Isabel Aguilar · Josefa M. Alamillo
Francisco García-Olmedo · Pablo Rodríguez-Palenzuela

# Natural variability in the *Arabidopsis* response to infection with *Erwinia carotovora* subsp. *carotovora*

**Abstract** The natural variation in the response of *Arabidopsis thaliana* (L.) Heynh. to *Erwinia carotovora* subsp. *carotovora* has been studied in seven ecotypes and two mutants. The susceptibility of all the plant types was investigated by (i) macroscopic symptoms, (ii) fluorescence microscopy using green fluorescent protein (GFP) and (iii) bacterial growth in planta. Although all the plants were susceptible to the bacterium, there was no correlation in the degree of infection as ascertained by the three methods. The induction, upon infection, of several genes known to be involved in defense was analyzed by RNA blot hybridization. The patterns of expression of these genes differed according to the genotype. These results suggest that both salicylic and jasmonic acid play a role in the response of *Arabidopsis* to this bacterium.

**Keywords** *Arabidopsis* (soft-rot) · *Erwinia* · Plant-pathogen interaction · Soft-rot

**Abbreviations** GFP: green fluorescent protein · hpi: hours post-inoculation · JA: jasmonic acid · PAL: phenylalanine ammonia-lyase · SA: salicylic acid

## Introduction

Soft-rots are worldwide destructive diseases caused by bacteria of the genus *Erwinia*, mostly *E. carotovora* and *E. chrysanthemi*. They are non-specific plant pathogens, which under favorable conditions are able to infect a wide range of hosts, causing heavy losses in economic crops (Perombelon and Kelman 1980). The main pathogenic feature of the soft-rot erwinias is the secretion of hydrolytic enzymes (Collmer and Keen 1986), in particular, pectinases that degrade the plant cell wall and release compounds such as oligogalacturonides, which can act as elicitors of the plant defense response (Norman et al. 1999).

Plants defend themselves from pathogen attack through several types of defense response (Maleck and Dietrich 1999; McDowell and Dangl 2000; Thomma et al. 2001). Some of these are mediated by salicylic acid (SA), whereas others are SA-independent. Other compounds, such as jasmonic acid (JA) and ethylene are implicated in the latter (Dong 1998). In a recent report about the interaction between *Arabidopsis thaliana* and *Erwinia carotovora* strain SCC3193 (Norman-Setterblad et al. (2000) the authors conclude that *E. carotovora* triggers two types of defense pathway in *Arabidopsis*, one is JAdependent and is inhibited by SA. The other one is ethylene- and JA-dependent and is potentiated by SA. Thus, SA appears to play a dual role in this interaction.

In spite of its potential economic importance, a genetically defined defense to soft-rot erwinias has not been described yet, and related work suggests that the interaction is very complex (Palva et al. 1994; Vidal et al. 1997, 1998). As a first step in studying the genetic basis of resistance/susceptibility to this bacterium, we have analyzed the natural variability of the *A. thaliana*/erwinia interaction with respect to: (i) macroscopic symptoms, (ii) feasibility of green fluorescent protein (GFP) as a bacterial reporter molecule in planta, (iii) bacterial growth, and (iv) activation of defense genes. Our results indicate overall that: (i) there is no correlation between GFP expression, bacterial growth and macroscopic symptoms and (ii) there is considerable variation in the defense responses among the ecotypes.

## Materials and methods

### Plant material

Seven *Arabidopsis thaliana* (L.) Heynh ecotypes (Col-0 = Columbia, Cvi-0 = Cape Verdi Islands, L*er*-0 = Landsberg *erecta*,

Ws-0 = Wassilewskija, Le-0 = Leiden, St-0 = Stockholm Nd-0 = Niederzenz), transgenic NahG plants (in Col-0 background), which contain an enzyme able to degrade SA (Delaney et al. 1994), and the *jar-1* mutant (for *j*asmonic *a*cid *r*esistant), which is mutated in a key component of the JA pathway (Staswick et al. 1998), were used for this study. Plants were grown in soil for 4–5 weeks in a growth chamber with cycles of 8 h light (150 µmol photons $m^{-2}$ $s^{-1}$)/16 h dark, 22/18 °C and 70% relative humidity (RH). Under these conditions the plants were at the rosette stage of development.

### Plant inoculations and bacterial growth estimation

Plants were challenged with the NCPPB 312-type strain of *Erwinia carotovora* subsp. *carotovora*, previously transformed with the commercial plasmid pEGFP containing the GFP sequence as reporter gene under the *lac* promoter (Clontech, Palo Alto, Calif., USA). The stability of this plasmid in planta was checked (data not shown). Bacteria were grown in King's B (KB) medium overnight and their concentration determined spectrophotometrically. Bacterial cultures were washed and resuspended in 10 mM $MgCl_2$ for in planta infiltrations. Culture containing $10^6$ colony-forming units was infiltrated by syringe into two leaves per plant, and 30 different plants per ecotype/mutant. After bacterial infiltration, plants were maintained at 28 °C and 100% RH for 2 days. Six leaves were collected at 0, 24 and 48 h post-infiltration and the bacterial population was estimated by tissue grinding and colony plating onto KB medium supplemented with 100 µg/ml ampicillin. The expression of the reporter EGFP was analyzed in the inoculated leaves, using fluorescence microscopy (Zeiss; excitation = 460 nm, emission > 478 nm).

### RNA extractions and Northern blot analyses

Total RNA was isolated using a standard phenol-SDS/LiCl procedure and subjected to RNA blot analysis using radioactively labeled, random-primed probes for phenylalanine ammonia-lyase (PAL), glutathione-*S*-transferase 1 (GST-1), pathogenesis related protein (PR-1), defensin (PDF 1.2) and thionin (THI) genes as described by Alamillo and Garcia-Olmedo (2001). *Arabidopsis* cDNA probes for GST and PAL were obtained by PCR using specific primers: PR-1 cDNA was from Uknes et al. (1992); PDF 1.2 and THI were obtained from the expressed sequence tag (EST) databank at the ABRC (Ohio State University). Ethidium bromide (EtBr) staining is shown as a loading control. RNA samples from three replicate experiments were analyzed in independent blots.

## Results and discussion

### Lack of correlation between GFP expression, bacterial growth and macroscopic symptoms

Symptoms of maceration were recorded 24 hours post-inoculation (hpi) in all the challenged plant types, with only minor differences at the macroscopical level (Fig. 1a). Among these differences, the Cvi-0 ecotype was the most sensitive to maceration. In the Ws-0 ecotype, a yellowish border appeared around the macerated area, whereas in the other cases general maceration symptoms were found. When lesions were observed under fluorescence microscopy, essentially two patterns of distribution of EGFP were found in the lesions. The first, characterized by a strong fluorescence signal inside the lesion, was found in Cvi-0, Ws-0 and the *jar-1* mutant (Fig. 1c, e, j). The second, characterized by a fluorescence signal localized in the area surrounding the lesion, was found in the Col-0, L*er*-0, Le-0, Nd-0 and St-0 ecotypes and NahG mutant (Fig. 1b, d, f–i). Apparently, this latter type of infection could be interpreted as the development of a bacterial front which leaves dead plant and bacterial cells behind, whereas in the first pattern an abundant bacterial population remains in the lesion. To further investigate this matter, the bacterial growth in the inoculated leaves of ecotypes and mutants was determined. Differences were found in the growth of the bacterial population in the different ecotypes and mutants tested, as is shown in Fig. 2. The most sensitive ecotype, according to this criterion, was Ws-0, in which the bacterial population had increased 2,500-fold by 48 hpi. The most resistant was Col-0 with and increase at 48 hpi of 54-fold. In all cases, steady bacterial growth was observed at 48 h. Both NahG and *jar-1* mutants were significantly more susceptible than the Col-0 ecotype. This result is apparently in contradiction to that published by Norman-Setterblad et al. (2000), although a different bacterial strain was used in that work.

The most important conclusion of these experiments is the lack of correlation between maceration symptoms, EGFP expression and bacterial population. A possible explanation for this phenomenon is that the activity of the EGFP plasmid could be lost or reduced after 24 hpi. Alternatively, the plant tissues from different ecotypes may differ in nutrient and toxic compounds, supporting different levels of bacterial population. Also, bacterial virulence factors could be modulated differently in different ecotypes. These results should be taken into account for future work on resistance of *A. thaliana* to *E. carotovora*.

### Variation in the defense response among ecotypes

Following these observations, we searched for differences in the defense responses of the ecotypes and mutants challenged with *E. carotovora* by analyzing the expression of key marker genes representative of different plant defense pathways in plants (Reymond and Farmer 1998). The results presented in Fig. 3 indicate that there is considerable variability in the defense responses triggered in the different ecotypes. In all the plants tested, except Ws-0, induction of PAL was observed at 4 hpi (Fig. 3). In Col-0, however, PAL accumulation was also observed at 24 hpi. Different levels of PAL transcript accumulation were found, being higher in Le-0, Nd-0 and St-0 ecotypes than in Col-0. PAL represents the phenylpropanoid pathway, which has several key functions in plant defense (Dixon and Paiva 1995) and has been shown to be induced early by bacterial infection, causing a hypersensitive response (Dong et al. 1991). Interestingly, in NahG and *jar-1* plants a different expression pattern from that of their Col-0 background was found. These plants showed higher accumulation of PAL transcripts at 4 hpi, and PAL

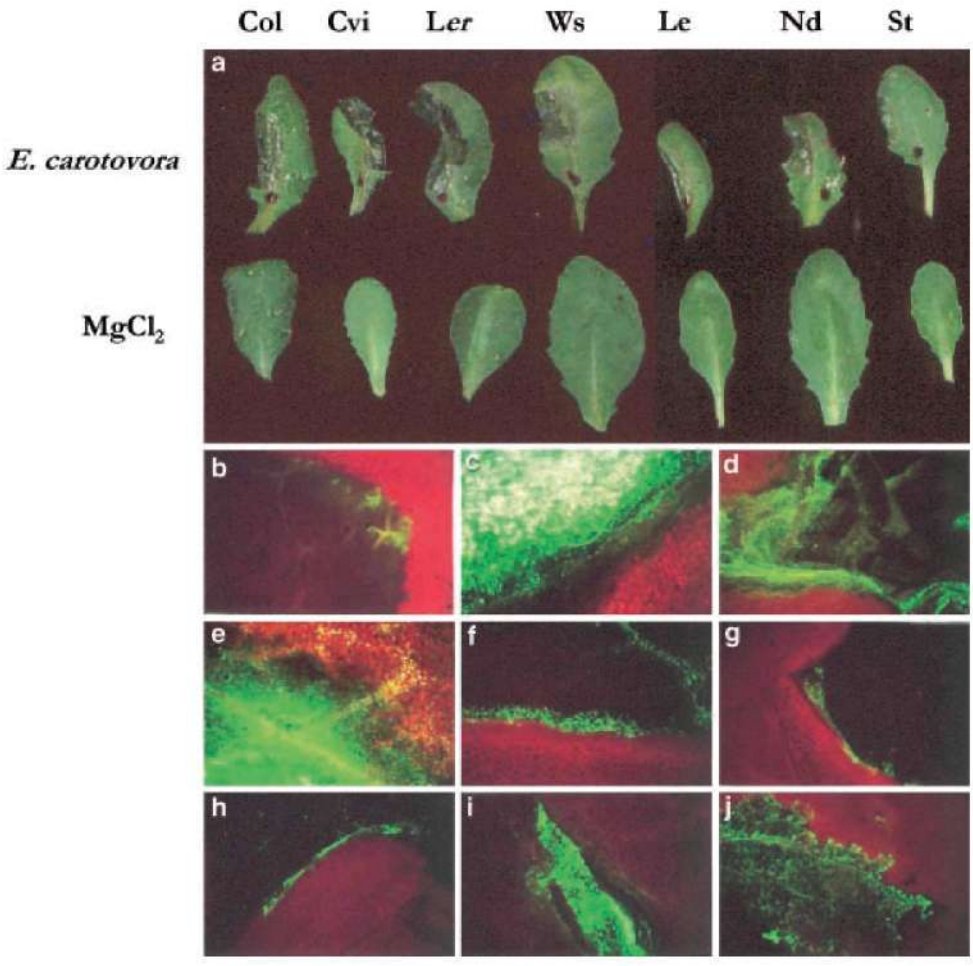


**Fig. 1a, j** Effects of *Erwinia carotovora* subsp. *carotovora* on different ecotypes and mutants of *Arabidopsis thaliana*. Plants were grown in a growth chamber (8 h light/70% RH/24 °C) for 4–5 weeks. Two leaves per plant were inoculated with 10 µl of bacterial culture (0.5 $OD_{600}$) and plants were maintained in a growth chamber (8 h light/100% RH/28 °C) for 3 days. This experiment was repeated five times and representative symptoms are shown. **a** Macroscopic symptoms of maceration in the different ecotypes 24 h after inoculation. As a negative control, plants were inoculated with 10 mM $MgCl_2$. **b–j** Microscopic effects of *E. carotovora* transformed with EGFP reporter plasmid. Leaves were observed under a fluorescence microscope 24 h after infection. *Bright green*, *E. carotovora* carrying plasmid EGFP; *red*, healthy plant cells; *yellow*, accumulation of phenolics; *black*, the completely macerated area. **b** Col, **c** Cvi, **d** L*er*, **e** Ws, **f** Le, **g** Nd; **h** St, **i** NahG, **j** *jar*

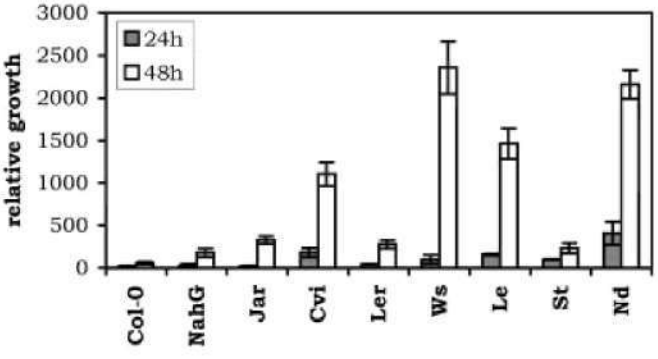


**Fig. 2** Relative growth of *E. carotovora* in all the *Arabidopsis* ecotypes and mutants tested 24 and 48 hpi. 4- to 5-week-old plants of all ecotypes and mutants were inoculated with 10 µl of *E. carotovora* (0.5 $OD_{600}$). At 0, 24 and 48 h, six leaves per time point were harvested and the amount of bacteria determined by plating serial dilutions of leaf extracts onto KB medium supplemented with 100 µg/ml ampicillin. The data correspond to the average of three independent experiments and are related to bacterial population at time point 0 h. The coefficient of variation at time zero was < 15%

induction was not detected at 24 hpi. Higher induction of PAL in NahG plants could be related to the fact that SA is located downstream in the phenylpropanoid biosynthetic pathway, which could lead to overexpression of PAL in these conditions.

The expression of the oxidative-stress-related gene, *GST-1*, reached maximum levels at 24 hpi in all the ecotypes and mutants analyzed except for Cvi-0, in which a transient expression at 4 h was found. A weak GST-1 induction was found in L*er*-0 and Ws-0. NahG and *jar-1* plants showed a different GST-1 expression pattern from Col-0: the same level of GST-1 accumulation was obtained in NahG plants at 4 h and 24 hpi, and in *jar-1*, transient GST-1 accumulation was found. We have recently described the production of an oxidative burst in tobacco plants infiltrated with the soft-rot *Erwinia chrysanthemi* (Miguel et al. 2000). The expression of GST-1 in all the ecotypes and mutants challenged with *E. carotovora* suggests the occurrence of an oxidative burst and the subsequent activation of a hydrogen peroxide detoxification mechanism. For Cvi-0, a different time course of GST-1 expression was observed, indicating a transient expression of this gene and a different reaction from the plant, consistent with the bacterial progression observed in vivo.

We also analyzed the expression of defensin (PDF 1.2) and thionin (THI), which encode well-known antimicrobial peptides with a likely role in defense

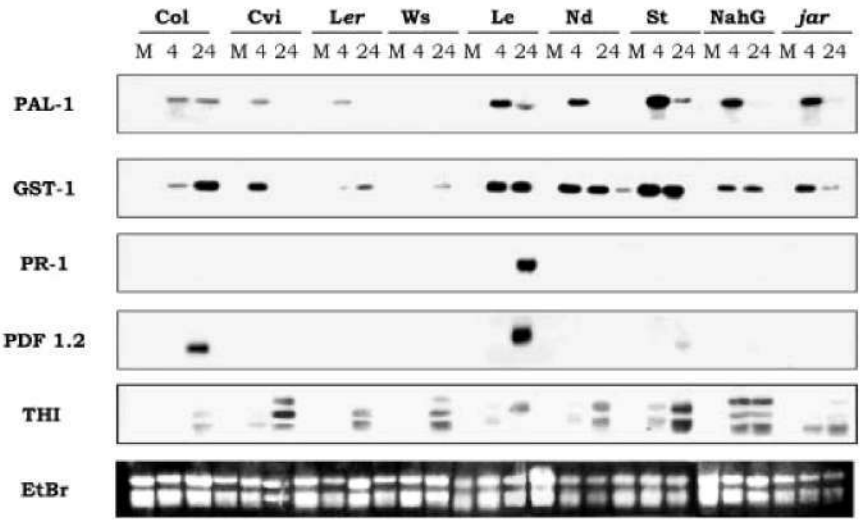


**Fig. 3** Steady-state levels of defense related genes induced in Arabidopsis in response to *E. carotovora*. Leaves from 4- to 5-week-old *Arabidopsis* ecotypes and mutants challenged with *E. carotovora* were taken at 4 and 24 hpi. RNA from infiltrated leaves was extracted and blots hybridized with probes for the following defense genes: phenylalanine ammonia lyase (*PAL*), glutathione-*S*-transferase 1 (*GST-1*), pathogenesis-related protein (*PR-1*), defensin (*PDF 1.2*) and thionin (*THI*). Ethidium bromide (*EtBr*) staining is shown as a loading control. RNA samples from three replicate experiments were analyzed in independent blots, one of which is shown. *M* Mock ($MgCl_2$ 10 mM)-inoculated leaves

(García-Olmedo et al. 1998). Induction of PDF 1.2 expression was detected at 24 hpi in Le-0 and Col-0, but not in NahG and *jar-1* plants, suggesting that both JA and SA are involved in the induction of this gene. The behaviour of the *jar-1* mutant was in agreement with that reported by Norman-Setterblad et al. (2000), whereas the behaviour of the NahG mutant was in contrast to that reported by these authors, since they found that PDF 1.2 expression is only marginally reduced in NahG plants. This apparent contradiction could be due to the fact that different bacterial strains were used in the two studies.

Thionin expression reached maximum levels at 24 hpi in all plant types but with different intensities. In all cases, more than a single band was detected, corresponding to different thionin RNA messengers recognized with this probe and may be due to the homology between different thionin genes (71% at nucleotide level for *Thi 2.1* and *Thi 2.2*) as previously reported (Epple et al. 1995). It should be pointed out that thionin expression is clearly higher in NahG plants than in Col-0 plants, and a lower mobility band, which probably corresponds to THI 2.1, seems to be specifically induced. This result suggests that SA represses thionin expression and is in accord with the fact that thionin genes are not induced by SA in *Arabidopsis*, as previously reported by Epple et al. (1995).

Induction of the PR-1 gene, used as a marker for the activation of SA-mediated defense pathways, was detected in the ecotype Le-0 at 24 hpi. This result was unexpected since defense pathways activated in the interaction with *E. carotovora* were thought to be mainly JA-dependent in the Col-0 ecotype (Norman-Setterblad et al. 2000), and SA is necessary for induction of PR-1 in response to pathogen attack (Ryals et al. 1996).

The most important conclusion of this study is that the defense response triggered by *E. carotovora* strain SCC3193 varies depending on the ecotype. Our results are in agreement with those published by Norman-Setterblad et al. (2000) for Col-0 with respect to the involvement of the JA defense pathway, revealed by the induction of defensin. However, defensin was induced only in two out of seven ecotypes tested in our work. Furthermore, our observation that PR-1 is induced in Leiden and that the NahG mutant is more sensitive than Col-0 indicates that SA plays a role in the *Arabidopsis-Erwinia* interaction.

The variability found should be taken into account for future studies of the *Arabidopsis-Erwinia* interaction, since the arbitrary election of one or another ecotype may lead to completely different results. Moreover, these results indicate that the interaction of *E. carotovora* with its hosts is more complex than previously thought.

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## References

Alamillo JM, Garcia-Olmedo F (2001) Effects of urate, a natural inhibitor of peroxinitrite-mediated toxicity, in the response of *Arabidopsis thaliana* to the bacterial pathogen *Pseudomonas syringae*. Plant J 25:529–540

Collmer A, Keen NT (1986) The role of pectic enzymes in plant pathogenesis. Annu Rev Phytopathol 24:383–409

Delaney TP, Uknes S, Vernooij B, Friedrich L, Weymann K, Negrotto D, Gaffney T, Gut-Rella M, Kessmann H, Ward E, Ryals J (1994) A central role of salicylic acid in plant disease resistance. Science 266:1247–1250

Dixon RA, Paiva NL (1995) Stress-induced phenylpropanoid metabolism. Plant Cell 7:1085–1097

Dong X (1998) SA, JA, ethylene and disease resistance in plants. Curr Opin Plant Biol 1:316–323

Dong X, Mindrinos M, Davis KR, Ausubel FM (1991) Induction of *Arabidopsis* defense genes by virulent and avirulent *Pseudomonas syringae* strains and by a cloned avirulence gene. Plant Cell 3:61–72

Epple P, Apel K, Bohlmann H (1995) An *Arabidopsis thaliana* thionin gene is inducible via a signal transduction pathway different from that for pathogenesis-related proteins. Plant Physiol 109:813–820

García-Olmedo FJ, Molina A, Alamillo JM, Rodríguez-Palenzuela P (1998) Plant defense peptides. Biopolym Pept Sci 47:479–491

Maleck K, Dietrich RA (1999) Defense on multiple fronts: how do plants cope with diverse enemies? Trends Plant Sci 4:215–219

McDowell JM, Dangl JL (2000) Signal transduction in the plant immune response. Trends Biol Sci 25:79–82

Miguel E, Poza-Carrión C, López-Solanilla E, Aguilar I, Llama-Palacios A, García-Olmedo F, Rodríguez-Palenzuela P (2000) Evidence against a direct antimicrobial role of $H_2O_2$ in the infection of plants by *Erwinia chrysanthemi*. Mol Plant Microbe Interact 13:421–429

Norman C, Vidal S, Palva ET (1999) Oligogalacturonide-mediated induction of a gene involved in jasmonic acid synthesis in response to the cell-wall-degrading enzymes of the plant pathogen *Erwinia carotovora*. Mol Plant Microbe Interact 12:640–644

Norman-Setterblad C, Vidal S, Palva ET (2000) Interacting signal pathways control defense gene expression in *Arabidopsis* response to cell-wall degrading enzymes from *Erwinia carotovora*. Mol Plant Microbe Interact 13:430–438

Palva TK, Hurtig M, Saindrenan P, Palva ET (1994) Salicylic acid induced resistance to *Erwinia carotovora* subsp. *carotovora* in tobacco. Mol Plant Microbe Interact 7:356–363

Perombelon MCM, Kelman A (1980) Ecology of the soft rot erwinias. Annu Rev Phytopathol 18:361–387

Reymond P, Farmer EE (1998) Jasmonate and salicylate as global signals for defense gene expression. Curr Opin Plant Biol 1:404–411

Ryals JA, Neuenschwander UH, Willits MG, Molina A, Steine HY, Hunt MD (1996) Systemic acquired resistance. Plant Cell 8:1809–1819

Staswick PE, Yuen GY, Lehman CC (1998) Jasmonate signaling mutants of *Arabidopsis* are susceptible to the soil fungus *Pythium irregulare*. Plant J 15:747–754

Thomma BPHJ, Penninckx IAMA, Broekaert WF, Cammue BPA (2001) The complexity of disease signaling in *Arabidopsis*. Curr Opin Immunol 13:63–68

Uknes S, Mauch-Mani B, Moyer M, Potter S, Williams S, Dincher S, Chandler D, Slusarenko A, Ward E, Ryals J (1992) Acquired resistance in *Arabidopsis*. Plant Cell 4:645–656

Vidal S, Ponce de León I, Denecke J, Palva ET (1997) Salicylic acid and the plant pathogen *Erwinia carotovora* induce defense genes via antagonistic pathways. Plant J 11:115–123

Vidal S, Eriksson ARB, Montesano M, Denecke J, Palva ET (1998) Cell wall-degrading enzymes from *Erwinia carotovora* cooperate in the salicylic acid-independent induction of a plant defense response. Mol Plant Microbe Interact 11:23–32