

## Spread of Fire Blight in Austria and Hungary – Variability of *Erwinia amylovora* Strains

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### Abstract

First outbreaks of fire blight were detected in Austria in 1993 and in Hungary in 1995. 137 *Erwinia amylovora* isolates were compared by PCR-RFLP, sequencing of the repeat region of the PstI fragment of plasmid pEA29, RAPD and AFLP analysis. Differences of repeat numbers in first isolates were observed. Six isolates showed different RAPD profiles. In AFLP analysis, so far, no differences in fragment pattern were determined. Pathogenicity tests with micropropagated apple cultivars revealed differences in plant susceptibility and in virulence of isolates. Responses of Malus tissue were visualized by the use of epifluorescence microscopy through the localization and the production of substances involved in cellular defence mechanisms (e.g. flavonoids, lignin) in various susceptible cultivars. The effect of bacterial strains differing in pathogenicity is shown by cellular peroxidase and flavonoid production.

**Keywords:** *Erwinia amylovora*; Austria; Hungary

### INTRODUCTION

Fire blight, caused by the necrogenic bacterium *Erwinia amylovora*, is a devastating disease of pome fruits and ornamentals belonging to the family of *Rosaceae*. First infections were detected in Austria, in the most westerly province, in 1993 (KECK 1994) and in Hungary, which has a common boarder with Austria in the East, in 1996 (HEVESI 1996). The aim of the common study was to compare *Erwinia amylovora* isolates of both countries by molecular techniques and pathogenicity tests in order to clarify whether the dissemination of fire blight progressed from west to east or *vice versa*. For testing strain aggressiveness the localization of peroxidase activity, flavonoid and lignin production in plant tissue of *in vitro* plants of different fireblight susceptibility was investigated by histochemical techniques.

### MATERIALS AND METHODS

#### Bacteria

137 *Erwinia amylovora* isolates were collected in different locations, host plants and years (Figures 1

and 2). Bacteria were cultivated on King's medium B for approximately 18 h at 26°C. Investigations on the stability of the repeat region of the PstI fragment were performed with strain 295/93, which has 14 copies of the 8-bp repeat. 295/95 was the first *Erwinia amylovora* isolate in Austria. Pathogenicity tests were achieved with selected strains 324/98, 328/98, 329/98, 495/98, 295/93 from Austria and Ea01, Ea10, Ea 12, Ea 17, Ea 28 from Hungary. For histochemical studies *Erwinia amylovora* strain 295/93 and 329/98 differing in aggressiveness were used. Suspensions were adjusted to 10<sup>7</sup> cfu/ml in sterile water for infections of *in vitro* plantlets.

#### PCR-restriction fragment length polymorphism analysis (RFLP)

Bacterial suspensions were prepared in sterile distilled water (10<sup>6</sup> cells/ml), heated to 95°C for 10 min and immediately frozen at -20°C for at least 30 min. PCR was performed according to BERESWILL *et al.* (1992) using primer A/B, encoding for the PstI fragment of plasmid Ea 29. RFLP studies were conducted according to LECOMTE *et al.* 1997 using the restriction enzyme MspI (Roche).

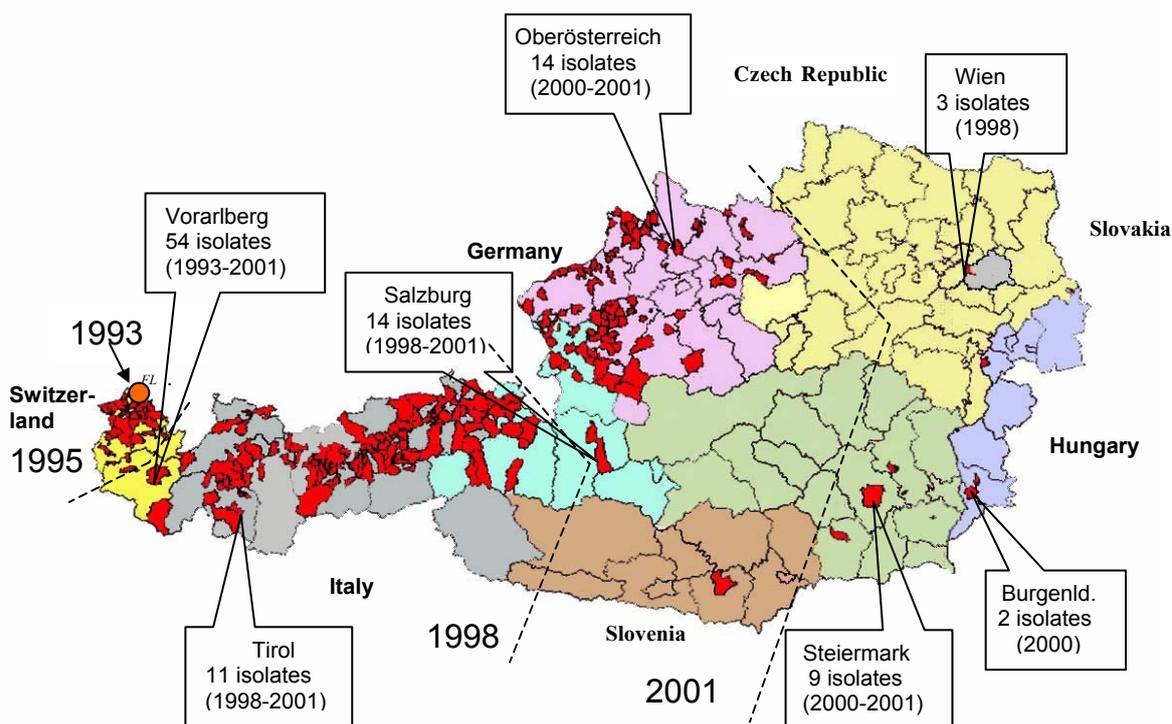


Figure 1. Spread of fire blight in Austria from 1993 to 2001

### DNA-sequencing

The repeat region of the PstI fragment of the pEA29 plasmid was amplified by the use of primer sA (5'-CAGGAAACAGCTATGACCCGTTTT-TAACGCTGGG) and primer sB (5'-TGTA AAC-GACGGCCAGTGGGCAAATACTCGGATT). The amplified DNA fragments were ethanol precipitated and sequence analysis was performed by the use of the SequiTherm Excel II Cycle Sequencing Kit (Biozym) with fluorescent labeled primers M13 univ. and T7 (Licor 4200S, MWG-Biotech).

### Random amplified polymorphic DNA fragments analysis (RAPD)

12 different primers were chosen for the analysis: Cugea1, Cugea2, Cugea3, Cugea4, Cugea5, Cugea6, according to MOMOL *et al.* (1997), P2 according to FITZIMONS *et al.* (1999) and M06, M10, M12, N05 purchased by Roth, Germany.

Bacterial DNA was extracted with DNAzol following the instructions of the manufacturer (Invitrogen). PCR amplifications were carried out as described by MOMOL *et al.* (1997) with minor modifications: Re-

action volume of 50  $\mu$ l containing 30 ng of genomic DNA, 30 pmol of primers, and Sigma RedTaq Ready Mix. 15  $\mu$ l of the amplified products were analyzed on 1.5% agarose gels (0.5% NuSieve GTC agarose, FMC; 1% SeaKem agarose, Biozym). At least two independent genomic DNA preparations and two independent amplification reactions were carried out for each strain to verify the obtained results.

Amplified restriction fragment polymorphism (AFLP): 250 ng of genomic DNA were used for each AFLP reaction. The AFLP microorganism Kit (Invitrogen) was used and the reactions were performed as described by the manufacturer with the exception that specific PCR reactions were performed with IRD 800 labeled EcoRI primers (MWG Biotech). The amplified products were analyzed on a 8% sequencing gel Licor 4200S (MWG-Biotech) using an automated DNA sequencer Licor 4200S (MWG-Biotech). Band detection and analyses was performed using the GeneImageIR software (Licor).

### Plant material

*In vitro* explants (Golden Delicious, Florina, Freedom, Jonagold, Royal Gala, MM 106) were cultivated

on Murashige and Skoog (supplemented with BAP 0.5 mg/l and AIB 0.1 mg/l and sucrose 30 g/l) and kept at 25°C, 16 h light. Plants were transferred on fresh medium one week before each experiment.

Artificial inoculations were achieved by cutting the upper leaves with scissors previously dipped into a bacterial suspension and checked daily (1–14 days, respectively 6 weeks) for the development of symptoms. Day plants were evaluated for symptoms according to HEVESI *et al.* (2000):

- 0 no fire blight symptom
- 1 browning of leaf tissues near the cut part
- 2 browning of leaf tissues near the cut part and the vein
- 3 browning of 2–3 other leaves near the cut leaf or the apex of the shoot
- 4 browning of half part of the shoot
- 5 browning of the whole stem and appearing bacterial exudate on the shoot.

**Visualization of peroxidase activity, formation of flavonoids and lignin in plant tissue**

*In vitro* plantlets of differing fire blight susceptibility (Florina: least susceptible, Golden Delicious: moderately susceptible; MM 106: highly susceptible) were inoculated with *Erwinia amylovora* strain 295/93 or

329/98 (pathogen suspension density:  $1.8 \times 10^6$  cfu/ml, artificial inoculation per leaf cutting). After 6 days, stem tissue from diseased plants was sampled in a distance of 0.3 mm from necrotic spots, was subsequently dissected into little, 1 mm<sup>2</sup> large pieces and fixed after vacuum infiltration ( $P = 55$  mbar) for 2 h in a freezing compound (Tissue Freezing Medium, Jung). Sections of tissue, 12 µm thick, were then cut with a freezing-stage microtome (Leica CM 1800). After staining with different reagents (*peroxidase*: 3,3',5,5'-tetramethylbenzidine (TMB, Sigma), *flavonoids*: Neu's reagent; *lignin*: phloroglucinol-HCL; according to DAI *et al.* (1995), sections were mounted in glycerine: water (15:85, v/v) and examined by light microscopy (Nikon Labophot-2) with two filter sets, a UV filter set with 330–380nm excitation and a 420nm barrier filter and a blue filter set with 450–490nm excitation and a 520nm barrier filter.

**RESULTS**

**Dissemination of *Erwinia amylovora***

After the first outbreak of fire blight in Austria, the disease remained located in a small area during the first four years (1993–1997). In the last years the disease migrated progressively from west to east (Figure 1).

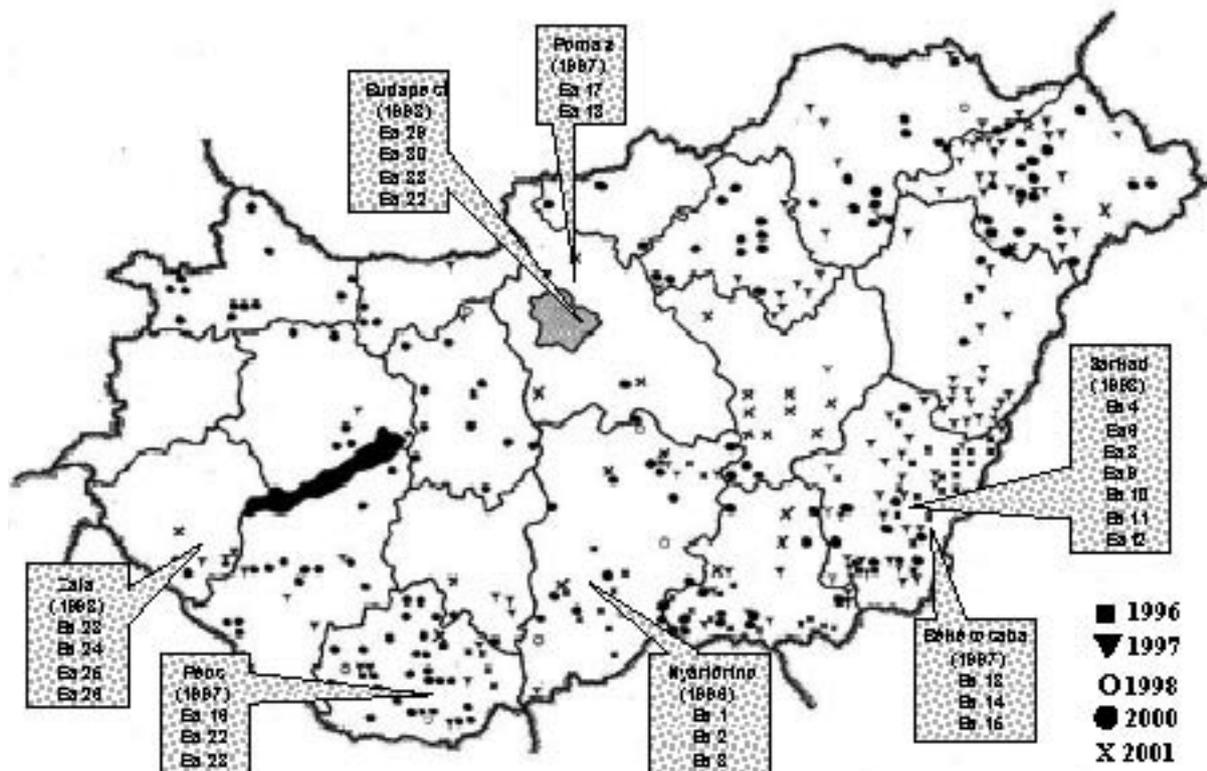


Figure 2. Occurrence of fire blight in Hungary

Table 1. Classification of *Erwinia amylovora* isolates by the number of copies of the 8-bp repeat of PstI fragment

	% of strains with repeats		
	14–10	9–6	5–3
Austria/Vorarlberg 1993–1997	94	6	0
Austria/Vorarlberg 1998–2001	28	72	0
Rest of Austria	0	21	79
Hungary 1996/2001	0	25	75

The spread of fire blight in Hungary seemed to be different (Figure 2). Two years after the first outbreak isolates were collected in very distant regions of the country (NEMETH 1999).

**Comparison of strains**

**Stability of repeats in fragment PstI.** When strain 295/93 with 14 repeats was transferred more than 160 times on culture media sequencing in regular intervals showed that the repeat region remained stable. The major part of analysed colonies had 14 repeats.

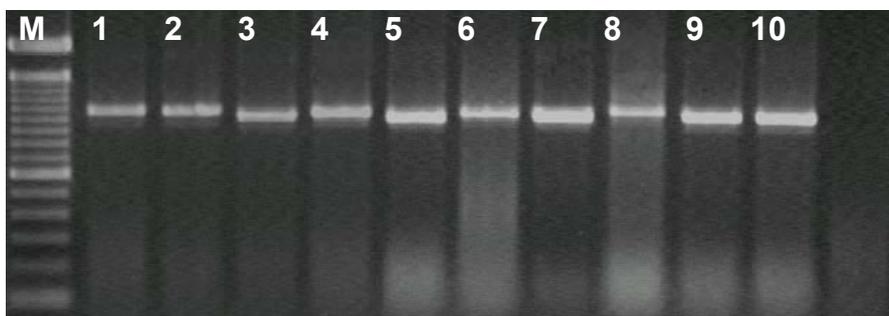
In minor cases number of copies were 23, 12 and 10. Strain 295/93 kept also the initial number of repeats, when it was inoculated in plants several times and sequenced after reisolation. Furthermore isolates collected in the area, where the first outbreak occurred, showed similarities with strain 295/93 and had mainly a high number of repeats (Table 1).

**Grouping of strains using repeat numbers**

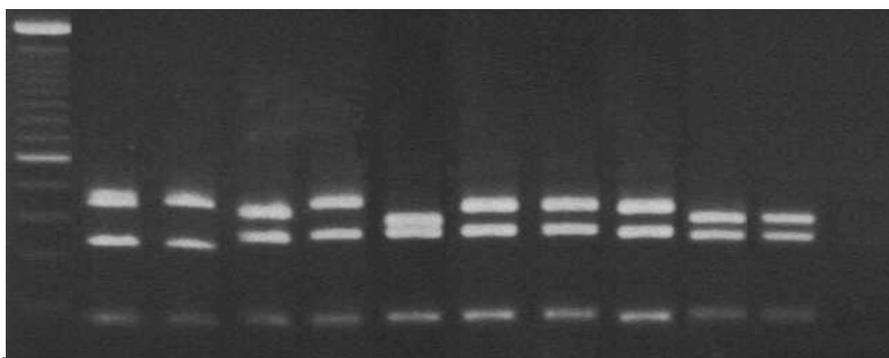
Marked differences in the length of the enriched fragment (900–1200 bp) can be seen by PCR and

*E. amylovora* strains                      273   295   324   328   2628   2447   495   499   1186   668

PCR



RFLP/Msp I



Number of repeats in PstI fragment of plasmid pEA 29  
 –(GAATTACA)<sub>n</sub>–

12   14   6   9   3   7   6   8   5   4

Figure 3. Comparison of *Erwinia amylovora* isolates by means of PCR, PCR-RFLP and sequencing of the repeat region of PstI fragment of the pEA29 plasmid

PCR-RFLP (Figure 3). After sequencing isolates were divided into three groups according to the number of copies of the 8-bp repeat: group 1: 14–10 repeats, group 2: 9–6 repeats and group 3: 5–3 repeats (Table 1).

Most of the Austrian and Hungarian isolates are classified into group 3 (3–5 repeats). However during the first five years of *Erwinia amylovora* infections in Austria isolates had mainly 14 repeats. From Hungary no isolate showed a high repeat number.

**Comparison of strains by RAPD and AFLP analysis.**

From all the primers used only 6 (5 Austrian and one Hungarian) out of 120 *Erwinia amylovora* isolates showed different profiles with primers Cugea4 and M6. In AFLP analysis, so far no differences in the fragment pattern could be determined.

**Pathogenicity of strains and reaction of apple tissue to infections.** Differences in plant susceptibility and in virulence of isolates were observed. In most experiments Jonagold and Royal Gala were more susceptible than Freedom. A good differentiation of pathogenicity of strains were obtained in Golden Delicious (Figure 4). Two of the selected strains were distinctively less pathogenic.

Fire blight symptoms were distinct in *in vitro* plants according to the susceptibility of apple cultivars. After 6 days of inoculation, symptoms on Florina were

distributed in the upper region of the stem; uninoculated leaves were symptomless. In Golden Delicious, symptoms on leaves and stem were found up to the middle of the stem whereas in MM 106 only the basis of the stem and the lower leaves remained healthy. Plants infected with virulent strain 329/98 exhibited more pronounced symptoms than those with 295/93. Stem parenchyma cells and leaf tissue of plants infected with both strains respectively reacted with TMB, indicating peroxidase activity. Cell walls and protoplast emitted a yellowish-blue color under UV and a blue fluorescence under blue light. Staining of both cell parts, especially of protoplast, was intensified in plant tissue infected with the more virulent strain 329/98. The amount of positive cell reaction was diminishing from MM 106 to Golden Delicious. Peroxidase activity was poor in Florina compared to Golden Delicious. After treatment with Neu's Reagent, parenchyma cells emitted a lemon-yellow fluorescence under blue light. Staining intensity of all cultivar tissues resembled that in peroxidase test. But, in contrary to peroxidase reaction, flavenoid activity was increasing in tissue of plants inoculated with the more aggressive strain 329. Even the differences between cultivars of divergent susceptibility were more apparent. Staining with phloroglucinol-HCL was positive in stem tissue of plants infected with both strains. There were less

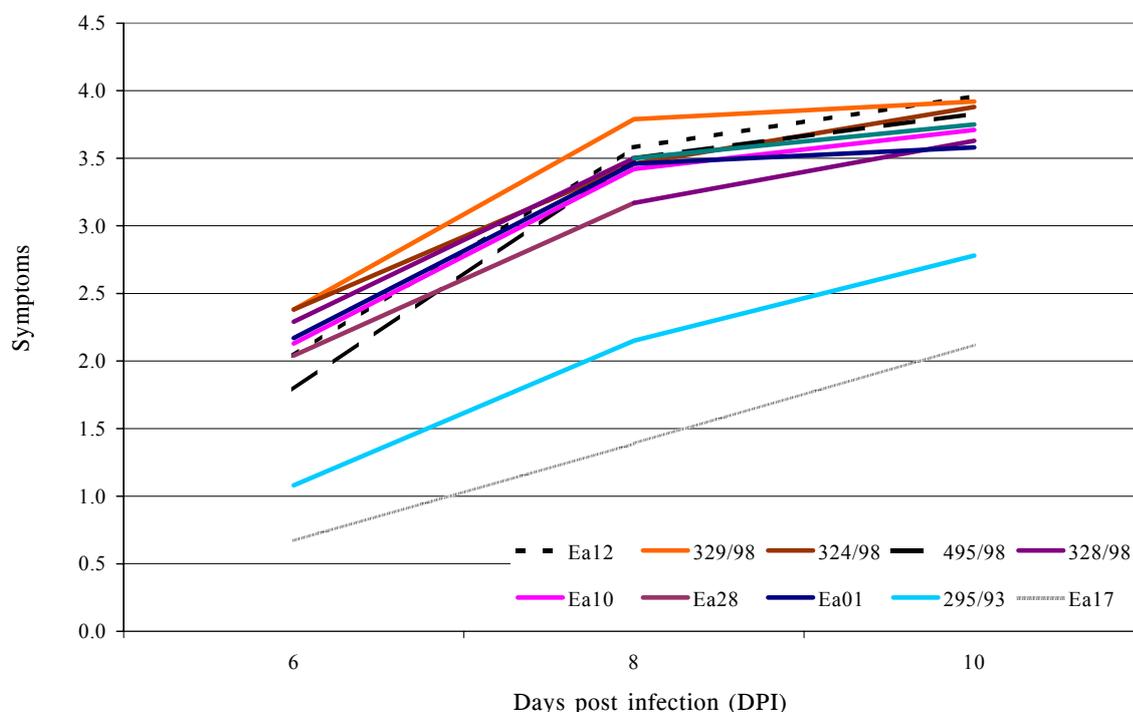


Figure 4. Pathogenicity of Austrian and Hungarian *Erwinia amylovora* isolates in *in vitro* plantlets cv. Golden Delicious

Table 2. Histochemical analysis of stem tissue of infected *in vitro* plants of different *Erwinia amylovora* susceptibility

Reagent		Stem after 6-day inoculation		
		Florina	Golden Delicious	MM 106
<b>Peroxidase</b>				
No treatment	Yellow	–	–	–
TMB	<i>E. amylovora</i> strain 295	–/+	+	++
blue uorescence	<i>E. amylovora</i> strain 329	+	++	+++
<b>Flavonoids</b>				
No treatment	Yellow	–	–	–
Neu	<i>E. amylovora</i> strain 295	–/+	+	++
lemon yellow uorescence	<i>E. amylovora</i> strain 329	+	++	+++
<b>Lignin</b>				
No treatment	Yellow	–	–	–
Phloroglucinol + HCl	<i>E. amylovora</i> strain 295	–/+	++	+++
red uorescence	<i>E. amylovora</i> strain 329	+	++	+++

differences between the amount of lignin neither in Golden Delicious/295 and MM 106/295 nor in Golden Delicious/329 and MM 106/329. Process of lignification in Florina was rather low. No reaction was observed in sections of all control plants (Table 2).

### DISCUSSION

The stability of the repeat region of the PstI fragment of plasmid pEA29 has been discussed (LECOMTE *et al.* 1997; SCHNABEL & JONES 1998). Our investigations with strain 295/93 proved that the number of copies remained stable and that this fragment can be used for the classification of *Erwinia amylovora* strains into three groups as proposed by LECOMTE *et al.* (1997). A comparison of first Austrian and Hungarian isolates with this marker showed no similarity between the isolates. Differences in isolates from Hungary and Austria were also found by ZHANG *et al.* (1998) and GEIDER (2001 – pers. commun.) by the use of Pulsed-Field Gel Electrophoresis. In pathogenicity tests first isolates of both countries revealed also to react differently. In summary the present study suggests that fire blight occurred independently in Austria and Hungary.

Histochemical tests showed that infected *in vitro* plants contained abundant isoenzymes of peroxidases. Positive reaction with TMB is indicative of the endogenous generation of hydrogen peroxide at infection

sites. Peroxidase activity was detected in the same parts of the tissue where accumulation of flavonoids also occurred. It may be therefore possible that the response to *E. amylovora* infections may be derived by the action of peroxidases and flavonoids in an intensified process of lignification postulated to contribute to plant-cell wall strengthening during plant-pathogen interaction (MÄDER & AMBERG-FISHER 1982; MÄDER & FÜSSL 1982; STICH & EBERMANN 1988). The differences in increase of lignification, peroxidase and flavonoid activity in plants in response to infections with *E. amylovora* strains of distinct virulence may be due to plant susceptibility and to differences in the degree to which the strain affect biochemical plant cell processes.

**Acknowledgements:** We want to thank E. JAMBOR-BENCZUR, Szent Istvan University, Budapest, Hungary for providing us apple explants.

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