17, 18, and 25). Although the CTV deletion mutants induced mild symptoms in most other citrus species (26,27), in this study, we were surprised to find that these deletion mutants affected stem-pitting symptoms in *C. macrophylla*.

Different isolates of CTV induce a myriad of phenotypes in different citrus varieties and species (9, 11, 14). Vein clearing, leaf cupping, and temporary yellowing and stunting of young seedlings are phenotypes used in greenhouse diagnosis, but these symptoms have little effect on the growth and yield of large trees. The major concern today around the world is stem pitting, which makes citrus production not economical. Production in areas where isolates that cause severe stem pitting are endemic can be

FIG 3 CTV deletion mutants progressively induced increased stem-pitting symptoms with the maturity of the stem in *C. macrophylla*. (A) Schematic diagram of a *C. macrophylla* plant showing the positions of cross sections used for examination for the presence of GFP fluorescence. A series of cross sections from the young (top) toward the mature (bottom) side branches at 3 months postinoculation was observed under a fluorescence microscope. The position of grafted bud (inoculum) from infected plants onto healthy *C. macrophylla* is indicated. (B) Cross sections of *C. macrophylla* branches infected with CTV9-GFP (top row), CTV9Δp33-GFP (middle row), and CTV9Δp33Δp18Δp13-GFP (bottom row). Note that cross sections from CTV9-GFP-infected plants showed the uniform localization of fluorescence mostly restricted to the phloem-related tissue in the bark tissue. In contrast, cross sections from GFP-tagged deletion mutant-infected stems showed progressively increased accumulation of fluorescence outside the phloem-related tissue as the result of severe stem pits elicited by the deletion mutants.
economical only if susceptible varieties, normally oranges and grapefruit, are not grown or by preinoculation of trees with isolates inducing mild pitting to reduce losses. Areas where isolates causing severe pitting are not endemic invest in quarantine measures to prevent their introduction. A major limitation is identification of an isolate causing severe stem pitting. Isolates of CTV that cause severe stem pitting can be identified in greenhouse tests by inoculating indicator hosts, but this assay takes about a year and requires large amounts of greenhouse space and labor. A major goal has been to identify viral sequences specific to severe stem pitting so that detection can rely on rapid antibody-based or PCR assays. In fact, it has been proposed that sequencing of many isolates of the virus and comparison of the sequences of stem-pitting isolates with those of non-stem-pitting isolates would define the causal sequences. However, the increase in stem pitting by CTV in C. macrophylla did not result from a certain sequence that could be targeted for identification. Instead, severe disease resulted from removal of sequences. The full-length virus that caused minimal stem pitting contains all of the sequences of the severe stem-pitting deletion mutants.

The formation of pits was apparently due to the inhibition of production of new xylem in the localized affected area, as shown by Brlansky et al. (4). The normally developing surrounding areas continue to grow, leaving a depression or pit at the affected area. What was unexpected was that in severely pitted areas, GFP fluorescence as a marker of virus replication was observed in regions normally made up of mature xylem or wood. It has been shown that the result of deletions in other viruses is to allow the virus to lose restriction to the phloem and move into nonvascular mesophyll cells (16) or alter the intestinal tropism within its insect vector (3). However, the CTV deletion mutants did not move into nonvascular mesophyll cells. Instead, they were found in a group of cells that appeared to be on the woody side of the phloem. In normally developing trees, most of the cells in this area differentiate into tracheary elements, which essentially consist of dead cells with thick walls connected into vessels for water transport. Interspersed in this area are live ray cells that transport nutrients from the phloem. In the full-length virus-infected trees, the fluorescence of GFP was always limited to the phloem ring outside the wood. However, increased stem pitting was associated with virus-infected cells in areas not normally infected. Since CTV multiplies and produces GFP only in living cells and free GFP was not found in noninfected adjacent cells (7), it would not be expected that the virus could produce GFP in mature xylem cells without virus replication or that GFP made in other cells could accumulate in xylem. However, it should be kept in mind that this is a process that occurs over a period of time and the stem increases in girth as the plant grows in the presence of the viral infection. These results suggest that the process of forming a stem pit not only involves the lack of production of new xylem in the affected area, resulting in a depression in the wood, but also affects development and causes cells within the pitted area to continue living and to be susceptible to CTV invasion and replication. Brlansky et al. (4) found that phloem-associated ray cells in citrus stem pitted by CTV were abnormal and disorganized. Virus-induced stem pitting might parallel regeneration after bark girdling where xylem cells dedifferentiate and transdifferentiate to form new cambium and phloem cells (28). Since CTV is normally restricted to phloem-associated cells, these masses of infected cells might be phloem-related cells. However, these cells apparently do not develop into functional phloem sieve elements that move carbohydrates, first, because CTV does not replicate in those cells and, more importantly, because the phenotype of severely stem-pitted trees is reduced phloem function. Further study at the microscopic anatomical level will be necessary to determine how the differentiation of the stem cells is altered by the severe stem-pitting mutants to cause pitting and reduction of tree growth. However, the CTV deletion mutants provide defined mutants that induce defined stem-pitting phenotypes that should be useful tools for further analysis of this phenomenon.

How did deletions in CTV induce severe stem pitting? One possibility was that the deletions caused an increase in expression of other CTV genes, which in turn induced the stem pitting. A common rule of expression of genes of viruses that express multiple genes via subgenomic RNAs is that genes positioned nearer the 3′ terminus tend to be produced in larger amounts, and movement of the genes closer to the 3′ terminus by deletion of intervening genes increases levels of expression (5). CTV follows this pattern. When the lowly expressed p33 or p6 gene was moved to near the 3′ terminus by deletion of all of the intervening genes, their mRNAs were increased many fold (1, 20). The largest deletion that would be expected to increase production of the upstream genes (CP, CPm, p61, HSP70h, p33, and p6) would be the deletion of both the p13 and p18 genes. However, this deletion induced the least amount of stem pitting. Increased stem pitting was often associated with deletion of the p33 gene. However, the p33 gene is positioned most 5′ of the genes expressed by subgenomic mRNAs, and its deletion does not increase expression of any other genes. Thus, it seems unlikely that the increased stem pitting due to deletions in the CTV genome was due to changes in expression of other viral genes.

Deletion of the p13 ORF tended to be correlated with reduced stem pitting. Thus, deletion mutants that retained the p13 gene (deletion of p33, p18, or p33 plus p18) tended to have the most stem pitting, which might suggest that the p13 gene product was involved in induction of stem pits. However, the triple-deletion mutant, which did not have the p13 gene, induced severe stem pitting, demonstrating that interpretation is not so simple. In contrast, increased stem pitting was generally associated with deletion of the p33 ORF. Mutants with the absence of the p33 ORF (deletion of the p33 plus the p18 ORFs and the p33 plus the p18 and p13 ORFs) induced severe stem pitting. Thus, mutants retaining the p33 gene (deletions of p13, p18, or p13 plus p18) had the smallest amounts of stem pitting. These results suggest that the presence of the p33 protein could be correlated with reduced stem pitting (and its absence increases it). However, the mutant with the deletion of the p18 ORF (with p33 and p13 retained) induced moderate stem pitting. Overall, the production of stem pits or no stem pits appears to be related more to a balance between expression of the p33 and p13 genes and possibly p18 genes.

In general, deletions in CTV resulted in a substantial increase in the stem-pitting disease of citrus examined here. Yet, there are different phenotypes of stem pitting. Some trees have large stem pits that are readily visible in tree trunks and limbs without removing the bark. Other trees exhibit “cheesy bark” stem pitting, which is a high density of very small pits. There is a continuum of levels in between. Some cause a rapid decline of tree growth and yield, while others cause little damage to the tree. Additionally, there is the extreme specificity between virus isolates and different citrus species and varieties. It should be noted that most of the
other hosts examined did not form stem pits when infected with these mutants (27; results presented here). There is no reason to think that all of the different stem-pitting phenotypes in different citrus hosts would be caused by the same virus-host interactions. Moreover, it should be kept in mind that within the host with stem pitting, much of the infected tissue is normal. It is only the occasional area that becomes a pit. This is a complex disease. Yet, here we have been able to associate viral alterations with one phenotype of stem pitting as a first step in understanding this process.

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