Regulation of ethylene biosynthesis in virus-infected tobacco leaves

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Proefschrift

Ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. C. C. Oosterlee,
hoogleraar in de veeteeltwetenschap,
in het openbaar te verdedigen
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STELLINGEN

1. Zowel de basale als de door virusinfektie geïnduceerde ethyleenprodukties in tabaksbladeren verlopen volledig via de methionine-biosyntheseweg.

   Dit proefschrift (Hoofdstukken I en II).

2. Het *N* gen in tabak dat verantwoordelijk is voor de overgevoeligheidsreaktie na infektie met enigerlei stam van tabaksmozaïekvirus, is slechts betrokken bij de initiatie en niet bij de realisatie van deze reaktie.

   Dit proefschrift (Hoofdstuk VI).

3. Slechts een deel van de fotosynthetische elektronentransportketen is betrokken bij de lichtremming van de omzetting van 1-aminocyclopropaan-1-carboxyl-zuur in ethyleen, en deze lichtremming kan niet verklaard worden door oxidatie van SH-bevattende enzymen welke essentieel zijn voor deze omzetting.

   Dit proefschrift (Hoofdstuk V).
   Gepstein, S. en Thimann, K.V. (1980) Planta *149*, 196-199.

4. Omdat het niet mogelijk is om met behulp van remmers van de DNA-afhankelijke RNA-synthese, zoals actinomycine D, in tabak de overgevoeligheidsreaktie op tabaksmozaïekvirus te doen overgaan in een systemische infektie treedt bij het tot expressie komen van de overgevoeligheidsreaktie geen transkriptie van het *N* gen op.

   Van Loon, L. C. (1981) in: Active defence mechanisms in plants (R. K. S. Wood, red.) pp. 247-274 Plenum Press, Londen.

5. Het fysiologisch belang van het door Konze beschreven systeem, waarbij 1-aminocyclopropaan-1-carboxylzuur *in vitro* wordt omgezet in ethyleen, is twijfelachtig.

   Konze J. R. en Kende, H. (1979) Planta *146*, 293-302.

6. De accumulatie van phytoalexinen is een van de reakties van de plant op stress die primair bijdragen tot verhoging van het aspecifieke resistentieniveau.

7. Het achterwege blijven van een afweerreaktie in wortels van leguminosen na infektie door Rhizobiumbakteriën kan verklaard worden door remming van de ethyleenproduktie door rhizobitoxine.

   Owens, L. D., Lieberman, M. en Kunishi, A. (1971) Plant Physiology *48*, 1-4.

8. Bij bestudering van het effekt van toevoer van fotosynthaten naar de wortelknollen in erwten op de relatieve efficiëntie van de stikstofbinding hebben Bethlenfalvay en Phillips zich niet gerealiseerd dat hun resultaten in tegenspraak zijn met bepalingen aan geïsoleerd nitrogenase.

   Bethlenfalvay, G. J. & Phillips, D. A. (1977) Plant Physiology *60*, 419-421.

9. Het produktieve effekt van een vaccin dient niet te worden afgemeten aan de stijging in antistoftiter na vaccinatie.

   Rijkers, G. T. (1980) Proefschrift, Wageningen.

10. Sportvissen is onsportief.

11. Vers is ten onrechte langer houdbaar geworden.

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met dank aan mijn ouders

voor Marianne

Voorwoord

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- Dorothée Brandenburg, jij hebt met grote inzet op een voortreffelijke manier een flink deel van het praktische werk voor je rekening genomen.
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- Manette Schönfeld die in het kader van haar doktoraalstudie aan het onderzoek heeft meegewerkt.

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Inhoudsopgave

Algemene inleiding

Wanneer tabak (*Nicotiana tabacum* L.) geïnfekteerd wordt door tabaksmozaïekvirus (TMV) kunnen zich verschillende typen van symptomen ontwikkelen (Bos, 1978). Enerzijds kan het virus zich sterk vermenigvuldigen en verbreiden door de gehele plant. Hierbij ontstaan veelal de klassieke mozaïeksymptomen, lichtgroen/donkergroen schakeringen in de jonge, zich ontwikkelende bladeren. Anderzijds kan zowel de vermeerdering als de verspreiding van het virus sterk beperkt blijven, waarbij het weefsel rondom de infectieplaats snel afsterft. Dit leidt in de praktijk tot resistentie tegen het virus. Het al dan niet optreden van zo'n nekrose-vormende overgevoeligheidsreaktie ("hypersensitive reaction") is afhankelijk van de genetische konstitutie van zowel de waardplant als het virus (Van Loon, 1980). Van tabak zijn verschillende natuurlijke en door kruising en selektie verkregen variëteiten beschikbaar, die verschillend reageren op infektie met de gewone stam van TMV. Daarnaast zijn er van TMV diverse stammen beschreven die verschillen in de symptomen die ze bij één en dezelfde tabakskultivar teweeg brengen. De overgevoeligheidsreaktie is niet exklusief voor TMV. Zij treedt bijvoorbeeld ook op na infektie van tabak met enkele andere virussen zoals het tabaksnekrosevirus (TNV) en na infektie met bepaalde schimmels en bakteriën (Goodman *et al.*, 1967).

Genetische aspekten van tabak in verband met de symptoomontwikkeling na infektie met TMV

Algemeen wordt aangenomen dat tabak ontstaan is uit een amphidiploïd van de huidige soorten *Nicotiana sylvestris* Spegazzini en Comes en *Nicotiana tomentosiformis* Goodspeed (Valleau, 1952; Holmes, 1955). Het al dan niet optreden van een nekrotische overgevoeligheidsreaktie in tabak na infektie met TMV wordt bepaald door een groep allelen aangeduid met varianten van de letter "n" (=necrose): N' (=EN), n^s, n' of n. Hierbij zijn de cultivars die het N' gen bezitten resistent tegen alle stammen van TMV behalve U1; het n^s gen geeft slechts een overgevoeligheidsreaktie na infektie met enkele zwakke stammen van TMV zoals Holmes' Ribgrass (U8) (Van Loon, 1972).

Na infektie met de agressieve stam U1 werden vroeger in alle tabaksvariëteiten de klassieke mozaïeksymptomen waargenomen. Dit vormde een ernstig praktijkprobleem. In 1929 ontdekte Holmes dat *Nicotiana glutinosa* L. en enkele andere *Nicotiana*-soorten met nekrotische vlekken reageerden op TMV U1. Het vermogen van deze soorten om overgevoelig te reageren op alle stammen van TMV werd toegeschreven aan het dominante *N*-alleel dat in *N. tabacum* niet voorkomt. In 1931 toonde Samuel aan dat de fenotypische expressie van het *N*-gen temperatuurgevoelig is; boven 28°C worden planten die het *N*-gen bezitten systematisch geïnfecteerd.

In 1938 verkreeg Holmes uit de variëteit Samsun van *N. tabacum* via de amphidiploïd *N. digluta* (*N. glutinosa* X *N. tabacum*) en na voortdurend terugkruisen met de oorspronkelijke cultivar Samsun, de substitutielijn Samsun NN, een nieuwe *N. tabacum* variëteit,

waarin beide H-chromosomen, met daarop het recessieve "n" gen, vervangen zijn door de functioneel homeologe Hg-chromosomen van *N. glutinosa* met daarop het dominante *N*-gen.

Uit recent onderzoek van Spurr & Burk (1979) is gebleken dat het vermogen om overgevoelig te reageren op infektie met TMV inderdaad een monogene eigenschap is. Deze onderzoekers beschreven de tabakskultivars "Aurea S" en "Aurea N", welke lijnen afgezien van hun verschillende monogene reaktie op TMV volledig isogeen zijn.

In Tabel 1 is een overzicht gegeven van de in dit onderzoek gebruikte waard/viruskombinaties en de daarbij optredende symptomen.

Tabel 1
Symptoomexpressie in Samsun en Samsun NN tabak na infektie met verschillende stammen van tabaksmozaïekvirus (TMV) of tabaksnekrosevirus (TNV).

Virus	Tabakskultivar	
	Samsun	Samsun NN
TMV W U1	systemisch lichtgroen/ donkergroen mozaïek	lokale bruine nekrotische vlekken
TMV Holmes' Ribgrass	semi-systemische gele kringvlekken	uitsluitend op jonge bladeren kleine witte nekrotische vlekjes
TNV	lokale bruine nekrotische vlekken	lokale bruine nekrotische vlekken

De overgevoeligheidsreaktie

Hoewel de overgevoeligheidsreaktie specifiek is voor bepaalde kombinaties van waard en virus, vertoont ze in alle gevallen dezelfde kenmerken. Een skala van fysiologische en biochemische processen verloopt op identieke wijze in alle overgevoelige kombinaties en vertoont overeenkomst met die welke optreden bij kunstmatige veroudering, verwonding of stress (Van Loon, 1980). Uit onderzoek bij verschillende waard/viruskombinaties is gebleken dat de ongeveer 2 dagen na inokulatie optredende nekrose geen voorwaarde is voor de lokalisatie van het virus. Zo treedt bijvoorbeeld lokalisatie van boneschерpmozaïekvirus op in chlorotische vlekken bij *Tetragonia expansa* (Gáborjányi *et al*, 1971). Ook kan een nekrotische reaktie optreden zonder dat het virus gelokaliseerd wordt. Dit is bijvoorbeeld het geval bij de systemische nekrose op tabak na infektie met tabaksratelvirus (TRV). Zulk een ontkoppeling tussen nekrosevorming en viruslokalisatie is echter in de door ons onderzochte tabak/virus kombinaties niet aangetoond.

Door vergelijking van de snelheid van virusvermenigvuldiging in systemisch en in overgevoelig reagerende tabak (Otsuki *et al.*, 1972) is gebleken dat in de overgevoelig reagerende variëteit pas 24 uur na infektie met de toename van de hoeveelheid extraheerbaar virus achterblijft bij die in de systemisch reagerende kombinatie. Dit tijdstip komt redelijk overeen met een extrapolatie naar vlekdiameter 0 van de lineaire toename van de vlekdiameter in overgevoelig reagerende tabak, die uitkomt op ongeveer 18 uur (Takahashi, 1974). De eerste fysiologische en biochemische veranderingen zijn echter pas waarneembaar vanaf enkele uren voor het verschijnen van de vlekken, ongeveer 48 uur na inokulatie.

Specifiek voor de overgevoeligheidsreaktie zijn de volgende biochemische veranderingen:

- Reeds kort vóór of bij het verschijnen van de lokale vlekken treedt, als gevolg van de sterke aktiviteitstoename van de sleutelenzymen fenylalanine ammonia-lyase en kaneelzuur-4-hydroxylase, een stimulering van de aromatische biosynthese op (Legrand *et al.*, 1976).
- Niet alleen in de geïnokuleerde bladeren, maar in de hele plant neemt na het verschijnen van de vlekken de peroxidase-aktiviteit toe. Bovendien wordt in de geïnokuleerde bladeren een nieuw peroxidase-isoënzym aangetroffen (Van Loon & Geelen, 1971).
- Vanaf het optreden van de lokale vlekken, verschijnt in de geïnokuleerde bladeren, en enkele dagen later ook in de andere plantedelen, een tiental nieuwe eiwitcomponenten (Van Loon & Van Kammen, 1970; Van Loon, 1972). Deze eiwitten zijn later "pathogenesis-related proteins (PR's)" genoemd (Antoniw *et al.*, 1980). Zij onderscheiden zich van de normale plante-eiwitten doordat zij bij pH 3 selectief geëxtraheerd kunnen worden en resistent zijn tegen proteolytische afbraak. Enzymatische aktiviteit van deze, voornamelijk extracellulair gelokaliseerde eiwitten is tot dusver niet aangetoond (Van Loon, 1981).
- Vlak vóór het verschijnen van de lokale vlekken vindt een enorme stimulering van de ethyleenproduktie plaats (Pritchard & Ross, 1975).
- Al vrij vroeg in de overgevoeligheidsreaktie (zelfs vanaf ongeveer 7 uur voor het zichtbaar worden van de vlekken) neemt de uitlek van ionen en dus de permeabiliteit van de membranen aanzienlijk toe (Weststeyn, 1978).

Niet geïnokuleerde plantedelen vertonen geen symptomen en er kan geen virus in worden aangetoond. Niettemin blijkt dat als gevolg van de eerdere infektie van de plant na een tweede, zgn. "challenge" inokulatie met TMV van nog ongeïnfekteerde bladeren de vlekken op deze bladeren significant kleiner blijven en meestal ook minder talrijk zijn. Dit verschijnsel wordt verworven systemische resistentie genoemd. Ross (1966) heeft aannemelijk gemaakt dat het mechanisme van viruslokalisatie in systemisch resistente bladeren identiek is met het ook na een eerste infektie optredende lokalisatiemechanisme. In systemisch resistente bladeren zou dit lokalisatiemechanisme sneller en in sterkere mate geaktiveerd worden. Deze bladeren vertonen een verhoogde peroxidaseaktiviteit en bevatten (relatief kleine hoeveelheden) PR's. Door Van Loon (1976) is aangetoond dat de verhoogde peroxidaseaktiviteit geen rol speelt bij de verworven resistentie. Hoewel er een goede korrelatie bestaat tussen de resistentie en de aanwezigheid van PR's, is nog niet bewezen dat hun aanwezigheid oorzakelijk met deze resistentie verbonden is (Van Loon, 1980).

Ethyleenproduktie tijdens de overgevoeligheidsreaktie.

Het plantehormoon ethyleen is betrokken bij de regulatie van een groot aantal processen in verschillende stadia van groei en ontwikkeling. Door Abeles (1973) en Lieberman (1979) werden de effecten van ethyleen uitvoerig beschreven. Vrijwel alle planteweefsels zijn in staat geringe hoeveelheden ethyleen te produceren. In bepaalde stadia van groei en ontwikkeling kan de produktie sterk toenemen (bv. in rijpend fruit, verwelkende bloemen of bij abscissie van i.h.b. knoppen). Ethyleenproduktie kan ook sterk gesti-

Tabel 2
Overzicht van de literatuurgegevens met betrekking tot de ethyleenproduktie in met virus geïnfekteerde planten.

Referentie	combinatie waardplant/ virus	Symptomen[a]	C_2H_4 produktie	
			wel/niet gestimuleerd	vóór/na optreden van symptomen
Ross & Williamson (1951)	Diverse combinaties, o.a. N. tabacum, N. glutinosa, Physalis floridana, Phaseolus vulgaris/TMV, lucernemozaïekvirus, tabakskringvlekkenvirus, aardappelvirus X of Y.	s.m.	niet	
		of		
		l.n.v.	wel C_2H_4??	
Balázs et al. (1969)	N. tabacum/komkommermozaïekvirus	s.m.	niet	
	N. tabacum/TMV	l.n.v.	wel	
Nakagaki et al. (1970)	N. glutinosa, N. tabacum (cv. Samsun nc.), Phaseolus vulgaris/TMV	l.n.v.	wel	na
	N. tabacum (cv. Bright Yellow)/ TMV	s.m.	niet	
Gáborjányi et al. (1970)	N. tabacum (cv. Xanthi nc.)/TMV	l.n.v.	wel	voor
	Tetragonia expansa/bonescherpmozaïekvirus	l.c.v.	wel	
Pritchard & Ross (1975)	N. tabacum (cv. Samsun NN)/TMV	l.n.v.	wel	voor
Koch et al. (1980)	Beta vulgaris/bietenvergelingsvirus of bietenmozaïekvirus	s.m.	niet	

a systematisch mozaïek = s.m.; lokale nekrotische vlekken = l.n.v.; lokale chlorotische vlekken = l.c.v.

muleerd worden door externe faktoren zoals kunstmatig met diverse chemicaliën, inclusief auxinen en andere regulatoren, en in het algemeen als reaktie op stress.

Al in 1951 toonden Ross & Williamson aan dat er tijdens de overgevoeligheidsreaktie van verschillende planten op virusinfektie een "fysiologisch aktieve komponent" vrijkwam. Later is door verschillende onderzoekers dit verschijnsel nader onderzocht in diverse waard/viruskombinaties (Tabel 2). Door Balázs *et al* (1969), Nakagaki *et al* (1970) en Gáborjányi *et al* (1971) werd aangetoond dat het hier het plantehormoon ethyleen betrof.

Uit de kinetiek van de ethyleenproduktie van al dan niet afgeplukte bladeren konkludeerden sommige onderzoekers dat de ethyleenproduktie begint ná, en het gevolg is van, de vorming van nekrotische vlekken (Nakagaki *et al.*, 1970). Anderen vonden reeds een toename in de ethyleenproduktie vóórdat vlekken zichtbaar werden (Gáborjányi *et al.*, 1971); Pritchard & Ross, 1975). Pritchard & Ross (1975) suggereerden dat het door infektie geïnduceerde ethyleen de vlekontwikkeling zowel kan stimuleren als remmen. Deze effekten van ethyleen bleken o.a. afhankelijk van lokale koncentraties en van het moment van produktie.

Van de biochemische en fysiologische processen welke karakteristiek zijn voor de overgevoeligheidsreaktie blijkt een aantal door ethyleen te kunnen worden gereguleerd.

Zo zijn de toename van membraanpermeabiliteit (Lyons & Pratt, 1964), de stimulering van de aromatische biosynthese (Abeles, 1973) en de induktie van bepaalde andere enzymaktiviteiten (Gahagan 1968; Abeles & Florrence, 1970; Abeles & Holm, 1966) als fysiologische effekten van ethyleen beschreven. Mede gezien het feit dat, wellicht met uitzondering van de toename in membraanpermeabiliteit, de lokale ethyleenproduktie het vroegst optredende fenomeen is tijdens de overgevoeligheidsreaktie, wordt verondersteld dat het lokaal geproduceerde ethyleen functioneel bij de pathogenese betrokken is (Pritchard & Ross, 1975). Deze hypothese werd in hoge mate versterkt door onderzoek van Van Loon (1977). Door blaadjes aan te prikken met naalden die gedoopt waren in ethephon (2-chloorethylfosfonzuur), waaruit in de plant ethyleen wordt vrijgemaakt, konden nagenoeg alle met het optreden van lokale vlekken gekorreleerde fysiologische en biochemische veranderingen worden nagebootst: lokale nekrosen, induktie van PR's, kwantitatieve en kwalitatieve veranderingen in peroxidasen, zelfs de induktie van systemische resistentie tegen virusinfektie. Hieruit bleek dat de sterke lokale ethyleenproduktie verantwoordelijk gesteld kan worden voor de veranderingen in het metabolisme tijdens de overgevoeligheidsreaktie.

Biosynthese van ethyleen

Bij het onderzoek naar de prekursor van ethyleen werd aanvankelijk gebruik gemaakt van niet-fysiologische modelsystemen. In aanwezigheid van metaalionen en een vrijeradikalengenererend systeem was men aldus in staat linoleenzuur, methionine, methional, propanal en keto-methylthiobutrytaat in ethyleen om te zetten (Lieberman, 1979). Later werd m.b.v. tracer-experimenten en het gebruik van specifieke remmers zoals rhizobitoxine (Lieberman *et al*, 1975) de ethyleenproduktie *in vivo* bestudeerd.

In rijpend fruit en verwelkende bloemen werd aangetoond, dat methionine de belangrijkste prekursor is (Yang, 1974). Door Kato (1976) werd echter gesuggereerd dat het ethyleen dat tijdens de overgevoeligheidsreaktie van cowpea op komkommermozaiekvirus infektie geproduceerd wordt, gevormd zou worden door peroxidatie van linoleenzuur.

Adams & Yang toonden in 1977 in appelweefsel aan dat S-adenosylmethionine (SAM) een intermediair is in de omzetting van methionine naar ethyleen. In 1979 werd door dezelfde groep de ethyleenbiosyntheseweg volledig opgehelderd: 1-aminocyclopropaan-1-carboxylzuur (ACC) bleek de direkte ethyleenprekursor, en de aktiviteit van het ACC-producerend enzym, ACC-synthase, bleek snelheidsbepalend voor de ethyleenproduktie (zie fig. 1). De rol van ACC als direkte prekursor van ethyleen en als intermediair

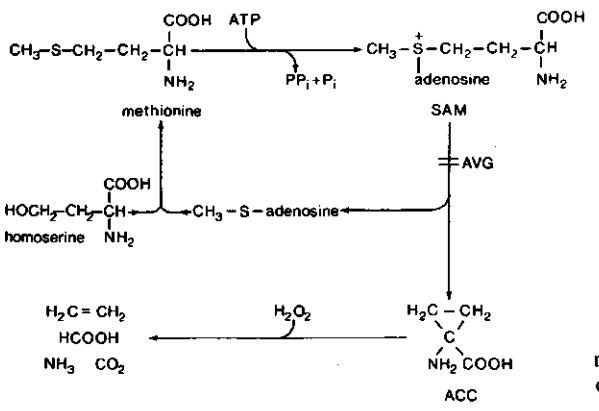

Fig. 1. Biosynthese van ethyleen. SAM=S-adenosylmethionine; AVG=aminoethoxyvinylglycine; ACC=1-aminocyclopropaan-1-carboxylzuur.

in de omzetting van methionine naar ethyleen werd gelijktijdig in een onafhankelijk onderzoek vastgesteld door Lürssen (1979).

Probleemstelling

De probleemstelling bij dit onderzoek omvat de vraag a) hoe de zeer snelle, sterke ethyleenafgifte tot stand komt en wordt gereguleerd, b) in hoeverre deze gerelateerd is aan de aktiviteit van het *N*-gen dat verantwoordelijk is voor de overgevoeligheids reaktie, en c) of deze lokale ethyleenproduktie funktioneel bij de viruslokalisatie en de induktie van systemische resistentie betrokken is.

In de hoofdstukken I en II is nagegaan in hoeverre methionine de voorloper is van ethyleen in zowel gezonde als overgevoelig op TMV-infektie reagerende tabaksbladeren. Naar analogie met het werk van Hanson & Kende (1976) werden hiertoe in met L-[U-^{14}C]-methionine gemerkte bladeren de specifieke radioaktiviteiten van enerzijds de methionine-pool en anderzijds het geproduceerde ethyleen met elkaar vergeleken.

Hoofdstuk I is toegespitst op de problemen die zich kunnen voordoen bij de kwantitatieve bepaling van een prekursor/produktrelatie, wanneer sprake is van een niet-homogene verdeling van de gemerkte prekursor en/of de plaats in het blad waar het produkt gevormd wordt.

In hoofdstuk II wordt naar aanleiding van de in het eerste hoofdstuk verkregen informatie de labelingsprocedure aangepast. Hierdoor, en door gebruik te maken van aminoethoxyvinylglycine (AVG), een methionine-analoog die specifiek de omzetting van SAM in ACC blokkeert, is de rol van methionine als voorloper van zowel de basale als de door virusinfektie geïnduceerde ethyleenproduktie vastgesteld.

Hoofdstuk III is gewijd aan de regulatie van de ethyleenbiosynthese in overgevoelig reagerende tabak. Het verloop van de koncentraties van metionine, SAM en ACC, en van de aktiviteiten van de verschillende bij de ethyleenproduktie betrokken reakties zijn hiertoe bepaald.

In hoofdstuk IV is onderzocht hoe de ethyleenproduktie gereguleerd is in genetisch verschillende waard/viruskombinaties. Op deze manier is bepaald of de stimulering van de ethyleenproduktie a) gerelateerd is aan de genetische konstitutie van de waard (bv. al dan niet in bezit van het *N*-gen), b) specifiek is voor bepaalde virussen, of c) verbonden is met de symptoomexpressie. Bovendien is getracht een faktor te vinden die verantwoordelijk zou kunnen zijn voor de initiatie van de verhoging van de ethyleenproduktie tijdens de overgevoeligheidsreaktie. Daarnaast is de regulatie van de ethyleenproduktie vergeleken in bladeren met en zonder verworven resistentie om een inzicht te krijgen in het mechanisme van de systemische resistentie.

De laatste stap in de ethyleenproduktie, de omzetting van ACC in ethyleen, bleek sterk geremd te worden door licht. De oorzaak hiervan en de konsequenties voor de door TMV gestimuleerde ethyleenproduktie zijn behandeld in hoofdstuk V.

In hoofdstuk VI is de invloed van een aantal faktoren (temperatuur, lichtkondities en bladleeftijd) op zowel de stimulering van de ethyleenproduktie als de ontwikkeling van lokale vlekken bestudeerd in Samsun NN-tabak na infektie met TMV. Getracht is om hieruit konklusies te trekken omtrent de betekenis van de vroege en late stimulering van de ethyleenproduktie voor de viruslokalisatie.

De resultaten van dit onderzoek worden nader bediskussieerd in de algemene diskussie. De nadruk is hierbij gelegd op de betekenis van de stimulering van de ethyleenproduktie in de overgevoeligheisreaktie en de funktie van het *N* gen.

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HOOFDSTUK I

Complications in Interpreting Precursor/Product Relationships by Labeling Experiments Methionine as the Precursor of Ethylene in Tobacco Leaves*)

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Summary

The role of methionine as the precursor of ethylene in healthy and tobacco mosaic virus-infected tobacco leaves was studied by comparison of the specific radioactivities of methionine and ethylene after labeling leaves with L-[U-^{14}C]methionine by petiolar uptake.

The specific activity of the ethylene produced was far less than expected on the basis of the methionine pool size within the leaves and the amount of label applied. This was caused, on the one hand, by an irreversible binding of the applied methionine to the cell wall fraction and, on the other hand, its rapid metabolization.

The rise in ethylene production due to virus infection was associated with a corresponding decrease in specific activity, suggesting a second precursor or another (unlabeled) methionine pool to be used for virus-induced ethylene production.

However, virus-induced ethylene production occurred only in the interveinal leaf parts where the specific radioactivity of methionine was (relatively) low. Hence, the decrease in the specific radioactivity of the ethylene resulted from the nonhomogeneous distribution of the applied ^{14}C-labeled methionine within the leaf rather than from another precursor.

Key words: Nicotiana tabacum, ethylene, methionine, virus infection, TMV.

Introduction

Methionine is a main precursor of ethylene in higher plants through a pathway involving S-adenosylmethionine (SAM) and 1-aminocyclopropane-1-carboxylic acid (ACC) (Adams and Yang, 1979). Such ethylene synthesis is blocked by aminoethoxyvinylglycine (AVG), which specifically inhibits the conversion of SAM into ACC (Lieberman, 1975). The large burst of ethylene production by tobacco leaves

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Abbreviations: ACC = 1-aminocyclopropane-1-carboxylic acid; AVG = aminoethylvinylglycine; SAM = S-adenosylmethionine; TMV = tobacco mosaic virus.

reacting hypersensitively to infection with tobacco mosaic virus (TMV) by the formation of local necrotic lesions, is inhibited for 95 % by AVG (De Laat et al., 1981). This suggests that most, if not all of the increased production of ethylene is derived from methionine.

However, labeling experiments with L-[U-^{14}C]methionine, involving comparison of the specific activities of the methionine pool and of the ethylene produced, showed consistent deviations from the expected values, inferring that methionine might not be the main precursor. Explanations for these discrepancies were sought by examining the fate of the labeled methionine after uptake by the leaf and by taking into account the localized nature of the increase in ethylene production after TMV infection.

Materials and Methods

Tobacco plants (*Nicotiana tabacum* L. cv. Samsun NN) were grown in a greenhouse for 10 or 11 weeks as described before (De Laat et al., 1981). Almost fullgrown leaves were selected, dusted with carborundum, inoculated by rubbing them with a gauze pad with either water or TMV (strain WU1) at a concentration ensuring maximal stimulation of ethylene production, and rinsed with tap water. At specific times leaves were detached for experimental use.

Leaves were labeled by putting a droplet containing the desired amount of L-[U-^{14}C] methionine (285 mCi/mmol; The Radiochemical Centre, Amersham, England) on the cut surface of the petiole. After uptake, the leaf was placed in a 5 mM solution of KCl for 20 h to avoid excessive adsorption of the labeled methionine to the conducting vessels (Hill-Cottingham et al., 1973).

Uptake and distribution of the label were studied by macroautoradiography and by combustion of different leaf parts in a Packard Sample Oxidizer. For macroautoradiography, a leaf was put between two pieces of cardboard and quickly frozen in liquid nitrogen. A Kodak X-ray film was pressed onto the leaf and exposed for 10 days at –20 °C. Due to the relative thickness of the veins, contact between the film and leaf was not assured for all leaf parts.

For determination of the specific activity of the methionine pool within the leaves free amino acids were extracted with either water or 70 % methanol and analyzed with a Beckman amino-acid auto-analyzer (type 4255), as described earlier (De Laat et al., 1981). The concentration of free methionine was determined by ninhydrin reagent; methionine-containing fractions eluted from the amino-acid auto-analyzer were collected and tested for radioactivity by liquid scintillation counting. Corrections for counting efficiency were made by using an external standard.

For determination of the specific radioactivity of the ethylene produced, leaves or leaf halves were incubated in 750 ml water-locked Petri dishes under controlled conditions (20 °C, photoperiod 16 h). The ethylene produced was quantitatively trapped by mercuric perchlorate. Part of the trapping solution was used for determining ethylene production; the other part was used for measuring radioactivity in ethylene. The specific radioactivity of the ethylene produced was then calculated as outlined earlier (De Laat et al., 1981). If all the ethylene produced is derived from methionine, the specific activity of the ethylene is expected to be 0.4 times that of the methionine pool, because two out of the five ^{14}C-atoms of methionine end up in ethylene.

Results and Discussion

Fate of L-[U-^{14}C]methionine in the non-infected leaf

Since vacuum infiltration induced stress ethylene and influenced the hypersensitive reaction of tobacco leaves to TMV infection by delaying lesion appearance by about 10 h, leaves were labeled by uptake through the petiole. After uptake, radioactive ethylene was produced during the subsequent 24 h. However, taking into account the amount of label taken up by the leaf (10^6 dpm = c. 1.4 nmol) and the total pool of methionine within the leaf (about 100 nmol), the amount of label found in ethylene was about 100 times less than expected if methionine were the only precursor (Table 1).

Table 1: Comparison of, on the one hand, the specific radioactivity in ethylene espected on the basis of the methionine pool size in the leaf and the amount of L-[U-^{14}C]methionine applied and, on the other hand, the specific radioactivity found in the ethylene produced.

methionine pool size	115 nmol/leaf
amount of L-[U-^{14}C]methionine applied	10^6 dpm = c. 1.4 nmol
specific radioactivity methionine (calculated)	8700 dpm/nmol
specific radioactivity C_2H_4 expected	3480 dpm/nmol
specfic radioactivity C_2H_4 found	44 dpm/nmol

Possible explanations for this observation were investigated. When whole leaves were extracted with either water or 70 % methanol after the 20 h period on KCl, recovery of the label was only 20 to 40 % of the amount taken up by the petiole. Since addition of 10 mM unlabeled methionine did not liberate any of the remaining activity from the residue after the initial extraction with water, it can be assumed that this methionine is not available for ethylene production. Further extraction of the residue with 2 % Triton-X-100 liberated 10 to 15 % of the remaining radioactivity, apparently from membranes. Successive extractions with 0.5 M HCl, 5 M HCl, 0.5 M NaOH, and 5 M NaOH, each for 30 min at 80 °C, destroyed the cell walls and liberated all of the remaining 50 %.

Amino acid analysis of the 70 % methanol extracts from labeled leaves revealed that radioactive methionine was quickly metabolized. Only 5 to 10 % of the radioactivity in the extracts made 20 h after the ^{14}C-pulse, was still eluted at the methionine position.

Autoradiography of intact leaves 20 h after uptake of the L-[U-^{14}C]methionine revealed that the distribution of the label within the leaf was far from uniform (Fig. 1); separation of the veins and the interveinal tissue and combustion of leaf parts showed that, particularly in the midrib proper, radioactivity was considerably higher than in the interveinal tissue.

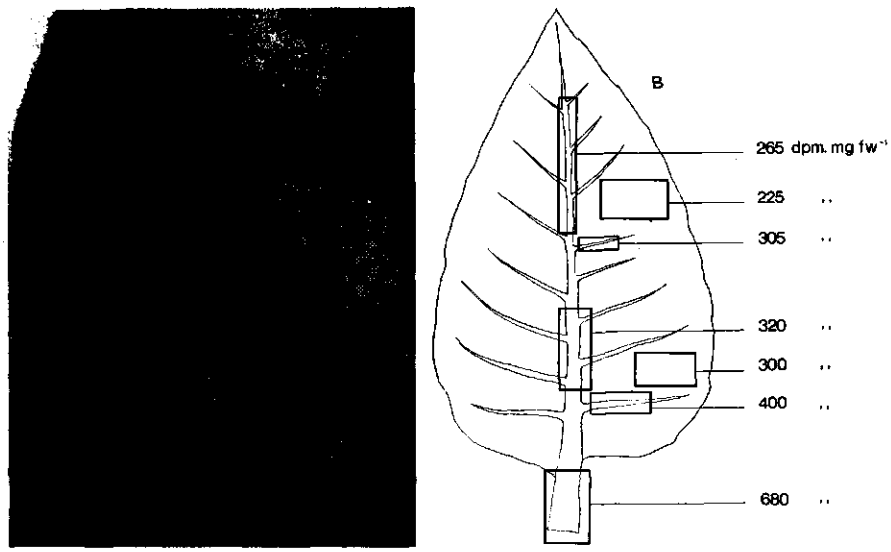

Fig. 1: Distribution of radioactivity in a tobacco leaf 20 h after labeling with 2 μCi L-[U-^{14}C]methionine by petiolar uptake. A) Autoradiogram of a leaf part. The labeled leaf was quickly frozen in liquid nitrogen and exposed to a Kodak X-ray film at −20 °C. Contact of the leaf to the film was not assured for all leaf parts. B) Radioactivity per g fresh weight estimated by combustion of different leaf parts in a Packard Sample Oxidizer.

Comparison of the specific radioactivities of methionine and ethylene in non-infected and TMV-infected leaves

After TMV infection, lesions develop only in the interveinal areas of the leaf, and ethylene is produced mainly by the tissue surrounding the local lesions. Because after uptake through the petiole a major part of the labeled methionine was held back in the main veins (cf. Fig. 1), the specific radioactivity of the ethylene produced by the leaf was compared with the specific radioactivity of the methionine pool within the interveinal tissue. To this end, the main veins were removed before the extraction of the amino acids.

Leaves were labeled for 20 h one day after inoculation and incubated afterwards for 24 h. Since under these conditions changes in the methionine pool were found to be minor, the specific radioactivity of the methionine was calculated as the average of the specific activities at the beginning and at the end of the incubation period during which the ethylene was trapped.

As demonstrated in Table 2, ethylene production of the virus-infected leaves was increased about 20-fold. However, the specific radioactivity of this ethylene was about ten-fold lower than that of the ethylene produced by the water-inoculated controls, whereas the specific radioactivity of the methionine pool within the TMV-

Table 2: Specific activities of the methionine pool in the interveinal tissue and of the ethylene produced by water- or TMV-inoculated leaves after uptake of ^{14}C-methionine through the petiole. Data are average values from 19 pairs of leaves. The leaves were labeled for 20h one day after inoculation. Ethylene was trapped afterwards for 24 hours. The specific activity of the methionine pool is taken as the average of the specific activities at the beginning and at the end of the incubation period.

	H_2O-inoculated	TMV-inoculated
conc. methionine (nmol · g fw^{-1})	50.6	34.6
spec. act. methionine (dpm · nmol^{-1}) (in interveinal tissue)	45	104
expected spec. act. C_2H_4 (dpm · nmol^{-1})	18	42
C_2H_4 production (nmol)	0.41	9.5
spec. act. C_2H_4 (dpm · nmol^{-1})	311	37.5

inoculated leaves was two to three times higher than that in the water-inoculated ones.

The specific activity of the ethylene produced by the TMV-inoculated leaves agreed quite well with that expected if all of the ethylene were derived from methionine. However, in various experiments the specific radioactivity of the ethylene produced by the water-inoculated leaves was 9 to 38 times *higher* than expected on the basis of the specific activity of methionine in the interveinal tissue.

On the basis of these results alone, it could have been concluded that the exogenously applied, radioactive methionine was preferentially used for ethylene production in the water-inoculated leaves, implying the existence of more than one methionine pool. The large decrease in the specific activity of ethylene accompanying the increase in ethylene production during the hypersensitive reaction could then be explained either by assuming a second precursor to be involved in virus-induced ethylene production, or by a loss of methionine compartmentation, leading to the equilibration of, on the one hand, a small pool of highly-labeled methionine within the ethylene-synthesizing cell compartment (for example the cytoplasm) and, on the other hand, a large pool of less highly-labeled methionine pool in a different compartment (e.g. the vacuole). Such a model for the regulation of ethylene production in ageing flowers was proposed by Kende and Baumgarter (1974) who suggested that the availability of methionine in the ethylene-synthesizing cell compartment would be a regulatory factor in ethylene production. Such a model might also apply to TMV-infected tobacco leaves, because Weststeyn (1978) found increased leakage from tobacco leaf discs at a very early stage of the hypersensitive reaction.

However, as an alternative explanation, it could be envisaged that the differences between the specific activities of the ethylene produced by the water- and the TMV-inoculated leaves resulted from the nonhomogeneous distribution of the labeled methionine within the leaf. Therefore, the contributions of the main veins and the interveinal tissue to the production of ^{14}C-labeled ethylene were compared after

physical separation, immediately following labeling, by cutting out the interveinal tissue alongside the main veins (De Laat et al., 1981). Under these conditions, the veins were found to contribute substantially (about 35 %) to the total ethylene production of the non-infected leaves. Since a major part of the labeled methionine was held back in the veins, the specific radioactivity of the ethylene produced by this leaf part was about 100 times higher than that of the interveinal tissues (Table 3).

Table 3: Comparison of the specific radioactivities of the methionine pools within, and the ethylene produced by the main veins and the interveinal tissue after labeling water- or TMV-inoculated leaves with L-U-^{14}C methionine by petiolar uptake.

		main veins	interveinal tissue
H_2O-inoculated	spec. act. methionine (dpm/nmol)	925	23
	C_2H_4 production (nmol)	0.97	1.35
	spec. act. C_2H_4 (dpm/nmol)	422	3.5
TMV-inoculated	spec. act. methionine (dpm/nmol)	715	43
	C_2H_4 production (nmol)	0.81	5.71
	spec. act. C_2H_4 (dpm/nmol)	525	6.0

After TMV infection ethylene production increased only in the interveinal areas where local lesions developed, reducing the contribution of the veins to the total ethylene production to no more than about 10 %. The decrease in the specific activity of the ethylene produced by TMV-infected leaves could thus be explained by the observation that a far larger part of the ethylene was derived from the interveinal tissues, which produced ethylene with a relatively low specific activity. Since this specific activity was in accordance with that of the methionine in the interveinal tissues, it can thus be concluded that all the ethylene produced during TMV-infection is derived from methionine.

Concluding remarks

These observations clearly demonstrate that irreversible immobilization and rapid metabolization of a labeled precursor in different pathways can account for lower radioactivity in a product than expected on the basis of precursor uptake. Furthermore, nonhomogeneous distribution of the labeled precursor may give anomalous results with respect to the specific activities of its products. This especially holds for situations in which the rate of conversion is unequal in different parts of an organ or even a cell, or in which certain treatments result in a nonhomogeneous stimulation of the reaction.

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HOOFDSTUK II

Regulation of Ethylene Biosynthesis in Virus-Infected Tobacco Leaves[1]

I. DETERMINATION OF THE ROLE OF METHIONINE AS THE PRECURSOR OF ETHYLENE

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ABSTRACT

The hypersensitive reaction of Samsun NN tobacco leaves to tobacco mosaic virus (TMV) was accompanied by a large increase in ethylene production, just before necrotic local lesions became visible. Normal and virus-induced ethylene production were both largely inhibited by 0.1 millimolar aminoethoxyvinylglycine indicating that methionine is a main ethylene precursor.

The contribution of methionine to ethylene production was estimated by labeling leaves with L-[U-^{14}C]methionine and comparing the specific activities of methionine within and ethylene produced by the leaf. When taken up through the petiole, methionine was largely retained in the veins, leading to production of ethylene with a far higher specific activity in the veins than in the interveinal tissue. After TMV infection, ethylene production increased only in the interveinal tissue, resulting in a decrease in specific activity of the ethylene produced. In the interveinal tissue, the specific radioactivity of the ethylene was lower than expected if methionine were the only precursor. After labeling by vacuum infiltration, the specific activities of the ethylene produced by water- and TMV-inoculated leaves were both identical and in accordance with the specific radioactivity of methionine. Inasmuch as the content of 1-aminocyclopropane-1-carboxylic acid was increased severalfold two days after TMV infection, methionine can be considered to be the only ethylene precursor in healthy and in TMV-infected tobacco leaves.

The increase in ethylene production after TMV-infection was not accompanied by an increased concentration of free methionine within the leaf. Compartmentation of methionine does not appear to be a regulating factor since labeled methionine supplied to the leaf by vacuum infiltration is equilibrated very rapidly with any methionine pool within the leaf cells.

Tobacco cultivars carrying the *N* gene react hypersensitively to TMV[2] (9). Multiplication and spread of the virus are restricted to a zone around the infection site which rapidly necroses in about 48 h. The process is terminated 5 to 8 days later when the slow expansion of these local lesions comes to a halt.

Lesion development is accompanied by a large burst of ethylene emanation (18, 19) which reaches a maximum near the time that lesions become visible, and remains elevated during subsequent lesion growth. Ethylene production does not increase in tobacco leaves infected with viruses that invade the plant systemically and do not cause necrosis (6, 17). The ethylene generated when Samsun NN tobacco reacts hypersensitively to TMV may be responsible for all ensuing physiological and biochemical changes, including the so called "systemic acquired resistance" against further virus infection (22).

Methionine is a main precursor of ethylene in higher plants (14, 24), although additional precursors and pathways may occur in specific situations, *e.g.* fruit ripening (13). Abeles and Abeles (1) found that application of toxic chemicals increased ethylene evolution from tobacco leaves and also increased the conversion of [U-^{14}C]methionine into ethylene. In a pathological situation such as a virus-induced hypersensitive reaction, other precursors might likewise be envisaged. Kato (11) considers that in cowpea reacting hypersensitively to cucumber mosaic virus, ethylene is formed by peroxidation of linolenic acid. By labeling Morning Glory flower tissue with L-[U-^{14}C]methionine, Hanson and Kende (7) demonstrated that all the ethylene evolving during flower senescence was derived from methionine. Such ethylene synthesis is blocked by treatment with AVG, which specifically inhibits the conversion of *S*-adenosylmethionine into ACC, the immediate precursor of ethylene (3, 14). These approaches were adopted to investigate: (a) whether methionine is the only precursor of ethylene in healthy tobacco leaves and (b) as to how far methionine is the precursor of the ethylene emanated in tobacco reacting hypersensitively to TMV.

MATERIALS AND METHODS

Plant Material. Tobacco plants (*Nicotiana tabacum* L. cv. Samsun NN) were grown from seed in a greenhouse; photoperiod was maintained at 16 h by additional illumination from high pressure mercury halide Philips HPI/T lamps at 18 $w \cdot m^{-2}$. Minimum temperature was 20 C during the day and 19 C at night. When plants were 10- to 11-weeks-old, almost fully-grown leaves, about 18 cm in length, were selected.

In most cases leaves were inoculated on the plant with either TMV or water as a control. Inoculation was carried out by rubbing carborundum-dusted leaves with water or purified TMV WU I (100 μg/ml) using a gauze pad, and rinsing with water. The day of inoculation is further referred to as day 0. At regular intervals leaves were detached from the plants, and incubated in growth chambers at 20 C (21).

Labeling of Leaves. Leaves were labeled by either vacuum infiltration or uptake through the petiole. Leaves were infiltrated for 30 s at 1 mm Hg with an aqueous solution of L-[U-^{14}C]-

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[2] Abbreviations: TMV, tobacco mosaic virus; AVG, aminoethoxyvinylglycine; ACC, 1-aminocyclopropane-1-carboxylic acid.

methionine. Labeling through the petiole was carried out by putting a droplet containing the desired amount of methionine on the cut surface of the petiole. After uptake, the leaves were placed in 5 mM KCl for 20 h to avoid excessive adsorption of the labeled amino acid to the conducting vessels (8).

Extraction of Free Amino Acids and Amino Acid Analysis. Preparation of samples for amino acid analysis was carried out by freezing the leaf material in liquid N_2, grinding it in a mortar with pestle, and extracting the resulting powder two times for 30 min with water or 70% methanol. The extracts were clarified by centrifugation at 30,000*g* for 5 min. The combined supernatants were taken to dryness by rotary evaporation at 30 C. The resulting residue was dissolved in 2 ml 0.2 M sodium citrate buffer (pH 2.2) containing 1% thiodiglycol, 0.2% Brij, and 0.01% caprylic acid. After clarification at 30,000*g* for 5 min, the samples were used for amino acid analysis. Recovery of methionine in the final extract was always over 95%, as determined by adding [^{14}C]methionine as an internal standard.

One-half or 1 ml of the amino acid extract was used for amino acid analysis. Analysis was performed with a Beckman amino acid auto-analyzer (type 4255) using sodium citrate buffers of pH 3.22 and 4.25. The retention time for methionine was 84 min. The methionine concentration in the eluate was determined by ninhydrin reagent. Its specific radioactivity was determined by liquid scintillation counting of the methionine-containing fraction. Corrections for counting efficiency were made using an external standard.

Measurement of Ethylene Production. Detached leaves were incubated in water-locked 750 ml Petri-dishes on wetted filter paper under controlled conditions (21). To study the effect of AVG, leaves were cut parallel to the main vein into strips of about 1 cm wide, and incubated on either water (control) or 0.1 mM AVG. At specific times, 1-ml gas samples were withdrawn through a sealed hole in the lid and injected into a gas chromatograph equipped with an alumina column and a flame ionization detector.

To determine the specific radioactivity of ethylene produced by ^{14}C-labeled leaves, a small dish, containing 2 ml 0.25 M mercuric perchlorate in 2 M $HClO_4$, was placed in the Petri dish next to the leaf to absorb the ethylene (2). Efficiency of ethylene trapping was more than 95%. No effect of the presence of mercuric perchlorate on ethylene production or lesion development was noticed. After incubation, radioactivity in 1 ml of the trapping solution was determined by liquid scintillation counting in Lumagel (Lumac Chemicals AG). The other half of the mercuric perchlorate solution was injected into a 40-ml sealed serum flask containing 1 ml of 4 M LiCl. The flask was firmly shaken for 1 min during which time the ethylene was quantitatively released (more than 98%). Two 1-ml gas samples were taken from the flask to determine the ethylene concentration. After the ethylene had been released, the radioactivity of the mercuric perchlorate/LiCl mixture was also counted. This radioactivity consists of CO_2 and other gaseous metabolites that remain bound to mercuric perchlorate. More than 85% of the radioactivity released from the mercuric perchlorate by the addition of LiCl was absorbed by a solution of 0.1 M mercuric acetate in methanol, providing proof that this radioactivity is in ethylene (2).

The specific radioactivity of ethylene was then calculated according to the following formula:

$$\text{Specific radioactivity ethylene} = \frac{\text{Radioactivity } (Hg(ClO_4)_2) - \text{radioactivity } (Hg(ClO_4)_2/LiCl) \text{ mixture}}{\text{Ethylene production}}$$

ACC Assay. Leaf material was frozen in liquid N_2 and ground in a mortar with pestle. Two ml of 5% sulfosalicylic acid were added per g fresh weight and the mixture was stirred for 30 min at room temperature. After centrifugation of the homogenate for 10 min at 10,000*g*, the concentration of ACC in the supernatant was determined by chemical conversion into ethylene according to Lizada and Yang (15).

Chemicals. L-[U-^{14}C]methionine (285 mCi/mmol) was purchased from The Radiochemical Centre (Amersham, England). AVG was from Dr. R. Maag AG, Dielsdorf, Switzerland.

RESULTS AND DISCUSSION

Ethylene Production During Pathogenesis and Effect of AVG on Ethylene Production. Figure 1A shows that inoculation of hypersensitively reacting tobacco leaves with TMV gave rise to a large burst of ethylene just before lesions became macroscopically visible. Maximal production was usually attained between 1 and 2 days after inoculation and was proportional to the number of developing lesions. Ethylene production remained elevated for the rest of the incubation period during which the lesions slowly enlarged. In contrast, ethylene production of water-inoculated leaves remained low during the whole incubation period. The elevated production during the first hours of incubation was caused by wound ethylene produced as a result of the inoculation procedure.

AVG, recently shown to inhibit ethylene production in tobacco leaf discs (4), inhibited ethylene production for more than 75% in water-inoculated, and for about 95% in TMV-inoculated leaves (Fig. 1B). Methionine appears to be the main precursor of ethylene in uninfected as well as in hypersensitively reacting tobacco leaves. AVG did not inhibit lesion formation, but interfered with resulting metabolic alterations. This agrees with the conclusion by Van Loon (23) that ethylene is not responsible for the induction of local lesions but is a causative factor in redirecting plant metabolism during the hypersensitive reaction.

Labeling by Petiolar Uptake. The role of methionine as an ethylene precursor was further studied by labeling leaves with L-[U-^{14}C]methionine and comparing the specific radioactivities of the methionine pool within the leaf and the ethylene produced. Since only two of the five C-atoms of methionine end up in ethylene, the specific radioactivity of ethylene is expected to be 0.4 times that of the methionine if methionine is the only ethylene precursor. If other compounds also serve as a precursor, the specific radioactivity of the ethylene would be correspondingly lower.

Initially, leaves were labeled by uptake through the petiole. After uptake, radioactive ethylene was produced. Such production was completely suppressed by simultaneous feeding of 0.1 mM AVG, confirming that methionine is the main precursor of ethylene in tobacco.

When leaves were labeled 1 day before inoculation with either

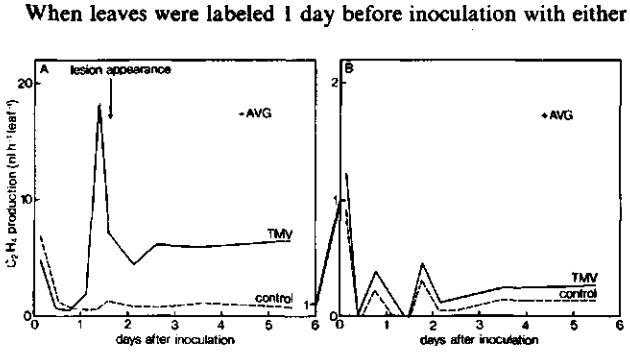

FIG. 1. Ethylene production of Samsun NN tobacco leaves incubated on water (A) or 0.1 mM AVG (B) after inoculation with water (- - -) or TMV (——). Leaf strips were incubated in water locked Petri dishes and ethylene production was measured at 8-h intervals during the first 2 days and at longer intervals during the subsequent period.

water or TMV, the specific radioactivity of the wound ethylene produced during the first day after inoculation was similar in both cases (Fig. 2). In water-inoculated leaves, the return of the ethylene production to a basal level was accompanied by a large increase in specific radioactivity. On the contrary, in TMV-infected leaves, the enhanced ethylene production by the time of lesion appearance was accompanied by a corresponding decrease in specific radioactivity of the ethylene. In both water- and TMV-inoculated leaves the specific activity of the ethylene declined at the same rate during the subsequent days, apparently due to an exhaustion of the label from the methionine pool. Taking into account the amount of labeled methionine taken up by the leaf (1 μCi = about 3.5 nmol) and the pool size of methionine within the leaf (about 100 nmol), the amount of label recovered in ethylene was only about 1% of that expected if all of the applied methionine were available for ethylene production. From autoradiography and extraction it became clear that most of the methionine taken up was held back in the veins, more than 50% of which was irreversibly bound to the cell walls, whereas the free radioactive methionine was quickly metabolized (*cf.* Table II).

Leaves were further labeled some hours after inoculation, and incubated during the subsequent day when local lesions became visible. Since changes in the methionine pool were found to be minor under these conditions, the specific radioactivity of methionine was calculated as the average of the specific radioactivities at the beginning and at the end of the period during which the ethylene was trapped. No differences were found between the specific radioactivities of methionine in water- or TMV-inoculated leaves. The specific radioactivity of the ethylene produced by water-inoculated leaves was slightly higher than expected on the basis of the specific radioactivity of methionine in the leaf. The specific radioactivity of the ethylene produced by TMV-inoculated leaves was far less (Table I).

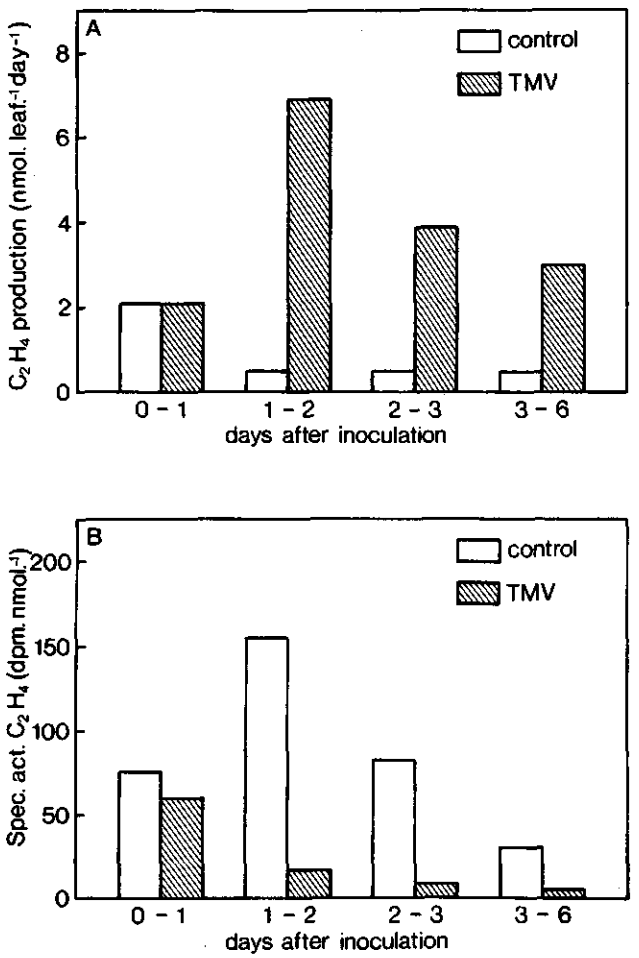

FIG. 2. Comparison of (A) the production and (B) the specific radioactivity of the ethylene produced by tobacco leaves inoculated with water or TMV 1 day after labeling with 1 μCi L-[U-^{14}C]methionine.

After TMV infection, lesions develop only in the interveinal areas and ethylene is produced mainly by the tissue surrounding the local lesions. Therefore the contributions of the main veins and the interveinal tissue to the production of [^{14}C]ethylene were compared after physical separation, immediately following labeling, by cutting out the interveinal tissue alongside the main veins. For water-inoculated leaves, amino acid analysis of extracts from either the vein parts or the interveinal tissue revealed that the concentration of methionine within the veins was two to three times lower than in the interveinal areas. Since most of the labeled methionine taken up was held back in the veins, the specific radioactivity in the veins was 15 to 30 times higher than in the interveinal tissue (Table I). The veins contributed to the ethylene production of the leaf for about one-third. The specific radioactivity of this ethylene was about 50 times higher than that from the interveinal tissue. The specific radioactivity of the ethylene produced by the veins was as expected for methionine being the sole precursor, that of the interveinal tissue was substantially less.

After TMV infection, ethylene production by the veins hardly changed, but ethylene production in the interveinal tissue increased severalfold, reducing the contribution of the veins to only 5 to 10%. As in uninfected leaves, the specific radioactivity in the vein parts was always far higher than in the interveinal tissue. The specific radioactivities of the ethylene produced by either the veins or the interveinal tissue were similar to those of uninfected leaves.

Table I. *Comparison of the Specific Radioactivities of the Methionine Pools Within, and the Ethylene Produced by the Whole Leaf, the Main Veins and the Interveinal Tissue, Respectively*

Leaves were labeled with L-[U-^{14}C]methionine by uptake through the petiole. Specific radioactivity of methionine was calculated as the mean between the specific radioactivities at the beginning and at the end of the incubation period. Ethylene was trapped during a 24-h period when local lesions became visible.

Material		H_2O-Inoculated	TMV-Inoculated
Whole leaf	SA[a] methionine, dpm·nmol^{-1}	240	245
	Expected SA C_2H_4, dpm·nmol^{-1}[b]	96	98
	C_2H_4 production, nmol·leaf^{-1}	1.2	30.0
	SA C_2H_4, dpm·nmol^{-1}	119	16.3
Main veins	SA methionine, dpm·nmol^{-1}	945	1504
	Expected SA C_2H_4 dpm·nmol^{-1}	379	602
	C_2H_4 production, nmol·leaf^{-1}	0.4	1.2
	SA C_2H_4, dpm·nmol^{-1}	349	238
Interveinal tissue	SA methionine, dpm·nmol^{-1}	64	55
	Expected SA C_2H_4, dpm·nmol^{-1}	25.7	22
	C_2H_4 production, nmol·leaf^{-1}	0.81	28.8
	SA C_2H_4, dpm·nmol^{-1}	7.9	7.4

[a] SA, specific radioactivity.

[b] The specific radioactivity of ethylene is expected to be 0.4 times that of methionine.

The decrease in the specific radioactivity of the ethylene produced by the whole leaf after TMV infection, seen in Figure 2 and Table I, can thus be explained by the fact that a far larger part of the ethylene is derived from the interveinal tissue, which produces ethylene with a relatively low specific radioactivity. The same situation appears to hold for the wound ethylene produced immediately upon inoculation (Fig. 2).

Thus, nonhomogeneous distribution of a label taken up through the petiole may give anomalous results with respect to the specific radioactivity of its product. This especially holds for situations in which a treatment such as localized wounding or TMV infection results in a nonhomogeneous stimulation of the reaction, such as the locally increased ethylene production.

The observation that the specific radioactivity of the ethylene produced by the interveinal tissue was substantially less than expected could still result from a nonhomogeneous distribution of the labeled methionine. Since the specific radioactivities in water- and TMV-inoculated leaves are the same, the pathways of ethylene production must be the same in healthy and virus-infected leaves. Whether methionine is the only precursor cannot be assessed on the basis of these results alone. The possibility that fatty acids can function as additional precursors was investigated by labeling leaves with 5 μCi [^{14}C]acetate. Under these circumstances [^{14}C]acetate was rapidly incorporated into lipids, as evidenced by extraction according to Roughan and Batt (20), but no radioactive ethylene was produced during the subsequent 7 days.

Labeling by Vacuum Infiltration. To circumvent unequal distribution of the labeled methionine, leaves were labeled by vacuum infiltration, although this treatment delayed lesion appearance by about 10 h. Leaves were inoculated on the plant and detached just before lesion appearance when ethylene production was rising to its maximum. The midrib was removed, leaf halves were infiltrated with [^{14}C]methionine solution, and ethylene was trapped during the subsequent 3 h. The specific radioactivities of methionine were similar in both water- and TMV-inoculated leaves and decreased rapidly during this period. No differences were found between the specific radioactivities of the ethylene produced by the water or TMV-inoculated leaves (Table II). Although the rapid decrease in specific radioactivity of the methionine pool makes precise determination impossible, the specific radioactivities of the ethylene can be considered in reasonable agreement with those expected if methionine were the only precursor, because the specific radioactivity of ethylene also decreased rapidly during incubation (Fig. 3).

When TMV-inoculated leaves were vacuum-infiltrated with labeled methionine after a 24-h preincubation in 0.1 mM AVG, ethylene production during the subsequent 5 h was inhibited for 98%. The specific radioactivity of the ethylene produced by these leaves was similar to that of controls preincubated in water. It can be concluded that the small amount of ethylene evolved in the presence of AVG is also derived from methionine. This indicates that methionine is the only precursor of ethylene in tobacco leaves.

Radioactive ethylene was produced without a lag phase (Fig. 3). Hence, a physical barrier for methionine between the intercellular space and the site of ethylene production is not apparent. This agrees with the possibility that (part of) the ethylene-producing enzyme system(s) is located at the plasmalemma (14, 16). Since the specific radioactivity of the ethylene produced during the first 3 h after vacuum infiltration was already in accordance with that of the methionine within the leaf, [^{14}C]methionine supplied by vacuum infiltration is equilibrated very rapidly with any methionine pool within the leaf cells. Regulation of ethylene synthesis through effects on the compartmentation of the ethylene precursor, as suggested by Kende and Baumgarter (12), can be questioned.

Endogenous Concentration of Free Methionine. To establish whether the increased ethylene production after TMV infection could result from an increased methionine concentration within the leaf, detached leaves were inoculated with water or TMV. Extracts were made daily to determine the concentration of free

Table II. *Comparison of the Specific Radioactivities of Ethylene Produced by Water- or TMV-Inoculated Leaves After Vacuum Infiltration with [^{14}C]Methionine*

Leaves were detached 2 days after inoculation, labeled and incubated during the subsequent 3 h.

	t = 0 h	t = 3 h
SA[a] methionine, dpm·nmol^{-1}	4450	240
Expected SA, C_2H_4, dpm·nmol^{-1}	1780	96
	H_2O-inoculated	TMV-inoculated
C_2H_4-production, nmol·leaf^{-1}	0.20	1.36
SA C_2H_4, dpm·nmol^{-1}	536	587

[a] SA, specific radioactivity.

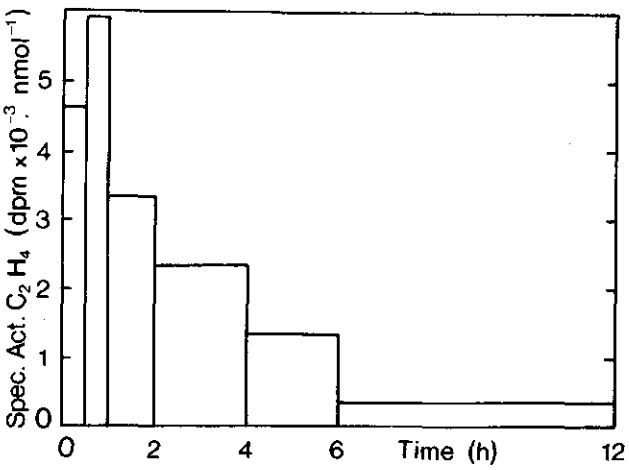

FIG. 3. Course of specific radioactivity of ethylene produced by tobacco leaf halves after vacuum infiltration with L-[U-^{14}C]methionine solution 2 days after TMV-infection.

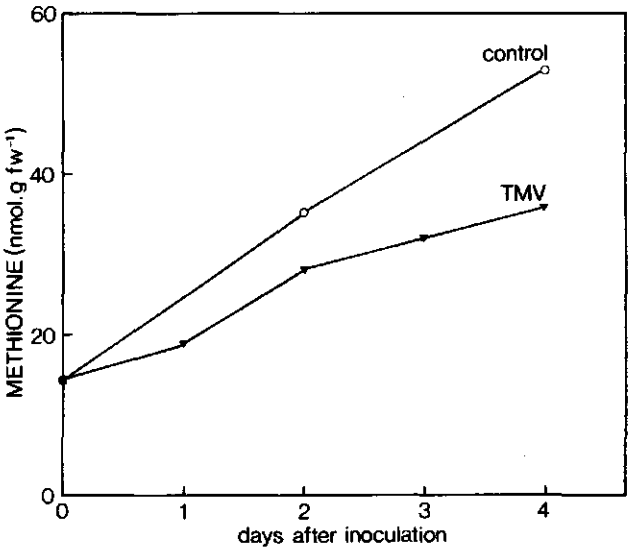

FIG. 4. Methionine concentration in tobacco leaves after water- or TMV-inoculation.

Table III. *Ethylene Production of and ACC Content in Tobacco Leaves 2 Days After Inoculation with Water or TMV*

Data are averages of two or three independent determinations for each experiment.

	Experiment 1		Experiment 2		Experiment 3	
	C_2H_4 (nmol/g fresh weight·h)	ACC (nmol/g fresh weight)	C_2H_4 (nmol/g fresh weight·h)	ACC (nmol/g fresh weight)	C_2H_4 (nmol/g fresh weight·h)	ACC (nmol/g fresh weight)
H_2O-inoculated	0.37	0.13	0.18	0.50	0.32	0.13
TMV-inoculated	2.65	0.52	1.18	2.60	1.51	1.68

methionine (Fig. 4). In water-inoculated leaves, the methionine concentration increased with time, probably due to proteolysis. In TMV-infected leaves the increase in methionine concentration was somewhat less than in the controls. Hence, the increase in ethylene production during the hypersensitive reaction cannot be accounted for by an increase in methionine content.

ACC Determination Experiments. If TMV infection stimulates the conversion of methionine to ethylene, it might be expected that the concentration of ACC, the immediate precursor of ethylene, is increased in infected tissues around the time of lesion appearance (5, 10). Leaf discs were cut from water- or TMV-inoculated leaves. Comparable discs were used either to measure ethylene production or to determine ACC content. As seen in Table III, the content of ACC in TMV-inoculated discs was increased severalfold. The increase in ACC concentration was well correlated with the increase in ethylene production.

It has been suggested (5, 25) that ethylene production is regulated by the activity of the enzymes involved in ethylene biosynthesis, particularly ACC-synthase. This possibility is now further investigated.

Conclusions. The inhibition of ethylene production by AVG, and the accordance between the specific radioactivities of methionine and ethylene after labeling with L-[U-^{14}C]methionine, indicate that methionine is the precursor of ethylene in tobacco leaves. Because similar results were obtained with virus-infected leaves and these leaves showed an increase in ACC content at the onset of the stimulation of ethylene production, virus-induced ethylene production must be likewise derived from methionine.

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HOOFDSTUK III

Regulation of Ethylene Biosynthesis in Virus-Infected Tobacco Leaves[1]

II. TIME COURSE OF LEVELS OF INTERMEDIATES AND *IN VIVO* CONVERSION RATES

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ABSTRACT

Ethylene production was stimulated severalfold during the hypersensitive reaction of Samsun NN tobacco to tobacco mosaic virus (TMV). Exogenous methionine or *S*-adenosylmethionine (SAM) did not increase ethylene evolution from healthy or TMV-infected leaf discs, although both precursors were directly available for ethylene production. This indicates that ethylene production is not controlled at the level of methionine concentration or availability, nor at the level of SAM production or concentration. In contrast, 1-aminocyclopropane-1-carboxylic acid (ACC) stimulated ethylene production considerably. Thus, ethylene production is primarily limited at the level of ACC production.

The regulation of ethylene production during the hypersensitive reaction to TMV was further studied by determining the time course of the concentrations of methionine, SAM, and ACC, as well as the course of their *in vivo* conversion rates. Endogenous concentrations of methionine and SAM remained unaffected until late in infection. On the contrary, the peak in ethylene production near the time of local lesion development was preceded by a large increase in ACC production. As a result of this increase, ACC accumulated in the leaf tissue. Only after local lesions became visible, the capacity to convert ACC into ethylene increased severalfold, associated with a sharp decrease in ACC content and a large increase in ethylene production.

Ethylene production in tobacco leaves reacting hypersensitively to TMV is thus regulated at the level of both the production of ACC and its conversion to ethylene.

Infection of *N* gene-containing tobacco cultivars with TMV[2] leads to the formation of necrotic local lesions within 48 h after inoculation (10). Such a hypersensitive reaction is accompanied by a sharp peak in ethylene production near the time of lesion appearance. Virus-stimulated ethylene production gradually subsides during subsequent lesion growth (6, 17).

In a previous paper, we demonstrated that methionine is the precursor of both normal and virus-stimulated ethylene in tobacco leaves (6). This conclusion was based on observations that AVG, known to inhibit methionine-derived ethylene production (9, 14), inhibited both normal and virus-induced ethylene production. Furthermore, after labeling leaves with L-[U-^{14}C]methionine, the specific radioactivity of the ethylene produced was in accordance with that of the methionine pool within the leaves. Moreover, the concentration of the intermediate, ACC (1, 16), increased severalfold 2 days after TMV infection, when ethylene production was maximal.

An increase in ethylene production may be the result of an increase in: (a) the concentration and/or availability of the ethylene precursor(s), and/or (b) the activity of one or more of the enzymes involved in ethylene biosynthesis. It has been suggested that in the biosynthetic pathway for methionine-derived ethylene production, ACC synthesis rather than its conversion to ethylene is the rate-limiting step (3, 11, 25). However, the accumulation of ACC after virus infection (6) indicates that the utilization of ACC may also be limiting. The present study was undertaken to analyze how, during the hypersensitive reaction of Samsun NN tobacco to TMV, the limiting factors change in the pathway from methionine to SAM to ACC to ethylene in order to enable the ethylene outburst.

MATERIALS AND METHODS

Plant Material and Incubation Conditions. Tobacco plants (*Nicotiana tabacum* L. cv. Samsun NN) were grown from seed in a greenhouse at a minimum temperature of 20°C; photoperiod was maintained at 16 h by additional illumination from high pressure halide Philips HPI/T lamps at 18 w/m^2. Almost full-grown leaves from 10- or 11-week-old plants were selected. The leaves were inoculated on the plant by dusting with carborundum, rubbing with either water (controls) or TMV W U1 (100 μg/ml) using a gauze pad, and rinsing with tap water. At specific times leaves were picked off for analyses. Subsequent incubations of the detached leaves were carried out in growth chambers under controlled conditions (20°C, 16 h photoperiod, 70% RH) (20).

Uptake of labeled substrates was accomplished by vacuum infiltration. Leaf discs (40 mm diameter) were immersed for 30 s at 1 mm Hg in aqueous solutions of either L-[U-^{14}C]methionine or SAM-[3,4-^{14}C]methionine, blotted dry with Kleenex tissues, and incubated in water-locked 750-ml Petri dishes (6).

Determination of Methionine Concentration. Leaf material was extracted and the concentration of methionine determined by amino acid analysis as described earlier (6). The specific radioactivity of methionine from [^{14}C]methionine- or [^{14}C]SAM-labeled

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[2] Abbreviations: TMV, tobacco mosaic virus; ACC, 1-aminocyclopropane-1-carboxylic acid; AVG, aminoethoxyvinylglycine; SAM, *S*-adenosylmethionine.

leaves was determined by liquid scintillation counting of the methionine-containing fractions eluted from the amino acid analyzer. Corrections for counting efficiency were made using an external standard.

Determination of SAM Concentration and *in Vivo* Conversion of Methionine to SAM. Leaf material was frozen in liquid, ground in a mortar with pestle, and 2 ml 6% $HClO_4$/g fresh weight were added. The homogenate was stirred for 30 min at 0°C and centrifuged for 10 min at 30,000*g*. The supernatant was adjusted to pH 4.5 at 0°C by slowly adding solid $KHCO_3$.

SAM was separated from other UV-absorbing compounds by subjecting the mixture to sulfopropyl (SP)-Sephadex ion-exchange column-chromatography according to Glazer and Peale (8). A comparison with authentic SAM by TLC (19) and thin-layer electrophoresis (18), confirmed that SAM was the only UV-absorbing compound within the SAM-containing fraction. The concentration of SAM was determined spectrophotometrically, assuming a molar extinction coefficient of 15,000 $cm^{-1} \cdot M$ at 260 nm (8). Recovery of SAM during the whole procedure was about 80%, as established by adding a known amount of SAM as an internal standard.

The rate of the *in vivo* conversion of methionine to SAM was estimated by labeling leaf discs with L-[U-^{14}C]methionine and determining the amount of radioactive SAM formed. The pH 4.5 extracts were passed through a Bio-Rex 70(H^+-form) column (20 × 5 mm) by elution with water to remove the labeled methionine. SAM was then eluted with 0.1 M HCl according to Schlenk (18) and its radioactivity determined by liquid scintillation counting.

Determination of ACC Concentration and *in Vivo* ACC Production. After grinding of the leaf material, 2 ml of 5% sulfosalicylic acid/g fresh weight were added. The mixture was stirred for 30 min at room temperature and centrifuged for 10 min at 30,000*g*. The concentration of ACC in the supernatant was determined by chemical conversion of the ACC to ethylene according to Lizada and Yang (15). ACC determinations were carried out in 40-ml sealed serum flasks; the amount of ACC was calculated from the concentration of ethylene reached after conversion.

To identify the ethylene-releasing compound as ACC, extracts were passed through a Dowex 50 (H^+-form) column (0.4 × 4 cm). After the column was washed with water, amino acids were eluted with 10 ml 2 M NH_4OH. This fraction was taken to dryness by rotary evaporation at 30°C. The small residue was dissolved in 96% ethanol and the amino acids were separated by TLC on silica gel plates using chloroform:methanol:(17%)ammonia (2:2:1, v/v/v) as the solvent system, according to Boller *et al.* (2). Different zones of the chromatogram were scraped off and subjected to ACC determination. More than 95% of the total amount of ethylene produced by these zones was derived from the one containing ACC, as established by co-chromatography with authentic ACC.

In all determinations, corrections were made for the efficiency of the conversion of ACC to ethylene by using authentic ACC as an internal standard. Ethylene recovery from ACC was generally about 80%.

Attempts to isolate ACC-synthase activity from either healthy or TMV-infected tobacco leaves by methods similar to those used by Boller *et al.* (2) and Yu *et al.* (23) for tomato fruits were unsuccessful. Probably ACC synthase is inactivated as a result of decompartmentation upon homogenization. Therefore, as a measure of ACC-synthase activity *in vivo*, ACC production was assayed by determining ACC accumulation during incubation of tobacco leaf discs under anaerobic conditions. Since the conversion of ACC into ethylene requires oxygen, anaerobiosis blocks the last step in ethylene biosynthesis (1). Discs from one leaf half were extracted immediately after detachment to determine the level of ACC at the start of incubation, whereas discs from the other half were placed in an atmosphere of N_2. To this end, a desiccator was 3 times evacuated and filled with oxygen-free nitrogen. This procedure reduced the O_2 concentration to less than 0.1%, resulting in an inhibition of ethylene production of more than 80%.

Preliminary experiments indicated that accumulation of ACC under these conditions was equal in light and darkness. The rate of ACC accumulation remained constant for at least 8 h. Thus, within this time period, any influence of anaerobical conditions on ACC-synthase activity, such as the induction found as a long-term effect of anaerobiosis in tomato roots (4) could be excluded.

Routinely, leaf discs were incubated for 4 h at 20°C in light. After opening of the desiccator, the leaf material was immediately frozen in liquid N_2 for extraction and determination of ACC. ACC production was expressed as the increase in ACC content in nmol/g fresh weight.

Measurement of Ethylene Production. Individual leaf discs (25 mm diameter) were incubated in 1 ml water in 40-ml sealed serum flasks at 20°C in fluorescent light (10 w/m^2). At specific times, 1-ml gas samples were withdrawn through the rubber seal. The concentration of ethylene was determined with a gas chromatograph, equipped with an alumina column, and a flame-ionization detector. Ethylene production was calculated as the mean of at least three independent incubations. Each experiment was repeated at least twice.

After labeling leaves with either L-[U-^{14}C]methionine or *S*-adenosyl-[3,4-^{14}C]methionine the ethylene produced was trapped with mercuric perchlorate and its specific radioactivity was determined as described earlier (6).

Determination of the *in Vivo* Capacity of the ACC-Converting Enzyme(s). This capacity should be assessed by exogenous application of a saturating amount of ACC. However, due to the low affinity of the ACC-converting enzyme system to ACC (13), saturation could not be reached (7). Nevertheless, it can be assumed that comparison of the ethylene production of leaf discs incubated in a high concentration of ACC will reflect their relative capacities for ACC conversion. To this end, discs were incubated in 1 ml 1 mM ACC solutions in 40-ml serum flasks. Ethylene production was measured during a 4-h incubation in light (6).

Chemicals. L-[U-^{14}C]methionine (285 mCi/mmol) was purchased from The Radiochemical Centre (Amersham, UK) and *S*-adenosyl-[3,4-^{14}C]methionine (about 40 mCi/mmol) from the "Commissariat à l'Energie Atomique" (CEA) (Gif sur Yvette, France). ACC was obtained from Bayer AG (Leverkusen, F.R.G.) and AVG from Dr. R. Maag AG (Dielsdorf, Switzerland).

RESULTS

Ethylene Production. When discs were punched out of freshly detached leaves at specific intervals after inoculation, ethylene production during the first 4 h showed a pattern identical to the one established earlier for whole detached leaves during continuous incubation (6). Discs from TMV-inoculated leaves showed a sharp peak in ethylene production with a maximum at 52 h after inoculation, 4 h after lesions became macroscopically visible (Fig. 1). The ethylene production by leaf discs from water-inoculated leaves remained at a steady low level.

Influence of Exogenously Applied Methionine, SAM, and ACC on Ethylene Production. Leaf discs, cut from freshly detached, noninoculated leaves, were vacuum infiltrated with, and further incubated in water or methionine solutions at concentrations varying from 10^{-5} to 10^{-2} M. At all concentrations tested, ethylene production was equal to that on water (about 0.3 nl/disc·h). Also, after preincubation of leaf discs in an humidified atmosphere for 24 h, application of methionine did not affect ethylene production.

Leaf discs were then cut 2 days after inoculation with either water or TMV, and infiltrated with and further incubated in solutions of methionine, SAM, or ACC, at a concentration of 1 mM. This is far higher than the endogenous concentrations of these ethylene precursors (see below). Methionine and SAM did

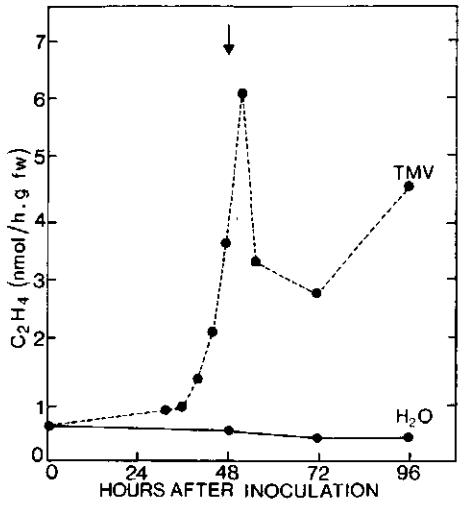

FIG. 1. Time course of ethylene production by leaf discs (25 mm diam), punched from water- (——) or TMV-inoculated (-----) leaves at the indicated times after inoculation. Ethylene production was measured during a 4-h incubation period at 20°C in light. The time that lesions became macroscopically visible is indicated by the arrow.

Table I. *Ethylene Production of Tobacco Leaf Discs, Cut Two Days after Inoculation with Either Water or TMV*

The discs were incubated in 1-ml solutions of 1 mM methionine, SAM, or ACC. Ethylene production was measured during a 5-h incubation period immediately after punching of the discs, and was expressed as the average (± SD) from four leaf discs.

Incubation	Ethylene Production	
	Water-inoculated	TMV-inoculated
	nl/disc·h	
Water	0.22 ± 0.03	3.13 ± 0.29
Methionine	0.18 ± 0.04	3.10 ± 0.24
SAM	0.21 ± 0.05	3.25 ± 0.38
ACC	1.05 ± 0.04	6.37 ± 0.40

not influence ethylene production in either the control, or in the virus-infected leaf discs. In contrast, the ethylene production of discs incubated in ACC was increased 5- and 2-fold, respectively (Table I). This indicates that ethylene production in tobacco is limited at the level of ACC.

After labeling leaves by vacuum infiltration with either L-[U-^{14}C]methionine or *S*-adenosyl-[3,4-^{14}C]methionine, radioactive ethylene was produced without any noticeable lag phase (Fig. 2), indicating that exogenously applied methionine or SAM are directly available to the ethylene-synthesizing enzyme system.

After labeling leaves with [^{14}C]methionine, the specific radioactivity of the methionine pool within the leaf decreased from 5,500 dpm/nmol at the beginning, to 250 dpm/nmol after 7 h of incubation, indicative of a high turnover rate. As a result, the specific radioactivity of the ethylene produced by these leaves decreased rapidly with time.

Although after vacuum infiltration, ethylene was produced from applied [^{14}C]SAM without a lag phase, the specific radioactivity of the ethylene was low and remained constant for at least 7 h. Apparently, SAM was taken up only gradually by the leaf cells. This prevented a reliable estimation of the specific radioactivity

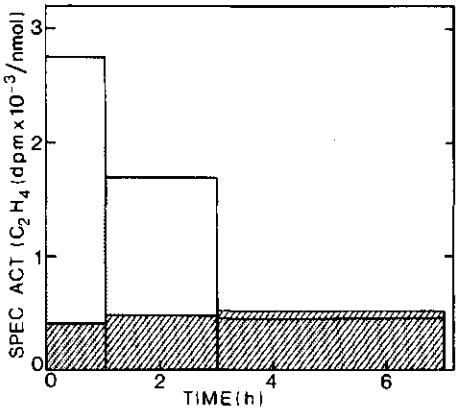

FIG. 2. Time course of the specific radioactivity of the ethylene produced by TMV-infected tobacco leaf discs 2 days after inoculation. Leaf discs were labeled with either L-[U-^{14}C]methionine (■) or SAM-[3,4-^{14}C]methionine (▨) at time 0.

of SAM within the tissue proper. Due to the labeling with [^{14}C]SAM, the specific radioactivity of the methionine pool increased from 30 dpm/nmol at the beginning to 80 dpm/nmol at the end of the incubation period, indicating that some of the [^{14}C]SAM was hydrolyzed in the tissue. However, since the specific activity of the ethylene produced from [^{14}C]SAM was about 450 dpm/nmol, the ethylene was produced directly from SAM and not via SAM-derived methionine.

Time Course of Methionine and SAM Concentration and the *in Vivo* Capacity to Convert Methionine to SAM During the Hypersensitive Reaction. In water-inoculated leaves, the methionine content remained stable at about 15 nmol/g fresh weight. No appreciable change occurred in TMV-infected leaves during the development of the lesions when ethylene production was rapidly rising (Fig. 3A).

Similarly, SAM content was unaffected during the hypersensitive reaction and remained at a level of about 7 nmol/g fresh weight during the first 3 days after inoculation, equal to the level of SAM in water-inoculated leaves (Fig. 3B). Only at 4 days after inoculation did the concentrations of both methionine and SAM increase significantly in the TMV-inoculated leaves, reflecting metabolic changes well after occurrence of the necrotic lesions.

To compare the conversion rates of methionine to SAM between water- and TMV-infected leaves, leaf discs were punched from leaves 48 h after inoculation, and labeled with L-[U-^{14}C]methionine. In a previous paper (6) we demonstrated that after labeling with L-[U-^{14}C]methionine by vacuum infiltration both healthy and virus-infected leaf discs produced ethylene with the same specific radioactivity, indicating uptake of the labeled methionine to be identical. This was confirmed by pulse-chase labeling: from 57,000 dpm L-[U-^{14}C]methionine introduced in the leaf discs by vacuum infiltration, after successive washings with 0.1 M KCl and 0.1 M unlabeled methionine 20 min after the pulse, 9,500 ± 1,100 dpm and 10,100 ± 1,500 dpm were retained in the water- and TMV-inoculated leaf discs, respectively.

Figure 4 shows that the amount of radioactivity recovered in SAM increased with time up to a maximum 30 min after labeling in both water- and TMV-inoculated leaf discs, apparently reflecting methionine uptake into the cells. At longer incubation times, the amount of radioactive SAM decreased, probably due to both the rapid decrease of the specific radioactivity of the methionine pool and the rapid turnover of SAM.

In each of three experiments, the peak in radioactive SAM formed was about 2 times higher in the virus-infected leaf discs

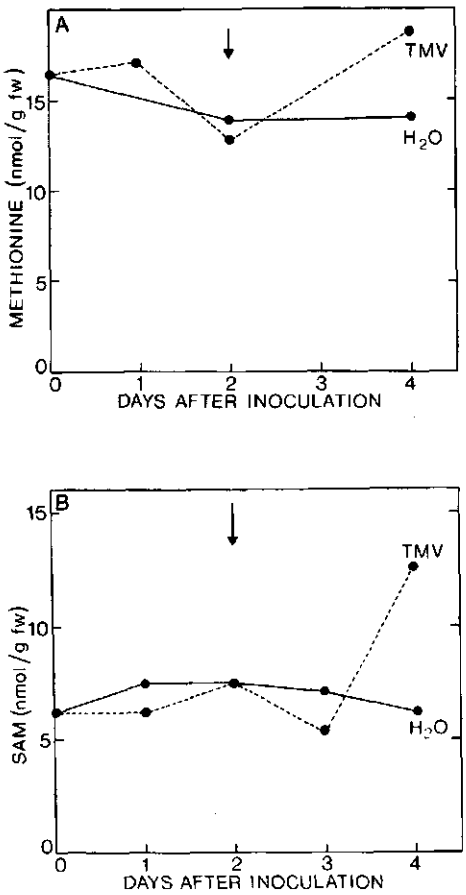

FIG. 3. Changes in endogenous concentrations of free methionine (A) or SAM (B) in extracts from tobacco leaves at specific times after inoculation with either water (——) or TMV (-----). The time of lesion appearance is indicated by the arrow.

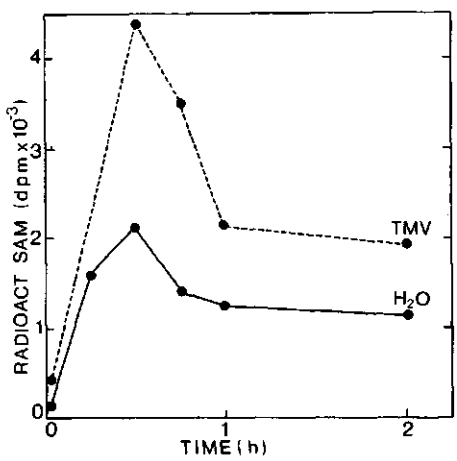

FIG. 4. Radioactive SAM formed after labeling leaf discs with L-[U-^{14}C]methionine. Discs were cut from leaves 2 days after inoculation with either water (——) or TMV (-----), and incubated at 20°C in light.

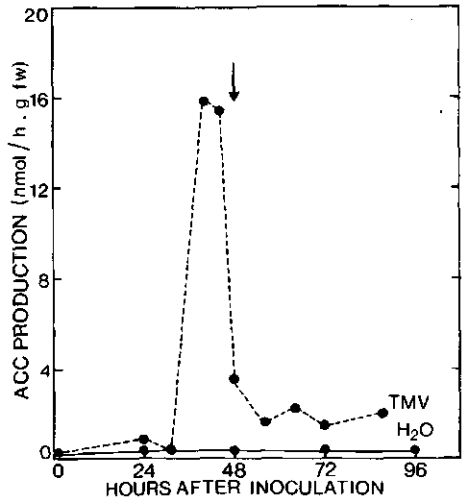

FIG. 5. ACC production at specific times after water (——) or TMV (-----) inoculation of leaves. ACC-synthase activity was calculated from the amount of ACC accumulated in leaf discs during a 4-h incubation in a nitrogen atmosphere. The time of lesion appearance is indicated by the arrow.

than in the water-inoculated controls. Since the SAM-pool size was not increased after TMV-infection (Fig. 3B), this indicates that the turnover of SAM is increased about 2-fold upon TMV infection.

Time Course of *in Vivo* ACC Production and ACC Content During the Hypersensitive Reaction. Figure 5 shows the time course of the *in vivo* ACC production after water or TMV inoculation. After water inoculation, the activity of ACC production remained at the level of about 0.3 nmol/g fresh weight·h, whereas, in TMV-infected leaves, it increased more than 50-fold (up to 16 nmol/g fresh weight·h), as early as 40 h after inoculation. At 48 h, when local lesions had become macroscopically visible, ACC production had fallen to about 2 nmol/g fresh weight·h. It remained at this comparatively high level during subsequent days.

In both water- and TMV-inoculated discs, the endogenous ACC content fully reflected ACC synthesis. After water inoculation, it remained about 0.5 nmol/g fresh weight during the whole period, whereas in the TMV-infected leaves it increased from 40 h after inoculation onwards (Fig. 6), with a sharp peak at 48 h (about 9 nmol/g fresh weight). In four different experiments, the maximal increase was 15-, 9-, 24-, and 12-fold, respectively. By 60 h, the ACC content had decreased to the control level, but it slowly increased again to about 2 nmol/g fresh weight from 3 days after inoculation on.

As described before (6), AVG, at a concentration of 0.1 mM, very efficiently inhibited ethylene production of water- or TMV-inoculated leaves. ACC-synthase activity of AVG-treated, noninfected leaves was nearly abolished, whereas the rate of ethylene production of leaf discs incubated in 1 mM ACC was not influenced by AVG (Table II). Similar effects of AVG were found in TMV-infected leaves. These results confirm the conclusions of Boller *et al.* (2) and Yu and Yang (24) that AVG inhibits methionine-derived ethylene production by blocking ACC synthesis, rather than by inhibition of the conversion of ACC into ethylene.

Conversion of ACC into Ethylene. The sharp peak in ACC production during the hypersensitive reaction caused a temporary accumulation of ACC within the leaf tissue (Fig. 6). Since the increased ethylene production during the hypersensitive reaction is confined to the ring of tissue immediately adjacent to the

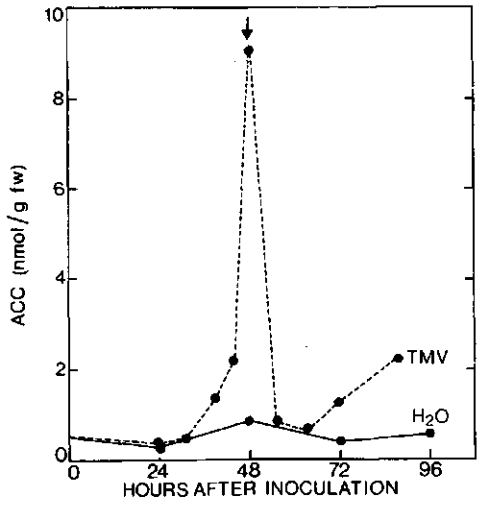

FIG. 6. Time course of ACC content in tobacco leaves after inoculation with water (——) or TMV (-----). The time of lesion appearance is indicated by the arrow.

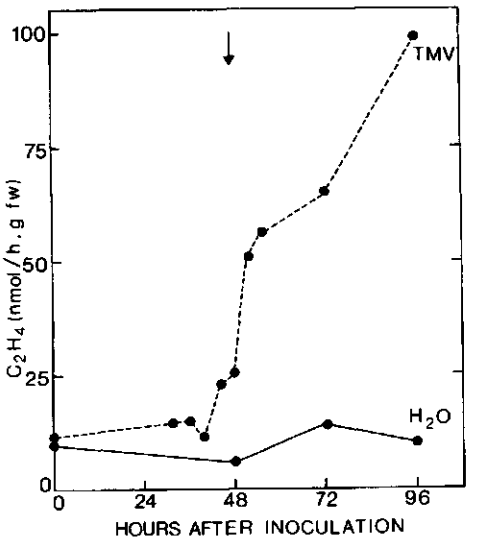

FIG. 7. Ethylene production of leaf discs, cut from water- (——) or TMV- (-----) inoculated leaves, upon incubation in 1 mM solutions of ACC at 20°C in light for a 4-h period. The time of lesion appearance is indicated by the arrow.

Table II. *Influence of (0.1 mM) AVG on Ethylene Production (Expt. 1) or ACC-Synthase Activity (Expt. 2)*

Ethylene production of noninfected leaf discs was measured between 2 and 7 h of incubation either in the presence or absence of 1 mM ACC. ACC-synthase activity was calculated from the amount of ACC accumulated during a 6-h anaerobic incubation following vacuum infiltration of the leaf discs with either water or 0.1 mM AVG.

Expt. No.	Incubation conditions	Ethylene Production	
		nl/disc·h	*% of control*
1	Water	0.115	100
	AVG	0.010	8.7
	ACC	0.813	707
	ACC + AVG	0.781	679
		ACC-Synthase Activity	
		nmol/g fresh wt·h	*% of control*
2	Water	0.285	100
	AVG	0.003	1.1

necrotic lesions, it is tempting to assume that ACC accumulated only in that area. However, the amount of ACC had already declined when lesions became clearly visible. This indicates that, in the lesion area, at least from 36 to 48 h after infection, the final step in ethylene biosynthesis was rate-limiting.

The time course of the capacity of leaf discs to convert ACC into ethylene was studied by incubating discs, cut from leaves at specific times after inoculation, in 1 mM solutions of ACC (Fig. 7). A comparison of Figure 1 with Figure 7 shows that the ethylene production of the water- and the TMV-inoculated leaf discs was increased manifold upon incubation in ACC. Starting around lesion appearance on day 2, the capacity of the TMV-inoculated leaf discs to convert exogenously applied ACC into ethylene increased 5- to 10-fold during the following days. Contrary to its production, ACC conversion kept increasing once local lesions had become visible.

DISCUSSION

After labeling leaves with L-[U-^{14}C]methionine, the specific radioactivity of the ethylene produced decreased rapidly with time, in accordance with the rapid decrease of the specific radioactivity of the methionine pool (*cf.* ref. 6). The specific radioactivity of the ethylene produced by *S*-adenosyl-[3,4-^{14}C]methionine-labeled leaves was low in comparison with that of the [^{14}C]methionine-labeled ones, but was far higher than that reached by the methionine pool within these leaves, indicating that the applied [^{14}C]SAM was not converted into ethylene via methionine, but that it was taken up as such and acted as an intermediate in methionine-derived ethylene production.

Since after labeling of the leaves with L-[U-^{14}C]methionine or *S*-adenosyl-[3,4-^{14}C]methionine, radioactive ethylene was produced without any lag time, exogenously applied methionine or SAM must have been directly available for the ethylene-synthesizing enzyme system. However, neither methionine nor SAM stimulated ethylene production of tobacco leaf discs when applied at 1 mM in the incubation solution. This indicates that the conversion rate of SAM to ACC is the limiting factor in ethylene biosynthesis as was shown to be the case in several other systems (3, 5, 11, 22, 24, 25). Furthermore, the increase in ethylene production 2 days after TMV infection was not accompanied by an increase in the pool size of either methionine or SAM. These results exclude the possibility suggested by Kende and Baumgarter (12) that ethylene production is regulated at the level of either the concentration or the availability of methionine or SAM.

Determination of the *in vivo* conversion of methionine to SAM in leaf discs, punched from leaves 2 days after inoculation with either water or TMV, revealed that the rate of [^{14}C]SAM production from [^{14}C]methionine was elevated in the TMV-infected leaves. This fits with our observations that, on the one hand, the SAM pool size remained constant during the hypersensitive reaction, whereas, on the other hand, SAM is converted to ACC at an increased rate.

As described earlier (6), ethylene production by tobacco leaf discs was increased severalfold upon incubation in ACC, in accordance with Cameron *et al.* (4), who demonstrated ACC to increase ethylene production in 16 plant species. This further demonstrates that ACC synthesis is the rate-limiting step in ethylene biosynthesis. As expected, the peak in ethylene production 52 h after inoculation with TMV was preceded by an increase in ACC production peaking at 40 h. Whether this stimulation of ACC production results from *de novo* synthesis of ACC synthase, as found for chemically- or mechanically-induced wound ethylene in tomatoes (25), or is caused by activation of a preexisting enzyme, was not investigated because inhibitors of protein synthesis inhibit virus multiplication and, hence, interfere with the virus-induced hypersensitive reaction and ethylene production.

In most experiments, the amount of ethylene evolved was less than expected on the basis of the ACC production. Inasmuch as accumulation of ACC during the 4-h incubation in the nitrogen atmosphere was linear, an effect of anaerobiosis as described by Bradford and Yang (4) for water-logged tomato plants is improbable. Perhaps the discrepancy between ACC and ethylene production results from an inhibition of other reactions in which ACC and/or ethylene are normally involved.

Apparently, as a result of the increase in ACC production, ACC accumulated within the leaf tissue. A maximal content (up to a 15-fold increase) was reached at 48 h after inoculation. Since ethylene production in TMV-infected tobacco leaves is increased only in the leaf cells surrounding the developing local lesions (6), the increase in ACC content is expected to be restricted to these cells only. Thus, the concentration of ACC within these cells must be excessively high. Under these conditions, the capacity of the ACC-converting enzyme system turned out to become limiting. Only around the time of lesion appearance did the capacity of the leaf discs to convert ACC into ethylene strongly increase, accompanied by a decrease in ACC-content and an increase in ethylene production during the first hours after lesions had become visible. Because the final step in methionine-derived ethylene production, the conversion of ACC into ethylene, is assumed to be mediated by a peroxidase-like enzyme (1), the increase in the ACC-converting enzyme activity is in good agreement with the increase in peroxidase activity after lesion appearance in TMV-infected tobacco leaves, observed by Van Loon and Geelen (21).

From the observations that ethylene production in noninfected leaves is increased severalfold upon incubation in ACC solutions and, the increase in ACC synthesis precedes the stimulation of ethylene production in hypersensitively reacting leaves, we can conclude that virus-stimulated ethylene formation is regulated primarily at the level of ACC production. However, inasmuch as ACC accumulates near the time of lesion appearance, and the peak in ethylene evolution occurs only when the capacity for conversion of ACC into ethylene is increased, ethylene production is also limited temporarily at the level of ACC oxidation.

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HOOFDSTUK IV

Regulation of Ethylene Biosynthesis in Virus-Infected Tobacco Leaves

III. THE RELATIONSHIP BETWEEN VIRUS-STIMULATED ETHYLENE PRODUCTION AND SYMPTOM EXPRESSION IN GENETICALLY DIFFERENT HOST-VIRUS COMBINATIONS.

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ABSTRACT

The regulation of ethylene biosynthesis was studied in Samsun and Samsun NN tobacco (*Nicotiana tabacum* L.), infected with tobacco mosaic virus (TMV) W U1, TMV Holmes Ribgrass (HR), or tobacco necrosis virus (TNV). Stimulation of ethylene production upon infection was determined neither by the genetic constitution of the host plant, nor by the infecting virus, but related to symptom expression.

A hypersensitive reaction (Samsun NN infected with TMV W U1 (20°C) or TNV, and Samsun infected with TMV HR or TNV), was accompanied by an increase in the production of the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), accumulation of ACC, and a burst of ethylene, and followed by an increase in the capacity to convert ACC to ethylene. None of these changes occurred in systemic infections (TMV W U1-infected Samsun NN at 30°C or Samsun at 20°C). These observations indicate that the increase in ethylene production during a hypersensitive reaction depends primarily on an increase in ACC-producing activity. Both enhanced ACC production and ACC accumulation were restricted to the necrotic areas. Consequently, virus-stimulated ethylene is produced locally within or just around the lesions.

After shifting TMV W U1-infected Samsun NN from 30 to 20°C, ethylene production started within 6 h. The accompanying increase in ACC production was blocked by inhibitors of RNA and protein synthesis. The rise in ACC content and in ethylene production could not be simulated, either by localized loss of membrane integrity or by application of extracts or phloem exudates from leaves in which ethylene production was stimulated.

After primary infection of hypersensitively reacting plants, the capacity to convert ACC to ethylene was increased systemically. After challenge inoculation ACC did not accumulate, but was immediately converted to ethylene. The ethylene thus produced may be responsible for limiting lesion enlargement in tobacco leaves exhibiting systemic acquired resistance.

INTRODUCTION

All tobacco (*Nicotiana tabacum* L.) varieties carrying the *N* gene have the inherent capacity to restrict multiplication and spread of tobacco mosaic virus (TMV) to a zone around the infection site which rapidly necroses. This hypersensitive reaction precludes further invasion of the host.

In 1951 Ross and Williamson (*16*) observed that a "physiologically active emanation" was produced during such virus-induced local lesion development which mimicked the "triple response" of etiolated pea seedlings to ethylene. That this emanation is ethylene, indeed, was demonstrated by Balász *et al.* (*2*), Nakagaki *et al.* (*14*) and Gáborjányi *et al.* (*7*). Both Nakagaki *et al.* (*14*) and Balász *et al.* (*2*) showed that ethylene production was increased several-fold over the basal level during hypersensitive local lesion formation, but was not stimulated at all during a systemic virus infection that did not involve necrosis.

That ethylene plays a role in the realization of the hypersensitive response, was put forward by Van Loon (*24*). Pricking Samsun NN tobacco leaves with needles moistened with the ethylene-releasing compound, 2-chloroethylphosphonic acid (ethephon), gave rise to necrotic spots resembling virus-induced local lesions. Moreover, treatment with ethephon resulted in changes in protein constitution and peroxidase isoenzymes, and in induction of a systemic acquired resistance, comparable to the effects of TMV in hypersensitively reacting Samsun NN tobacco. Since ethylene may also be responsible for other characteristics of the hypersensitive reaction (*25*), e.g. increases in oxidative and hydrolytic enzymes (*18,19*) and alteration in membrane permeability (*20,26,27*), this substance appears to be a causative factor in symptom expression and virus localization.

Both the basal ethylene produced by uninfected Samsun NN tobacco leaves and the virus-stimulated ethylene accompanying local lesion formation are derived from methionine according to the biosynthetic pathway: methionine →S-adenosylmethionine (SAM)→1-aminocyclopropane -1-carboxylic acid (ACC)→ethylene (*4,5*). Six hours before the appearance of local lesions on TMV W U1-infected leaves 48 after inoculation a large peak in ACC production occurred, resulting in accumulation of ACC to a sharp peak at the moment the lesions became visible. Subsequently, the capacity to convert ACC to ethylene increased severalfold, associated with a sharp decrease in ACC content and a rapid enhancement of ethylene emanation, which peaked 4h after lesions became visible. Ethylene production in TMV-infected Samsun NN tobacco leaves is thus regulated both at the level of ACC production and ACC conversion to ethylene.

Since infection of Xanthi-nc tobacco with cucumber mosaic virus (*2*) or Bright Yellow tobacco with TMV (*14*) resulted in systemic infections without stimulation of ethylene production, it became of interest to study the relationship between virus-stimulated ethylene production and symptom expression further, using both a single virus strain and near-isogenic host varieties that respond differentially to the virus, and a single tobacco variety that reacts differentially to different virus strains. The tobacco variety Samsun is susceptible to common strains of TMV such as W U1, developing systemic mosaic symptoms after infection. From this variety, Samsun NN was developed through substitution of the H chromosome by the Hg chromosome from *N. glutinosa* (*8*). The *N* gene on the Hg chromosome confers to the host hypersensitivity to all strains of TMV at temperatures below c. 28°C. Above this temperature also Samsun NN plants are systemically invaded by TMV W U1, indistinguishable from the reaction of Samsun plants, indicating that the activity of the *N* gene is temperature-dependent (*17*). After inoculation with the weak TMV strain, "Holmes Ribgrass" (U8) (*9*), Samsun NN remains virtually symptomless, whereas Samsun develops a semi-systemic ringspotting.

Furthermore, both tobacco varieties react hypersensitively to tobacco necrosis virus (TNV), indistinguishable from the reaction of Samsun NN tobacco to TMV W U1 below 28°C.

By studying how ethylene production is regulated in these combinations (see Table 1), it will be possible to distinguish whether genetically determined differences exist in the capacity of different host plant varieties to react to virus infection with a stimulation of ethylene production, or whether this stimulation depends primarily on the nature of the infecting virus strain or whether the type of symptom expression is the determining factor. Such an analysis will further indicate how general the regulation of virus-stimulated ethylene production at the level of ACC production and/or conversion to ethylene is, and may provide a clue as to the nature of the factor(s) inducing the increase in ethylene production.

Table 1 Symptom expression in several combinations of Nicotiana tabacum L. cultivars and viruses

Tobacco cultivar	Virus strain	Temperature	Symptoms
Samsun	TMV W U1	20°C	mosaic (systemic infection)
	TMV HR	20°C	yellow ringspotting (semi-systemic infection)
	TNV	20°C	local lesions
Samsun NN	TMV W U1	20°C	local lesions
	TMV W U1	30°C	mosaic (systemic infection)
	TMV HR	20°C	very small lesions on young leaves only
	TNV	20°C	local lesions

MATERIALS AND METHODS

Plant material, viruses, and incubation conditions

Experiments were performed using 10 to 12 weeks-old tobacco plants (*Nicotania tabacum* L. cv. Samsun or Samsun NN), grown in a greenhouse as described before (*4*). Two almost full-grown leaves per plant were inoculated by dusting them with carborundum, rubbing with either virus solutions or water (control) using a gauze pad, and rinsing with tap water. TMV W U1, TMV HR and TNV were the same strains as used previously (*21,22*). Viruses were purified according to standard techniques (*22*) and inoculated at a concentration of 100 $\mu g\ ml^{-1}$. After inoculation plants were transferred to a growth cabinet and kept under controlled conditions (20°C, 16 h photoperiod, 70% rel. hum.). At specific times, leaves were picked off and leaf discs were cut for further determinations.

All determinations were calculated as the mean of at least 3 independent incubations; each experiment was repeated at least twice.

To compare the pattern of ethylene production in systemically resistant and non-resistant leaves, two upper leaves of plants that were inoculated earlier on lower leaves with TMV or water were "challenge" inoculated seven days later.

Temperature shift experiments

The characteristics of virus-stimulated ethylene production were investigated by making use of the effect of a temperature shift on lesion formation in Samsun NN leaves. To study the localization of ethylene production, plants were inoculated with only 1 μg ml^{-1} TMV and kept at 20°C for two days. After a small number of lesions of about 1 mm diameter had appeared, the temperature was increased to 30°C for 3 days, then shifted back to 20°C. Within 20 h large secondary necroses of about 4 mm diameter developed around the primary lesions. The distribution of the precursor ACC was determined 7 h after the shift from 30° to 20°C, when virus-stimulated ethylene production was rapidly increasing. The leaves were put on ice, transferred to 2°C, and primary lesions (diam 1.5 mm), small rings of the surrounding tissues (3 and 8 mm diam., respectively, including the secondary necrosis), and discs of uninfected leaf parts (3 mm diam.) were punched out, immediately frozen in liquid nitrogen, and used for ACC determination. To determine the site of ACC synthesis, ACC was allowed to accumulate during incubation of leaves in a nitrogen atmosphere between 6 and 12 h after the shift from 30° to 20°C.

The effects of inhibitors of RNA (actinomycin D, cordycepin) and protein synthesis (cycloheximide, D-2-(4-methyl-2,6-dinitroanilino)-N-methyl-propionamide (MDMP) (*3*), chloramphenicol) on virus-stimulated ethylene production were studied in Samsun NN leaf discs (25 mm diam) taken from plants inoculated 2 days previously and kept at 30°C. After a 2 h pre-incubation in 40 ml serum flasks at 30°C, the temperature was lowered to 20°C and the ethylene production was measured over the subsequent 24 h period.

Induction of ethylene production

To establish whether a localized loss of membrane integrity could account for the increase in ethylene production in infected leaves, this condition was simulated by pricking non-infected leaves over their whole surface with needles (*24*) moistened with either liquid nitrogen or a 1% solution of the detergents Triton X-100, Nonidet P 40, Tween 20 or deoxycholate. Leaf discs were cut and incubated at 20°C for 24 h to measure ethylene production.

To investigate whether a factor stimulating ethylene synthesis is produced upon infection. TMV-inoculated leaves sampled at different points of time were frozen in liquid nitrogen, ground in a mortar with pestle, and extracted with an equal volume of water. The homogenate was centrifuged for 10 min at 10.000 *g*, and the resulting supernatant was vacuum-infiltrated into non-infected leaf discs. Extracts from water-

inoculated leaves served as controls. Alternatively, phloem exudates from infected and non-infected leaves were collected according to King and Zeevaart (*11*), and fed to non-infected, detached leaves by petiolar uptake. The leaves were incubated in large, water-locked petri dishes as described previously (*4*). Ethylene production was measured over the subsequent 24 h period.

Determination of ACC concentration and in vivo ACC production

Leaf material was frozen in liquid nitrogen, ground in a mortar with pestle, and 2 ml of 5% sulfosalicylic acid per g fresh weight were added. After stirring (30 min at room temperature) and centrifugation (10 min at 30.000 *g*) the concentration of ACC was determined directly by chemical conversion to ethylene according to Lizada and Yang (*12*), as described before (*4,5*). The production of ACC was measured *in vivo* by measuring accumulation of ACC during incubation of leaf discs in a nitrogen atmosphere that prevents its conversion to ethylene (*5*). After a 4 h incubation at 20°C in light, leaf material was immedeately frozen in liquid nitrogen for extraction and determination of ACC content.

Determination of the in vivo capacity to convert ACC to ethylene

As discussed previously (*5*), the ethylene production of leaf discs incubated in 1 mM ACC reflects their capacity to convert ACC to ethylene. Leaf discs were incubated in 1 ml solutions in 40 ml serum flasks at 20°C in light; ethylene production was measured during a 4 h incubation period.

Measurement of ethylene production

Individual leaf discs (25 mm diam) were incubated in 40 ml sealed serum flasks at 20°C in light. At specific times, 1 ml gas samples were withdrawn through the rubber seal. The concentration of ethylene was determined with a Varian 1400 gas chromatograph, equipped with an alumina column and a flame-ionization detector.

Chemicals

ACC was obtained from Bayer AG (Leverkusen, FRG) and D-MDMP from Shell Bioscience Laboratories (Sittingbourne, UK).

RESULTS

Ethylene production in TMV-infected Samsun NN leaves at 20° and 30°C

Upon infection of Samsun NN with TMV at 20°C local lesions became apparent 40 h after inoculation (Fig. 1). In accordance with our previous report (*5*), ACC production (Fig. 1A), ACC content (B), and ethylene production (C) were all peaking near the time of lesion appearance whereas the capacity for ACC conversion increased only afterwards (D).

At 30°C, however, none of these effects occurred and TMV W U1-inoculated leaves behaved similarly to water-inoculated controls, ACC production, ACC content, ethylene production and ACC-converting capacity continuously remaining at the low control levels (Fig. 1A-D). Consequently, virus-stimulated ethylene production in Samsun NN tobacco depends either on the activity of the *N* gene, on the specificity of the virus or on the type of symptoms produced.

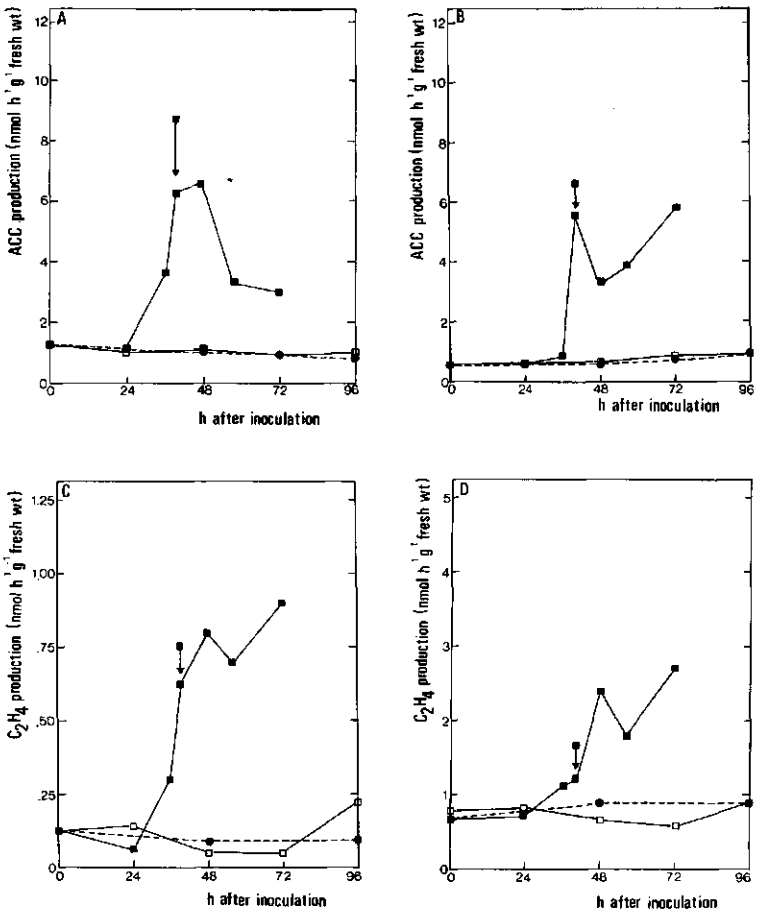

Fig. 1. Time course of (A) ACC production (determined by measuring accumulation of ACC during a 4 h anaerobic incubation), (B) ACC content, (C) ethylene production (mean production during a 4 h incubation period) and (D) the capacity of the tissue to convert ACC to ethylene (mean ethylene production of discs incubated in 1 mM ACC for 4 h). Samsun NN leaves were inoculated with water (●--------●) or TMV W U1 (————) and incubated at 20°C (■————■) or 30°C (□————□). Lesion appearance indicated by arrow.

Ethylene production in TNV infected Samsun NN leaves

To determine whether it is the infecting virus which influences the pattern of ethylene biosynthesis during a hypersensitive reaction, Samsun NN plants were inoculated with TNV (Fig. 2). Local lesions appeared 30 h after inoculation. As after infection with TMV W U1, lesion appearance was accompanied by a large increase in ethylene production, more than ten-fold, which remained at a high level afterwards; ethylene production by water-inoculated controls remained continuously at about 0.1 nmol h^{-1} g^{-1} fresh wt. (Fig. 2C). Comparable to the situation in TMV W U1-infected leaves, the peak in ethylene production coincidenced with a peak in both ACC production (Fig. 2A) and ACC content (Fig. 2B), and was succeeded by an increase in the capacity to convert ACC to ethylene (Fig. 2D). Hence, the pattern of ethylene biosynthesis is the same in Samsun NN leaves reacting hypersensitively to either TMV W U1 or TNV.

Ethylene production in virus-infected Samsun leaves

To determine in how far the *N* gene is involved in the stimulation of ethylene production, the *N* gene-lacking Samsun plants were inoculated with the mosaic-inducing TMV strain W U1, the ringspot-inducing TMV strain HR, TNV, which causes a hypersensitive reaction, or water as a control. As shown in Fig. 3, the time courses of ACC production, ACC content, ethylene production and ACC-converting capacity for Samsun leaves infected

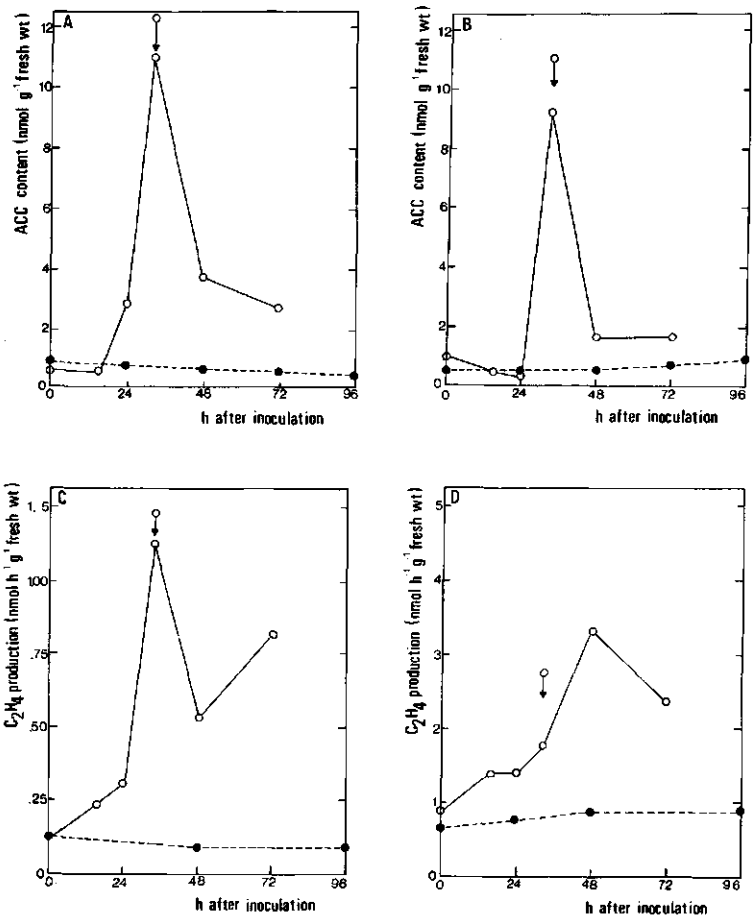

Fig. 2. Time courses of (A) ACC production, (B) ACC content, (C) ethylene production and (D) the ACC-converting capacity. Samsun NN leaves were inoculated with water (●-------●) or TNV (o————o), and incubated at 20°C at a photoperiod of 16h.

with TMV W U1 were comparable to those of TMV-infected Samsun NN leaves incubated at 30°C (Fig. 1A-D); neither the production of ACC and ethylene, nor the content and converting capacity of ACC exceeded the level of the water-inoculated controls. Also later after infection, when mosaic symptoms appeared on the young, developing leaves, ethylene production remained at the low control level (data not shown).

After infection with the HR strain, ringspotting became apparent after about 48 h. The formation of rapidly expanding necrotic rings was accompanied by a sharp increase in ACC production which remained at this high level for at least two days (Fig. 3A). ACC content (Fig. 3B), ethylene production (Fig. 3C), as well as ACC-converting capacity (Fig. 3D) kept increasing from lesion appearance onwards. Probably the increasing ACC content, ethylene production, and ACC-converting capacity reflect the expanding necrosis with the accumulation of ACC resulting from a loss of ACC-converting enzyme activity within necrotized cells (cf. Table 2).

After infection with TNV, local lesions appeared within 26 h. As in TNV-infected Samsun NN leaves, local-lesion appearance was accompanied by a sharp increase in ACC production, ACC content, ethylene production, and ACC-converting capacity (Fig. 3, cf. Fig. 1).

Since the development of necrosis in Samsun tobacco was associated with the same changes in the biosynthetic pathway of ethylene as in Samsun NN tobacco, the activity of the *N* gene cannot be involved. Rather, the amount of tissue affected during

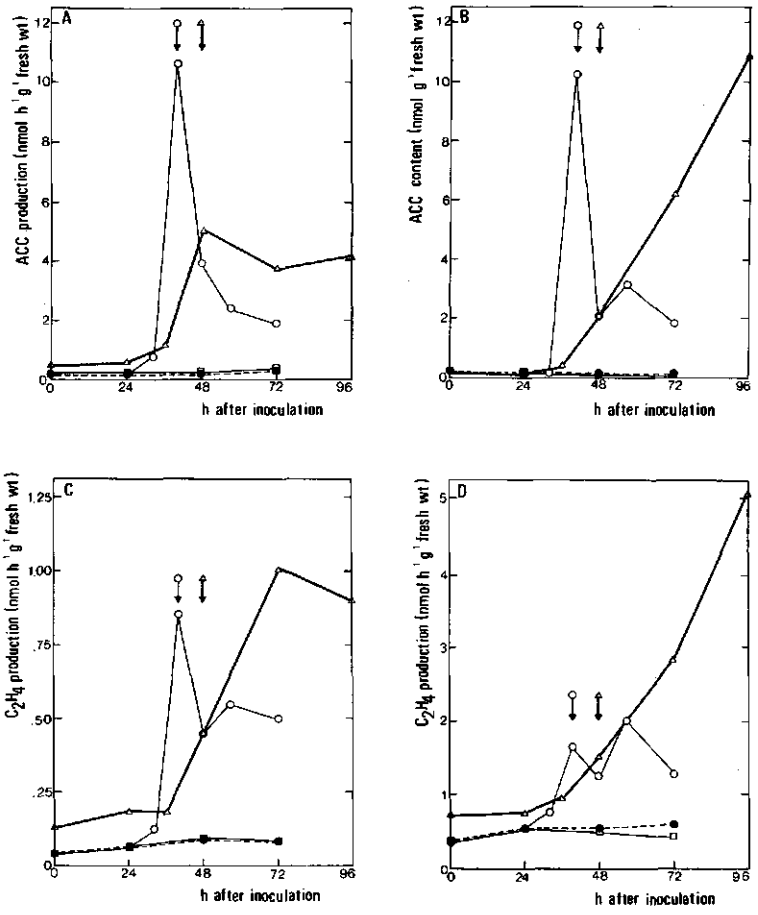

Fig. 3. Time courses of (A) ACC production, (B) ACC content, (C) ethylene production and (D) the ACC-converting capacity. Samsun leaves were inoculated with water (●--------●), TMV W U1 (□————□), TMV HR (Δ————Δ) or TNV (○————○) and incubated at 20°C.

necrogenesis determined the course and the extent of the stimulation, as evidenced by the differential effects provoked by TNV and TMV HR.

Comparison of ethylene biosynthesis in leaves with and without systemic acquired resistance

According to Pritchard and Ross (*15*), ethylene production starts earlier upon challenge inoculation of systemically resistant leaves on previously infected plants than on plants inoculated for the first time. Since ethylene also induces systemic acquired resistance (*24*), it may be a causative factor in the enhanced lesion limitation in systemically resistant and non-resistant leaves. Therefore, a comparison of the regulation of ethylene biosynthesis between systemically resistant and non-resistant leaves may provide additional information on the mechanism of acquired resistance.

A comparison between non-infected leaves from plants that had been inoculated previously with TMV, and comparable leaves from plants that had previously been treated with water, showed that the only consistent difference between the two was an increase in the former, systemically-resistant ones, in the capacity to convert ACC to ethylene. The increased capacity was evident in all the leaves above the primarily inoculated ones (Fig. 4), varying from 20 to 200% in different experiments. ACC-converting capacity was highest in the youngest leaves and relatively low in older ones. Interestingly, hardly any increase due to primary inoculation was observed in the older leaves.

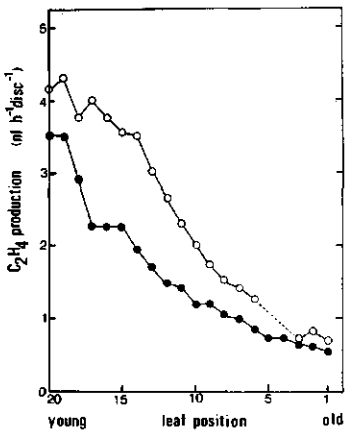

Fig. 4. ACC-stimulated ethylene production by discs from leaves of different age from non-resistant (●——●) or systemically resistant (o——o) Samsun NN plants. Leaf 4 and 5 were inoculated with TMV W U1 or water 7 days before; discs of all leaves were cut and incubated on 1 mM ACC in light. Ethylene production was measured over a 20h period.

When 7 days after primary infection the two leaves just above the inoculated ones were challenge inoculated with TMV, the patterns shown in Fig. 5 were obtained. In systemically resistant leaves, the moment of lesion appearance and the number of lesions were unaffected, but lesion size was decreased between 20 and 60%. ACC production was increased severalfold around the time of lesion appearance (Fig. 5A), but in most experiments it remained below the level of the non-resistant leaves. This is probably connected with the decrease in total lesion area. As a result of the increase in ACC production (Fig. 5A), high amounts of ACC accumulated in the non-resistant

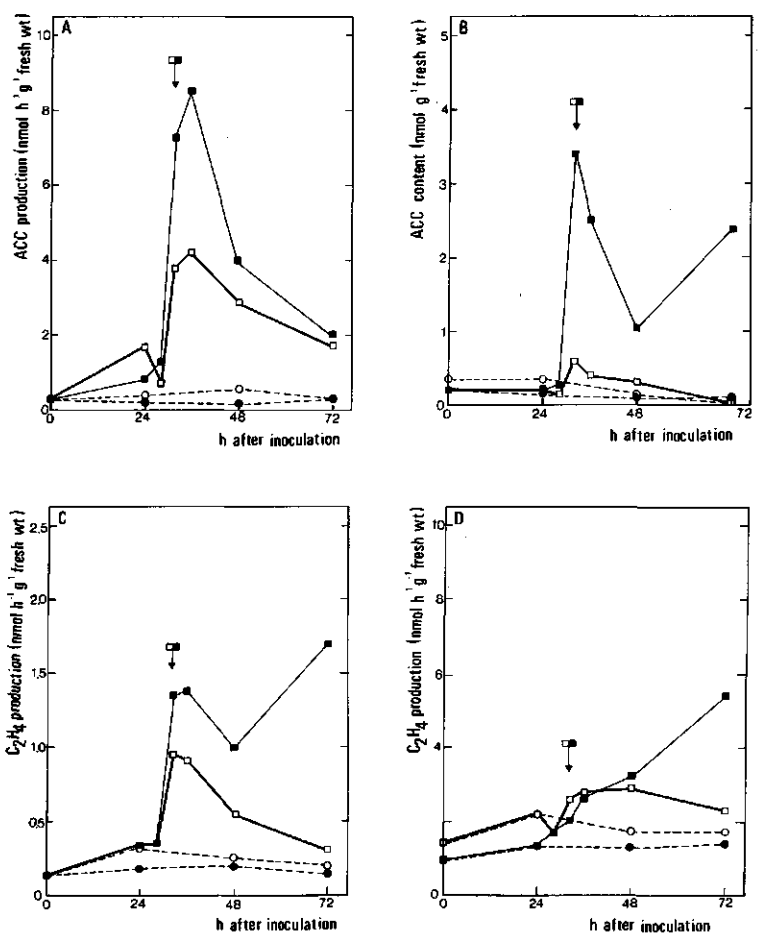

Fig. 5. Time course of (A) ACC production, (B) ACC content, (C) ethylene production and (D) the ACC converting capacity in systemically resistant (o,□) or non-resistant (●,■) Samsun NN leaves after inoculation with either water (------) or TMV W U1 (———).

leaves (Fig. 5B, cf. Fig. 1B) until the ACC-converting capacity had increased. However, in the systemically resistant leaves ACC accumulation was negligible, apparently as a result of their increased capacity for ACC conversion to ethylene (Fig. 5D). In contrast to the findings of Pritchard and Ross (*15*), virus-induced ethylene production in the systemically resistant leaves did not start detectably earlier than in the non-resistant leaves (Fig. 5C).

TMV-induced ethylene production in Samsun NN leaves after a shift from 30° to 20°C.

When Samsun NN leaves were infected with TMV W U1 at 30°C and shifted to 20°C 2 days after inoculation, within 8 h large necrotic lesions developed, associated with a rapid production of extremely high amounts of ethylene (Fig. 6). Virus-induced ethylene production started about 2 h after the shift and reached a maximum up to 50 nmol h^{-1} g^{-1} fresh wt 14 h later. Because under these conditions a) lesions were very large, b) the extent of virus-stimulated ethylene production was extremely high, and c) the timing of the increase in ethylene production was quite predictable, this type of experiment was adopted for studies on the characteristics of virus-stimulated ethylene production.

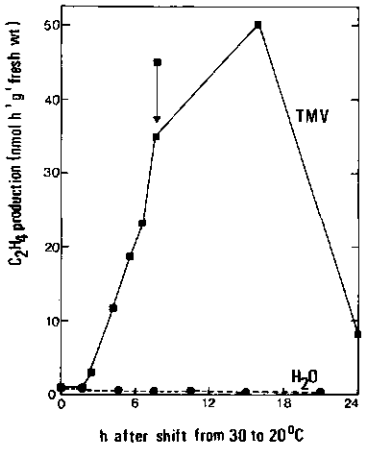

Fig. 6. Stimulation of ethylene production in Samsun NN leaves after a temperature shift from 30° to 20°C. Leaves were inoculated with either water (●--------●) or TMV W U1 (■————■) and incubated at 30°C for 3 days, then transferred to 20°C. Ethylene production was measured over the subsequent 24h period.

Localization of virus-induced ethylene production

Since the rate of virus-induced ethylene production is proportional to the number and size of the local lesions (*4,14*, cf. also Fig. 5), this ethylene is assumed to be produced locally within, or in the tissue just surrounding, the necrotizing cells. This assumption was tested by strip-inoculating 5 mm wide zones of Samsun NN leaves, and separating the lesion-free and lesion-containing strips 2 days later by cutting. Ethylene production was measured over the subsequent 24h period. As shown in Table 2, the virus-stimulated ethylene production was completely restricted to the lesion-containing areas.

Efforts to define the localization of virus-stimulated ethylene production more precisely by measuring ethylene production of 1 mm discs containing the necrotic lesions and rings enclosing the surrounding tissues, suffered from the substantial and unpredictable production of wound ethylene, and were therefore abandoned. As an alternative, the localization of both ACC content and ACC production was determined in leaves from infected plants that were kept for 2 days at 20°C, then 3 days at 30°C,

Table 2 Localization of ethylene production in Samsun NN leaves stripinoculated with TMV W U1 (100 μg ml^{-1})

	C_2H_4 production[a] ($nl\ h^{-1}\ g^{-1}$ fresh wt)	
	0-4 h	4-24 h
non-inoculated leaves	2.45	0.24
TMV-inoculated lesion-free strips[b]	2.13	0.36
" lesion-containing strips	8.39	4.11

[a] Ethylene production of the separated leaf parts was measured from 0-4 h (includes wound ethylene production due to the cutting) and from 4-24 h (wound ethylene effect subsided).

[b] Lesion-containing and lesion-free zones were separated by cutting 2 days after inoculation.

and subsequently shifted back to 20°C. Seven hours after the shift from 30° to 20°C, the distribution of ACC was determined by punching out 1.5 mm discs encompassing a primary lesion, 3 and 8 mm rings containing surrounding tissue with secondary necrosis, and 3 mm discs from between the necrotizing areas. The rate of ACC production was determined similarly after a 6 h anaerobic incubation between 6 and 12 h after the temperature shift. As shown in Table 3, the distribution of both ACC content and ACC production was far from uniform; the concentration of ACC in the primary lesions and in the surrounding tissue rings was 40, 30 and 5 times higher, respectively, than that in the non-infected leaf parts. ACC production in primary lesions and surrounding tissue was 8 and 4 times higher, respectively, than in the control tissue.

The nature of the virus-stimulated ACC production

In all host-virus combinations in which ethylene production was stimulated, the pattern was similar to that described previously for TMV W U1-infected Samsun NN (5). Ethylene production thus appears to be regulated primarily at the level of ACC production. To test whether the virus-stimulated ACC production involves RNA and protein synthesis,

Table 3 Distribution of ACC content and ACC production in leaves from Samsun NN plants kept at 20°C for 2 days after inoculation with TMV W U1 (1 μg ml^{-1}), then at 30°C for 3 days and subsequently shifted to 20°C

	Bore diam.	ACC content[a] (nmol g^{-1} fresh wt)	ACC production[b] (nmol $h^{-1}\ g^{-1}$ fresh wt)
primary lesion	1.5	12.4	10.5
surrounding tissue	3	9.0	-[c]
rings	8	1.4	5.1
non-infected leaf parts	3	0.3	1.3

[a] ACC content in primary lesions, surrounding tissues, and uninfected leaf areas was determined 7h after the shift from 30° to 20°C.

[b] Localization of ACC production was calculated from the increase in ACC content in the different tissue areas after anaerobic incubation of intact leaves from 6 to 12h after the temperature shift.

[c] not determined

Table 4 Influence of inhibitors of RNA and protein synthesis on ethylene production and ACC content[a]

	C_2H_4 production[b] (nmol day^{-1} $leaf^{-1}$)	% inhibition	ACC content[c] (nmol g^{-1} fresh wt)
H_2O	20.2	--	33.8
20ug·ml^{-1} chloramphenicol	18.4	9	32.5
0.1 mM cycloheximide	10.7	47	30.3
0.1 mM D-MDMP	13.7	32	26.2
10 ug·ml^{-1} actinomycin D	14.9	26	20.3
0.04 mM cordycepin	7.0	65	8.7

[a] Two days after inoculation with 100 µg ml^{-1} TMV W U1 Samsun NN leaf-discs were shifted from 30° to 20°C and floated on water or inhibitor solutions.

[b] Ethylene production was measured from 4 to 16 h after the temperature shift and calculated as the mean of 4 independent incubations.

[c] ACC content was measured at the end of the 16h incubation period.

inoculated leaf discs were incubated at 30°C, and then shifted to 20°C in the presence of inhibitors. A representative experiment is listed in Table 4. Due to the temperature shift and the resulting onset of necrotic lesion formation, ethylene production was increased c. 100-fold. With the exception of chloramphenicol, that inhibits protein synthesis in chloroplasts and mitochondria, all inhibitors tested decreased both ethylene production and ACC content. Cordycepin, presumed to specifically block m-RNA synthesis, proved to be the most effective inhibitor by far. As none of the inhibitors affected virus concentration for the duration of the experiment, these observations strongly suggest both RNA- and protein synthesis to be involved in virus-stimulated production of ACC and ethylene.

The nature of the trigger for virus-stimulated ACC production

Since loss of membrane integrity during a hypersensitive reaction is the earliest

Table 5 Influence of local membrane destruction on ethylene production and ACC content

Pricking[a]	C_2H_4 production[b] (nl h^{-1} g^{-1} fresh wt) 0-4 h	4-24 h	ACC content[c] (nmol g^{-1} fresh wt)
no treatment	3.1	1.8	0.31
water	1.9	1.4	0.40
liquid nitrogen	2.4	1.0	0.34
deoxycholate	2.1	2.8	0.38
Nonidet P 40	2.6	1.2	0.29
Triton X-100	1.9	1.4	0.28
Tween 20	2.3	1.6	0.40

[a] Samsun NN leaves were pricked with needles moistened with liquid nitrogen or 1% solutions of detergents.

[b] Ethylene production of cut leaf discs was measured from 0-4 and 4-24 h of incubation.

[c] ACC content was determined at the end of the incubation period.

event described (*26*), and may precede the increase in ACC production, the effect of point freezing of non-infected leaves and of local treatment with detergents on ACC content and ethylene production were determined. A representative experiment is given in Table 5. In three experiments, none of the treatments affected ethylene production in a consistant manner. Nor did ACC accumulate to any significant extent.

To see whether a chemical trigger was produced in virus-infected leaves, non-infected leaf discs were vacuum infiltrated with aqueous extracts from non-infected or TMV W U1-infected Samsun NN leaves, or with water. As shown in Table 6, no significant increase in ethylene production was observed. The slight increases due to infiltration with extracts made 8 or 24 h after the temperature shift were caused by ACC present in the extracts already. Phloem exudates from virus-infected leaves proved equally ineffective (data not shown).

Table 6 Ethylene production by Samsun NN leaf discs after vacuum infiltration with an extract from non-infected leaves, extracts from virus-infected leaves made 0, 4, 8 or 24h after a temperature (T) shift from 30° to 20°C 3 days after inoculation with TMV W U1 or with water

Vacuum infiltration	ACC content in the extract (nmol ml^{-1})	C_2H_4 production ($nl\ h^{-1}\ disc^{-1}$) 0-4 h	4-24 h
water	-	0.56	0.04
healthy leaf extract	0.04	0.28	0.10
virus-infected leaf extract			
0 h after T shift	0.03	0.21	0.07
4 h " "	0.32	0.20	0.07
8 h " "	5.04	0.40	0.14
24 h " "	25.48	0.38	0.35

DISCUSSION

In all host-virus combinations giving rise to a localized necrosis (Samsun NN tobacco inoculated with TMV W U1 or TNV, and Samsun tobacco infected with TMV HR or TNV), the appearance of symptoms was accompanied by a sharp rise in ethylene emanation. The regulation of virus-stimulated ethylene production was the same in hypersensitively reacting Samsun NN and Samsun plants, demonstrating both tobacco varieties to have the potential to react to virus infection with increased ethylene production. Such an increase was connected with the type of symptoms produced and did not depend on the nature of the infecting virus either. Upon systemic infection (e.g. TMV W U1-infected Samsun plants), the ethylene production was not increased. Under such circumstances the initial step in virus-stimulated ethylene synthesis, the increase in ACC production, did not occur. Therefore, it is the specific type of response, such as hypersensitive necrosis with which the increase in ethylene emanation is connected.

During a hypersensitive reaction, the increase in ethylene production is already evident prior to lesion appearance (*5, 15*). Ethylene production is likewise increased in *Tetragonia expansa* leaves that form chlorotic lesions in response to infection with bean yellow mosaic virus (*7*). Therefore, a wound response connected with necrosis as the cause of the virus-induced ethylene production can be excluded. Conversely,

necrotization is not a direct result of the increased ethylene production. By gassing tobacco leaves for 40 h with 300 ppm ethylene, Pritchard and Ross (*15*) were able to induce chlorosis, but not necrosis. However, once necrosis is initiated, ethylene may stimulate the expansion of necrotic lesions.

The question as to how, in a hypersensitive reaction, viral infection leads to an increase in ethylene production is a challenging problem. Apparently, the decision to initiate ethylene production in response to virus infection falls either at, or prior to, the level of the increase in ACC production. The use of inhibitors indicates that *de novo* synthesis of both RNA and protein is involved in virus-stimulated ACC and ethylene production. The increase in ACC production clearly precedes the rise in ethylene emanation by several hours (5). Thus, the initiation of the rise in ACC-synthase activity, depending on RNA and protein synthesis, must depend on events well preceding any visible sign of symptom development.

Several approaches were adopted in the search for an inducing factor that increases ACC-synthase activity. The only known event that may precede the stimulation of ethylene production is a local loss of membrane integrity (*26*). On the one hand, increased membrane permeability can be a causative factor in the increase in ethylene production as proposed by Kende and Baumgartner (*11*) for the abundant ethylene emanation during flower fading. On the other hand, a loss of membrane integrity has been correlated with a decrease in ethylene-producing capacity, for instance in leaf discs exposed to high temperatures (*6*), and in apple protoplasts after treatments with detergents (*13*). In our experiments, point freezing or local treatment with detergents did not enhance ethylene production. This indicates that a localized loss of membrane integrity does not play a role in triggering the increase in ACC production in tobacco leaves. The search for ACC synthase-inducing agents in extracts or phloem exudates from hypersensitively reacting leaves has so far been equally unsuccesful.

Both the virus-induced ACC production and the resulting endogenous ACC increase were localized in the tissues within and just surrounding the local lesions. Hence, the diffusion rate of ACC must be low. Consequently, virus-induced ethylene will be produced locally within the lesion areas. This explains why the extent of virus-stimulated ethylene production is proportional to total lesion area (4,15). The less pronounced stimulation of ethylene production in systemically resistant Samsun NN leaves infected with TMV W U1 (cf. Fig. 5) is in agreememt with this observation.

As a consequence of primary infection, the ACC-converting capacity increased from lesion appearance on in the infected leaves, but also systemically throughout the plant (Fig. 4). Both the local and the systemic increase of the ACC-converting capacity parallelled the increase in peroxidase activity reported earlier (*22*). This supports the hypothesis by Apelbaum *et al.* (*1*) that the ACC-converting enzyme is of a peroxidase nature.

Apparently as a result of their increased rate for ACC conversion, systemically resistant leaves do not accumulate large amounts of ACC when challenged. Since all the ACC produced can be immediately converted to ethylene, ethylene production, on a cell basis, will be quicker and higher. Especially an early rise in virus-stimulated ethylene is assumed to play a role in resistance and virus localization (*15*).

Thus, the increased capacity for ACC conversion to ethylene may be involved in

limiting lesion size in systemically resistant leaves. Such induced resistance has been found to be essentially limited to the younger leaves (*25*), supporting the view that the lack of stimulation of ACC-converting capacity in older leaves is a limiting factor in the expression of acquired resistance.

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HOOFDSTUK V

The modulation of the conversion of l-aminocyclopropane-l-carboxylic acid to ethylene by light

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Abstract. Endogenous ethylene production of tobacco leaves was similar in light and in darkness. However, the rate of conversion of exogenously applied l-aminocyclopropane-l-carboxylic acid (ACC) to ethylene was reversibly inhibited by light. Virus-stimulated ethylene production, during the hypersensitive reaction of tobacco leaves to tobacco mosaic virus, was likewise inhibited by light. Under such circumstances ethylene production is limited at the level of the conversion of ACC to ethylene. Inhibition of the increase in ACC-stimulated ethylene production by cycloheximide and 2-(4-methyl-2,6-dinitroanilino)-N-methylpropionamide after shifting leaf discs from light to darkness indicated that de novo protein synthesis was involved. Regulation of ACC-dependent ethylene production by reversible oxidation/reduction of essential SH groups, as suggested by Gepstein and Thimann (1980, Planta **149**, 196–199) could be excluded. Instead, regulation of the ACC-converting enzyme at the level of both synthesis/degradation and activation/inactivation is suggested. Phytochrome was not involved in light inhibition, but low intensities of either red or blue light decreased the rate of ACC conversion. Dichlorophenyldimethylurea counteracted the inhibitory effect of light, indicating that (part of) the photosynthetic system is involved in the light inhibition. The ethylene production of *Pharbitis* cotyledons grown in darkness or light, either in the presence or absence of the inhibitor of carotenoid synthesis, SAN 9789 (norflurazon), supported this view.

Key words: l-Aminocyclopropane-l-carboxylic acid – Ethylene – Light and ethylene production – *Nicotiana* – *Pharbitis* – Protein synthesis.

Abbreviations: ACC = l-aminocyclopropane-l-carboxylic acid; DCMU = dichlorophenyldimethylurea; MDMP = 2-(4-methyl-2,6-dinitroanilino)-N-methyl-propionamide; SAM = S-adenosylmethionine; SH groups = sulfhydryl groups; TCA = trichloroacetic acid; TMV = tobacco mosaic virus

Introduction

Formation of necrotic lesions during the hypersensitive reaction of *N* gene-containing tobacco cultivars to tobacco mosaic virus (TMV) is accompanied by a large burst of ethylene (Pritchard and Ross 1975). This ethylene is derived from methionine, as is ethylene in healthy tobacco leaves. The increase in ethylene production upon infection is associated with an increase in the content of l-aminocyclopropane-l-carboxylic acid (ACC) (de Laat et al. 1981).

Although ACC was isolated by Burroughs as early as 1957 from pear and apple fruits, it has only recently been identified as the direct precursor of ethylene in higher plants (Adams and Yang 1979; Lürssen et al. 1979), according to the following biosynthetic sequence: methionine→S-adenosylmethionine (SAM)→ACC→ethylene.

While studying the role and biosynthesis of virus-induced ethylene, we found that light strongly reduced ethylene production. Inhibition of ethylene production by light during phototropic curvature and elongation growth had been reported by Kang et al. (1967), Goeschl et al. (1967), and Samimy (1978). Since inhibition of ethylene production by red light was reversed by far-red irradiation, this light effect could be phytochrome-mediated (Goeschl et al. 1967; Samimy 1978). In contrast, Saltveit and Pharr (1980) found that light increased ethylene production in germinating cucumber seeds with regard to those held in darkness. Recently, De Greef et al. (1980) described a positive correlation between light intensity and ethylene production in bromeliads.

The conversion of ACC to ethylene can be experimentally blocked by $CoCl_2$. According to Yu and Yang (1979), such inhibition would result from an interaction between Co^{2+} and essential sulfhydryl (SH)-groups required by the enzyme to oxidize ACC to ethylene.

While this paper was in preparation, Gepstein and Thimann (1980) reported that light decreased the conversion of exogenous ACC to ethylene in tobacco leaf discs, ethylene production in the light being two to nine times less than in darkness. These authors reported that the conversion of ACC to ethylene in darkness was reduced by $CoCl_2$ to the level found in light. Moreover, the low ethylene production in light could be increased to the high level characteristic of darkness by adding free sulfhydryl-containing compounds, such as mercaptoethanol. From these results, they suggested that light modulated ACC-stimulated ethylene production at the level of the activity of the ACC-converting enzyme by oxidation-reduction of essential SH groups. However, these authors disregarded possible effects of $CoCl_2$ and of mercaptoethanol on ethylene production in the light and in darkness, respectively.

This report describes further investigations on the nature of the modulation by light of the ethylene production from ACC.

Materials and methods

Plant material. Tobacco plants (*Nicotiana tabacum* L. cv. Samsun *NN*) were grown from seed in a greenhouse at a minimum temperature of 20° C. Fully-grown leaves of about 10-week-old plants were selected for the experiments.

Seeds of *Pharbitis nil* Chois, cv. Violet (obtained from Marutana Co. Ltd., Kyoto, Japan) were imbibed and grown for 3 d at 20° C either in darkness or under "cool-white" fluorescent light (5 Wm^{-2}) (see Table 1). Plants grown in darkness were exposed to far-red irradiation (10 Wm^{-2}) during the last 24 h before the start of experimental treatments, in order to promote expansion of the cotyledons. To prevent accumulation of chlorophyll in light-grown plants, some of the seedlings were treated with the herbicide SAN 9789 (norflurazon), which inhibits carotenoid synthesis (Frosch et al. 1979). To this end, seeds were imbibed and incubated in 0.5 mM SAN 9789 for 24 h and the seedlings were subsequently watered with the same solution. In all cases, cotyledons of 6-day-old plants were used for the experiments.

Incubation conditions. Plant material was sampled around 9.00 h. Tobacco leaf discs (25 mm diameter; average weight 0.10 g) and cotyledons of *Pharbitis* seedlings were incubated in 40-ml sealed serum flasks at 20° C in either 1 ml water or 1 ml of a 1-mM ACC solution.

Incubations were carried out in darkness or under different light conditions (Table 1). Light intensities were measured with an UTD photodetector Model-80X (United Detector Technology, Santa Monica, Ca., USA).

To compare the effects of SH reagents and SH-containing compounds to the results of Gepstein and Thimann (1980) the leaf discs were incubated in different concentrations of $CoCl_2$ and mercaptoethanol, respectively.

To study the involvement of photosynthesis in light effects, the leaf discs were vacuum-infiltrated with and further incubated in 1 ml 100-μM solutions of DCMU.

The involvement of protein synthesis was studied by incubating tobacco leaf discs in 1 ml 0.1-mM solutions of either cycloheximide or the D- or L-isomer of 2-(4-methyl-2,6-dinitroanilino)-N-methyl-propionamide (MDMP). D-MDMP is a very potent, specific inhibitor of protein synthesis at the translational level, whereas its L-isomer is completely inactive in this respect (Baxter et al. 1973).

Table 1. Irradiation treatments

Irradiation	Light source	Filter	Wave length (nm)	Fluence rate
white	'cool white' fluorescent tubes (FR40 T12/CW)[a]	–	–	10 W m^{-2}
blue	250 W halogen/ quartz lamps	3 mm BG 23[b]/ 30 mm $CuSO_4$	550	10 W m^{-2}
red	,,	Baird Atomic 57-60-2[c]	661–673	0–20 W m^{-2}
far red	,,	Baird Atomic 57-60-2[c]	725–737	10 W m^{-2}

[a] Sylvania, Waltham, Mass., USA
[b] Schott and Gen., Mainz, FRG
[c] Baird Atomic, Redford, Mass., USA

The influence of light on ethylene production during the hypersensitive reaction of tobacco to TMV was studied using detached, whole leaves. Carborundum-dusted leaves were rubbed with a gauze pad with either TMV W U1 (100 μg ml^{-1}) or water (control). After a rinse with tap water, these leaves were incubated in water-locked, 750-ml Petri dishes at 20° C (De Laat et al. 1981).

Measurement of ethylene production. At specific times, 1-ml gas samples were withdrawn through the rubber seal of the incubation vials or through a septum on the pierced lid of the Petri dishes. The amount of ethylene in the sample was measured on a gas chromatograph equipped with an aluminum oxide column and a flame ionization detector. In each experiment, the ethylene production was determined in at least three replicate incubations. When incubations were continued for more than 24 h, the vials were flushed with fresh air to avoid accumulation of ethylene and CO_2.

Measurement of protein synthesis. Tobacco leaf discs were floated on water or MDMP solutions in the presence of $1.9 \cdot 10^4$ Bq L-[U-^{14}C]methionine in white light. At specific times, some leaf discs were frozen in liquid nitrogen, ground in a mortar with pestle, and 2 ml 0.1 M Tris-HCl pH 7.5, containing 0.2% ascorbate, were added per disc. The mixture was stirred for 30 min at 0° C and centrifuged for 10 min at 10,000 *g*. An equal volume of 10% TCA was added to the supernatant to precipitate the protein. After 24 h at 0° C, the precipitated protein was collected on a millipore filter and washed with a cold solution of 5% TCA containing 10 mM unlabeled methionine. The amount of radioactivity on the filter was determined by liquid scintillation counting in Lumagel.

Measurement of photosynthesis. Tobacco leaf discs were incubated at 20° C and irradiated with different intensities of red light. Net photosynthetic activity was calculated from CO_2 uptake, which had been determined katharometrically according to Pieters (1971).

Chemicals. ACC was a generous gift from Bayer A.G., Leverkusen, FRG. Samples of L- and D-MDMP were kindly provided by Shell Bioscience Laboratory, Sittingbourne, U.K. SAN 9789 was purchased from Sandoz A.G., Basel, Switzerland, and L-[U-^{14}C]meth-

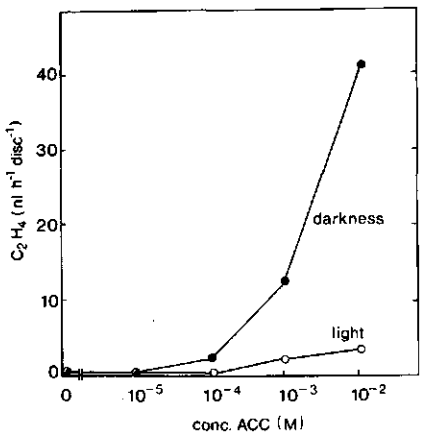

Fig. 1. Ethylene production of tobacco leaf discs incubated in ACC in continuous light (○) or continuous darkness (●). The rate of ethylene production was taken as the average from the first four hours of incubation

ionine (specific radioactivity $1.07 \cdot 10^{10}$ Bq $mmol^{-1}$) from The Radiochemical Centre, Amersham, U.K.

Results

Ethylene production by tobacco leaf discs in light and darkness. In the light, tobacco leaf discs floating on water produced between 0.09 and 0.39 nl ethylene h^{-1} $disc^{-1}$, corresponding to one-to-three nl h^{-1} g^{-1} fw. These values agreed well with those reported for tobacco leaf discs by Aharoni and Lieberman (1979). Endogenous ethylene production in darkness did not differ significantly from that in the light (cf. Fig. 7).

When 1 mM ACC was applied to discs incubated in the light, the ethylene production increased without a lag phase from 0.2 to 2.5 nl h^{-1} $disc^{-1}$ within the first 30 min and remained at this level for at least 4 h. However, in darkness ethylene production increased to 12.4 nl h^{-1} during the first 4 h of incubation. The effect of ACC on ethylene production was concentration-dependent (Fig. 1).

When leaf discs had been incubated in 1-mM solutions of ACC in continuous light for 24 h, and were then shifted to darkness, ethylene production increased immediately (Fig. 2A). In most experiments, this increase was more than tenfold within the first 2 h. Conversely, after 24 h of incubation in ACC in darkness, ethylene production decreased to the low level found in light within 6 h after the onset of illumination (Fig. 2B).

If ACC was omitted during incubation in darkness for several hours, and added when the discs were transferred to the light, the ethylene production was immediately at the low level characteristic for light conditions (data not shown). This indicates that the effect of darkness on the capacity to convert ACC to ethylene is immediately lost upon illumination.

Involvement of protein synthesis. The possibility that the increase in ACC-stimulated ethylene production in darkness depends on protein synthesis was examined. As shown in Table 2, the increase in ACC-stimulated ethylene production upon a shift from light to darkness was completely suppressed by 0.1 mM cycloheximide. However, because cycloheximide at this concentration inhibited protein synthesis by only about 60%, D-MDMP was chosen instead. D-MDMP, at a concentration of 0.1 mM, inhibited the

Table 2. Effect of cycloheximide (CH) on endogenous and ACC-stimulated ethylene production. Tobacco leaf discs were preincubated in the presence or absence of CH (0.1 mM) and ACC (1 mM) in light for 3 h, then incubated for 3 h in either light or darkness

	C_2H_4 production (nl h^{-1} $disc^{-1}$)	
	3 h light	3 h darkness
H_2O	0.28 ± 0.04	0.28 ± 0.05
ACC	1.69 ± 0.16	3.14 ± 0.38
CH	0.37 ± 0.04	0.22 ± 0.08
ACC + CH	1.47 ± 0.26	1.01 ± 0.30

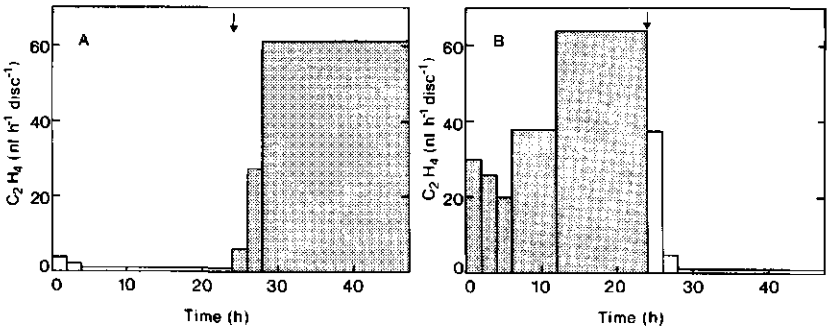

Fig. 2A, B. Influence of light-dark **A** and dark-light **B** shifts on the conversion of ACC to ethylene. Tobacco leaf discs were incubated in 1 mM ACC in continuous light (▭) or darkness (▨) and shifted to darkness and light, respectively, after 24 h (*arrow*)

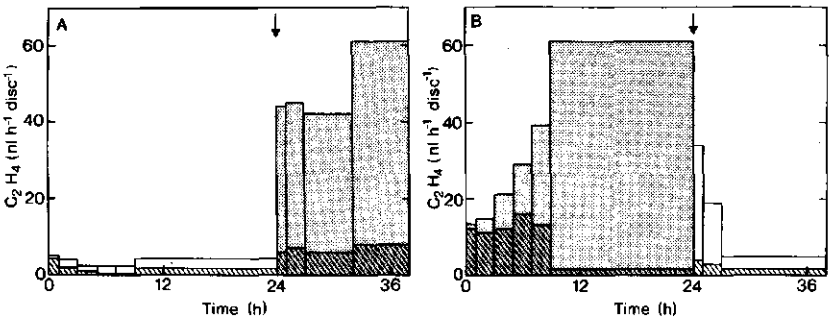

Fig. 3A, B. Influence of L- (▭) and D-MDMP (▨) on the conversion of ACC to ethylene. Leaf discs were incubated in **A** white light (▭, ▨) or **B** darkness (▩, ■). After 24 h of incubation, the light conditions were reversed (*arrow*)

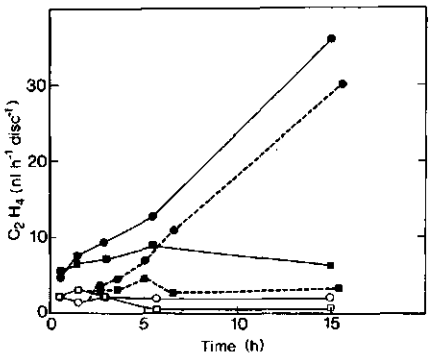

Fig. 4. Influence of D-MDMP on the course of ethylene production from ACC. Tobacco leaf discs were incubated in 1 mM ACC in the absence (○, ●) or presence (□, ■) of 0.1 mM D-MDMP. Incubations were carried out in light (○, □) or darkness (●, ■) continuously (——), or the flasks were shifted from light to darkness after 2 h (---)

incorporation of labeled methionine into protein by more than 90% within 2 h, whereas its L-isomer was completely inactive. The L-isomer was used as a control to correct for possible side effects, such as any stress ethylene that might be induced. However, no such stress-ethylene production was observed at this concentration of MDMP.

In the presence of 0.1 mM L-MDMP, a shift in light conditions effected the same increase or decrease of ACC-stimulated ethylene production as shown before (cf. Fig. 2). However, in the presence of the D-isomer, the increase in ACC-stimulated ethylene production was nearly abolished upon a change from light to darkness (Fig. 3A). D-MDMP also removed the increase in ACC-induced ethylene production in darkness upon the start of incubation (Fig. 3B). In the presence of D-MDMP, the ethylene production decreased from 14 to 2 nl h^{-1} $disc^{-1}$ within 24 h of dark incubation, possibly due to turnover of the enzyme(s) involved in the conversion of ACC to ethylene. Application of ACC to leaf discs at different times after pretreatment by D-MDMP demonstrated the half-life of the ACC-converting activity to be about 8 h. Under the various light conditions actinomycin D, at a concentration of 20 µg ml^{-1}, never affected the rate of ACC-stimulated ethylene production.

A requirement for protein synthesis was further indicated by observations made when leaf discs were shifted from light to darkness at different times after the start of incubation in 1 mM D-MDMP. Under continuous light, ACC-stimulated ethylene production reached a steady level of about 2 nl h^{-1} $disc^{-1}$ within 6 h. In continuous darkness, ethylene production started at 5 nl h^{-1} $disc^{-1}$ and increased almost linearly to 30 nl h^{-1} $disc^{-1}$ after 15 h (Fig. 4). This increase was nearly abolished by D-MDMP. When the leaf discs were shifted to darkness after 2 h incubation in the light, the rate of ethylene production immediately started to increase at a rate comparable to that in continuous darkness. However, in the presence of D-MDMP, ethylene production continued at the previous level in the light. Similar results were obtained when the shift from light to darkness was postponed for 5 h (data not shown).

Irradiation effects. When leaf discs were floated on a 1-mM solution of ACC, the ethylene production during the first two hours of incubation in darkness was 10.7 nl h^{-1}, on the average. In white light, ethylene production was inhibited by about 75% to 2.5 nl h^{-1} (Table 3, expt. 1). In red or blue light, ethylene production was decreased to a similar extent. However, far-red irradiation was ineffective in lowering the rate of ACC-stimulated ethylene production.

At a low intensity red light (2.5 W m^{-2}), the inhibition of ethylene production was 58% (Table 3, expt. 2). Simultaneous irradiation with high-intensity far-red (10 W m^{-2}) was ineffective in antagonizing the red-light effect; ethylene production remained unaffected at 42% of the dark control.

Figure 5 shows the effect of different intensities of red light on ACC-stimulated ethylene production and on net photosynthetic activity of tobacco leaf discs. Inhibition of ethylene production by red light was already saturated at 3 W m^{-2}, whereas photosynthesis was not yet saturated at about 20 W m^{-2}. To

Table 3. Influence of light of different wavelength on the production of ethylene by tobacco leaf discs incubated in 1 mM ACC. Ethylene was measured after a 2 h incubation period and compared to the production in darkness

Expt.	Irradiation	Relative C_2H_4 production (% of dark incubation)
1	white (10 W m^{-2})	24.5
	blue (10 W m^{-2})	22
	red (7.5 W m^{-2})	23.5
	far red (10 W m^{-2})	104
2	red (2.5 W m^{-2})	42.1
	red (2.5 W m^{-2}) + far red (10 W m^{-2})	41.8

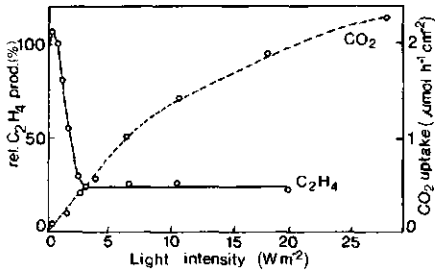

Fig. 5. Relative rate of ACC-stimulated ethylene production (o—o) and net photosynthesis (o---o) in tobacco leaf discs incubated under red light of different intensities. Discs were incubated in 1 mM ACC and ethylene production was related to the production of control discs kept in darkness. The photosynthetic activity was determined by measuring the uptake of carbon dioxide katharometrically

check for an effect of photosynthesis on ACC-stimulated ethylene production, the leaf discs were preincubated in either water or a 0.1-mM solution of DCMU for 3 h. Ethylene production was then determined in the presence or absence of 1 mM ACC in both light and darkness. As shown in Table 4, DCMU affected neither the endogenous nor the ACC-stimulated ethylene production in darkness. However, ACC-stimulated ethylene production in the light was increased by DCMU, well up to the level of the ACC-stimulated ethylene production in darkness.

The possibility, proposed by Gepstein and Thimann (1980), that light exerts its inhibitory effect by oxidation of essential SH groups of the ACC-converting enzyme was further examined. $CoCl_2$, presumed to block the essential SH groups, inhibited the conversion of ACC to ethylene in both darkness and light to similar extents. Moreover, mercaptoethanol, found by Gepstein and Thimann (1980) to increase the low ethylene production in light to the high level of darkness, at the same concentrations, did not affect ethylene production from ACC in either light or darkness.

Table 4. Influence of DCMU on endogenous and ACC-stimulated ethylene production by leaf discs incubated in light or darkness. Leaf discs were vacuum infiltrated and further incubated in either water or 0.1 mM DCMU for 3 h. After that, discs were incubated in the presence or in the absence of 1 mM ACC and 0.1 mM DCMU. The rate of ethylene production, measured after 4 h of incubation, was taken as the mean of four different incubations ± sd

	Preincubation	Incubation	Ethylene production (nl h^{-1} $disc^{-1}$)
Light	water	water	0.20 ± 0.04
	,,	ACC	1.04 ± 0.11
	DCMU	DCMU	0.27 ± 0.04
	,,	ACC + DCMU	7.46 ± 1.61
Darkness	water	water	0.24 ± 0.07
	,,	ACC	5.72 ± 1.60
	DCMU	DCMU	0.26 ± 0.07
	,,	ACC + DCMU	5.60 ± 1.35

Table 5. Ethylene production of *Pharbitis nil* cotyledons incubated in the presence or absence of ACC under different light conditions. Seedlings were grown from seed in continuous white light or in darkness, either in the presence or absence of SAN 9789. Ethylene production was measured after a 4-h incubation period

Growth conditions of seedlings	Incubation conditions	C_2H_4 production (nl h^{-1})	
		Light	Darkness
Light	water	0.08	0.09
	10^{-3}M ACC	1.47	7.38
Light;	water	0.10	0.08
$5 \cdot 10^{-5}$M SAN 9789	10^{-3}M ACC	4.27	4.61
Darkness	water	0.33	0.36
	10^{-3}M ACC	4.53	4.86
Darkness;	water	0.49	0.49
$5 \cdot 10^{-5}$M SAN 9789	10^{-3}M ACC	3.90	4.76

Ethylene production in cotyledons of Pharbitis nil. As in tobacco leaf discs, endogenous ethylene production by cotyledons of light-grown *Pharbitis* plants was the same in light and darkness (Table 5). However, the endogenous ethylene production by cotyledons from dark-grown plants was considerably higher than that by cotyledons from plants grown in the light. These results agree with those of Cameron et al. (1979) and Gepstein and Thimann (1980).

Plants grown in the light and treated with SAN 9789 did not contain any chlorophyll. However, endogenous ethylene production by these cotyledons was quite similar to that of green cotyledons from untreated, light-grown plants.

Cotyledons of *Pharbitis* also produced large amounts of ethylene during incubation in ACC. As in tobacco leaf discs, ACC-induced ethylene production in green cotyledons was inhibited by light. However, light did not appreciably inhibit the ethylene

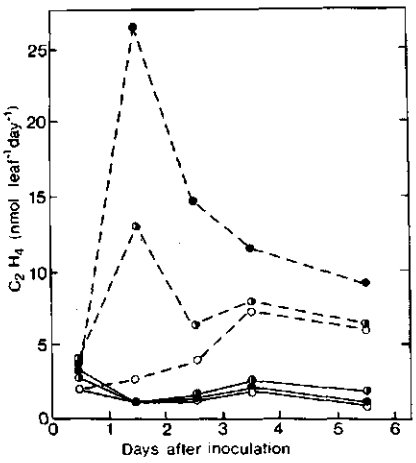

Fig. 6. Ethylene production of healthy (——) or TMV-infected (– – –) tobacco leaves incubated in continuous light (○), 12 h light/12 h darkness (◑), or in continuous darkness (●)

production in cotyledons from plants that lacked chlorophyll, whether they were grown in darkness or in the light in the presence of SAN 9789 (Table 5).

Influence of light on ethylene production during the hypersensitive reaction of tobacco leaves to TMV. Figure 6 shows the effect of different light conditions on ethylene production of healthy or TMV-infected tobacco leaves. The synthesis of ethylene by healthy leaves was not significantly affected by light. In this respect intact leaves reacted similarly to leaf discs floating on water (cf. Table 2). However, the large stimulation of ethylene production due to TMV infection was subject to modulation by light in the same way as ACC-stimulated ethylene production was. Under natural conditions (12 h light/12 h darkness), ethylene production was increased severalfold during the second day after inoculation. The increase was about two times higher in continuous darkness, whereas in continuous light the peak in the ethylene production was absent, and TMV-induced ethylene production only gradually reached a level characteristic of diseased leaves.

Discussion

In light, incubation in, e.g., 1 mM ACC, increased ethylene production of tobacco leaf discs about tenfold without any lag phase. Therefore, ACC synthesis rather than conversion of ACC to ethylene limited the rate of ethylene production in healthy tobacco leaf discs. Because the endogenous ethylene production was not influenced by light, the physiological importance of the phenomenon described here, namely the influence of light on the conversion of exogenously-applied ACC to ethylene, was not immediately clear. However, differences in the rate of ethylene production between light and darkness did become apparent under conditions involving a large stimulation of endogenous ethylene synthesis in tobacco leaves responding hypersensitively to TMV infection. Such leaves accumulated substantial amounts of ACC as the ethylene production was rapidly increasing (de Laat et al. 1981). Since virus-induced ethylene production and ACC-stimulated ethylene synthesis were both modulated by light in the same way, the pathways for the conversion of endogenous ACC and exogenously applied ACC appear to be the same. However, light modulation only became apparent when the ACC-converting enzyme could no longer cope with the endogenous or exogenous supply of ACC, and ACC accumulated. Because under normal conditions the rate-limiting step is ACC synthesis, a modulation of the activity of the ACC-converting enzyme by light would not be expected to greatly affect the rate of normal ethylene production, as was indeed observed.

No lag phase for the inhibiting effect of light on the conversion of ACC to ethylene was apparent. The ACC-stimulated ethylene production increased with increasing ACC concentration up to at least 10 mM. This contrasts with the results of Gepstein and Thimann who found the reaction to be saturated at 0.2 mM. Our results are in good agreement with those of Konze and Kende (1979), who demonstrated the ACC-oxidizing enzyme to have a rather low affinity for ACC.

Experiments in which light conditions were changed, demonstrated that the light inhibition of the conversion of ACC to ethylene was fully reversible. However, since $CoCl_2$ inhibited ACC-stimulated ethylene production in both light and darkness and, moreover, since we did not observe any distinct effect of mercaptoethanol, the hypothesis of Gepstein and Thimann that light acts by the reversible oxidation of SH groups was not confirmed.

Both D-MDMP and cycloheximide prevented the increase in ethylene production in continuous darkness or when the leaf discs were transferred from light to darkness. However, D-MDMP proved to be a more efficient inhibitor of protein synthesis in tobacco leaf discs. Further experiments with D-MDMP demonstrated a) a requirement for continued protein synthesis for ACC-stimulated ethylene synthesis to proceed, b) that the increase in ACC-stimulated ethylene production upon a shift from light to darkness requires protein synthesis, and c) that the rate of this increase is not influenced by the duration of the previous light period. Transcription does not seem to be required, in accordance with the rapidity with which the stimulation is realized.

A rapid decrease in ethylene production was ob-

served when leaf discs incubated in ACC were transferred from darkness to light. The nature of this decrease could not be assessed. D-MDMP proved to be inefficient in inhibiting protein synthesis in leaf discs when added several hours after the start of incubation, apparently as a result of poor uptake in the humid environment. Since conversion of ACC to ethylene in the presence of D-MDMP decreased only slowly with time (Fig. 5), and the half-life time of the enzyme was found to be about 8 h, the turnover of the ACC-converting enzyme must be rather limited. Therefore, the rapid decrease in enzyme activity could not be explained by a decline in enzyme synthesis. This leaves the possibility that a regulating factor, itself turning over rapidly, might be synthesized which specifically inactivates the ACC-converting enzyme in light. Alternatively, the enzyme could be regulated at the level of both synthesis/degradation and activation/inactivation, or enzyme activity might depend on the presence of a co-factor which is rapidly inactivated in light. A similar situation has been shown to exist for the decrease in phenylalanine ammonia-lyase activity upon a shift from light to darkness (Creasy and Zucker 1974).

Since activation/inactivation cannot be explained by the reversible oxidation of SH groups, we further investigated the nature of the inhibition of ethylene production by light. Because irradiation of leaf discs with continuous far-red did not inhibit ethylene production, the high-irradiance reaction did not seem to be involved (Schäfer 1976). No phytochrome effect at all was apparent because red/far-red reversibility was absent. Because blue and red light inhibited ethylene production to the same extent, light inhibition of the reaction might be related to the photosynthetic system. Indeed, DCMU increased ACC-stimulated ethylene production by tobacco leaf discs incubated in light to the level at darkness. Since maximal inhibition of ACC conversion was attained at (very) low intensities of red light (about 3 W m^{-2}), whereas photosynthesis was not yet saturated at 20 W m^{-2}, only part of the photosynthetic system might be involved.

In cotyledons of *Pharbitis nil* seedlings grown in darkness or treated with SAN 9789, ethylene production from ACC was not influenced by light. Since these plants were photosynthetically inactive, this result agrees with the hypothesis that the light inhibition of the conversion of ACC to ethylene depends on (part of) the photosynthetic system. In contrast, differences in the endogenous ethylene production between green and etiolated plants, as also reported by Cameron et al. (1979), were not caused by photosynthetic activity, because the endogenous ethylene production in the photosynthetically inactive cotyledons from plants grown in light, but treated with SAN 9789, equalled that of green plants. The differences in endogenous ethylene production, found between light-grown and dark-grown plants, did not depend on differences in the activity of the ACC-converting enzyme, since ethylene production could be stimulated manyfold by the addition of ACC in both types of plants.

Hence, (part of) the photosynthetic system may be involved in the regulation of the capacity to synthesize ethylene from ACC. This regulation cannot be explained by the reversible oxidation/reduction of SH groups, but apparently involves both an increase in protein synthesis in darkness and a rapid inactivation mechanism upon illumination.

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HOOFDSTUK VI

Regulation of Ethylene Biosynthesis in Virus-Infected Tobacco Leaves

IV. INFLUENCE OF TEMPERATURE, LIGHT CONDITIONS AND LEAF AGE ON ETHYLENE PRODUCTION AND SYMPTOM EXPRESSION.

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ABSTRACT

To investigate the relationship between virus-induced ethylene production and symptom expression in Samsun NN tobacco, the influences of temperature, light conditions and leaf age on the hypersensitive reaction to tobacco mosaic virus (TMV) were studied.

Both the production of 1-aminocyclopropane-1-carboxylic acid (ACC) and its conversion to ethylene increased with increasing temperatures up to 35°C, and decreased with leaf age. Light inhibited only the conversion of ACC to ethylene.

Temperature, light conditions and leaf age greatly affected the pattern of virus-stimulated ethylene production and lesion development. Lesion expansion increased with increasing temperatures up to 28°C and in continuous light at intensities above 750 lux, but decreased with leaf age. The enhanced localization of the virus in darkness and in old leaves was associated with a sharp peak in ethylene production near the time of lesion appearance, whereas in continuous light or in young leaves virus-induced ethylene production only gradually increased from lesion appearance onwards. Hence, the early burst in ethylene production appears to be involved in the virus-localizing reaction.

Temperature-shift experiments indicated that activity of the temperature-sensitive *N* gene is required for at least 6 h up to 24 h after inoculation to induce both an increase in ethylene production and development of necrotic local lesions at 30°. The *N* gene is thus involved only in the initiation, and not in the realization of the hypersensitive reaction.

INTRODUCTION

During the hypersensitive reaction of tobacco (*Nicotiana tabacum* L.) cv. Samsun NN to tobacco mosaic virus (TMV), the appearance of necrotic local lesions is accompanied by a large burst of ethylene (*4, 15*).

As during normal leaf development, this ethylene is synthesized from methionine via S-adenosylmethionine (SAM) and 1-aminocyclopropane -1-carboxylic acid (ACC), primarily due to an increase in ACC-synthase activity (*4, 6*). After lesions appear, the capacity to convert ACC to ethylene is also increased (*6*). In contrast, no change in ethylene production occurs when TMV-infected Samsun NN plants develop systemic mosaic symptoms at 30°C. At this temperature the *N* gene, conferring hypersensitivity towards TMV, is no longer expressed (*17*). However, the stimulation of ethylene produc-

tion is related to the type of symptoms expressed rather than to the genetic make-up of either host plant or virus (*7*).

In hypersensitively-reacting tobacco leaves, virus-stimulated ethylene production is assumed to be a causative factor in symptom expression and virus localization. Moreover, it may be responsible for the so-called systemic acquired resistance in which, after an inoculation of lower leaves with a necrotizing virus, upper leaves react to a challenge inoculation with smaller, and occasionally fewer lesions (*7, 15, 20*).

To further study the relationship between ethylene production and symptom expression, effects of temperature and light conditions, as well as the influence of leaf age were investigated.

MATERIALS AND METHODS

Plant material and incubation conditions

Tobacco plants (*Nicotiana tabacum* L.), cvs. Samsun NN and Samsun, were grown in a greenhouse and leaves were inoculated with TMV W U1, tobacco necrosis virus (TNV), or water, as described before (*7*). At specific times, leaves were detached and incubated in 750 ml Petri dishes on moist filter paper. Alternatively, leaf discs (25 mm diam.) were cut, put each into a 40 ml, sealed serum flask, and further incubated under the appropriate conditions of light and temperature in a growth cabinet.

All determinations were calculated as the mean of at least 3 independent inoculations; each experiment was repeated at least twice.

Influence of temperature, light conditions and leaf age

Just full-grown leaves of 12-weeks-old plants were used to investigate the influence of temperature and light. Leaf discs were each incubated in 1 ml distilled water, 1 mM ACC, or 0.1 mM indoleacetic acid (IAA), at temperatures varying from 10 to 40°C on a temperature-gradient table. The ethylene generated over a 24 h period was measured. Such measurements were extended for at least 3 days when leaf discs had been inoculated with TMV. Lesion size was determined 3 days after inoculation.

Leaves incubated in Petri dishes were either kept in darkness or illuminated continuously with "cool-white" fluorescent light. Varying light intensities were obtained by shading with one or more layers of a black netting; light intensity was measured with a Metrux-K photometer (Metrawatt GmbH, Nürnberg, FRG). Both lesion size and ethylene production were measured daily.

The effect of leaf age was investigated using all leaves of 14-weeks-old plants. Each day leaves from 3 plants were detached and used for determination of their ethylene production (mean production over a 24 h incubation period) after measuring lesion size.

Measurement of ethylene production

For measurement of endogenous ethylene production, leaf discs were each incubated in 1 ml water, whereas the capacity to convert ACC into ethylene was established by determining the ethylene production of leaf discs incubated in 1 ml 1 mM ACC (*6*). At

specific times after the start of incubation, 1 ml gas samples were withdrawn through a sealed hole in the lid of the petri dishes or through the rubber seal of the serum flasks. The concentration of ethylene in the samples was determined with a Varian 1400 gas chromatograph, equipped with an alumina column and flame ionization detector.

Measurement of virus spread and virus multiplication

To distinguish between effects of light on virus multiplication and virus spread, TMV-infected Samsun NN leaves were incubated at 30°C in continuous light or darkness. After 4 days, leaves were transferred to 20°C and a 16 h photoperiod. Under these circumstances the resulting necrosis reflected virus spread under conditions in which no limitation of virus multiplication occurred. The rates of virus multiplication in light and darkness were compared in leaves of the systemically reacting cultivar Samsun. After leaves were kept in continuous light or darkness for 4 days, they were homogenized in 2.5 vol (v/w) of water, using a mortar and pestle. The homogenate was stirred for 20 min at room temperature and clarified by centrifugation for 10 min at 10.000 *g*. The resulting crude extracts were assayed on 20 leaf halves of *Nicotiana glutinosa*. Relative virus content was expressed as the number of lesions developing per half leaf.

Measurement of lesion size

At specific times after inoculation, lesions were measured with a stereoscopic microscope equipped with an ocular micrometer at an enlargement of 10 times, and expressed as the mean of at least 50 lesion diameters. Differences were statistically analyzed using Student's *t*-test. Generally, average lesion sizes differing by more than 10% were significantly different at at least the 5% level (*19*).

RESULTS

Influence of temperature

Of the successive steps in the biosynthetic pathway from methionine to ethylene, the conversion of SAM to ACC is known to be stimulated by auxins like IAA, whereas the capacity to convert ACC to ethylene can be assayed in the presence of a large dose of the substrate, ACC. As shown in Fig. 1A, basal as well as IAA- and ACC-stimulated ethylene production increased with increasing temperatures up to 35°C. Above this temperature both the activity of ACC synthase and the conversion of ACC to ethylene sharply declined.

Maximum virus-stimulated ethylene production, reached 1-2 days after inoculation, increased with increasing temperature, but only up to 28°C (Fig. 1B). Within a further 2° temperature increase, virus-induced stimulation was completely abolished, concomitant with the transition from a hypersensitive reaction to a systemic response (*17*). Lesion size also increased with increasing temperature up to this transition (cf. ref. *11*).

By alternate incubations at 20° and 30°C, it was investigated at what time and

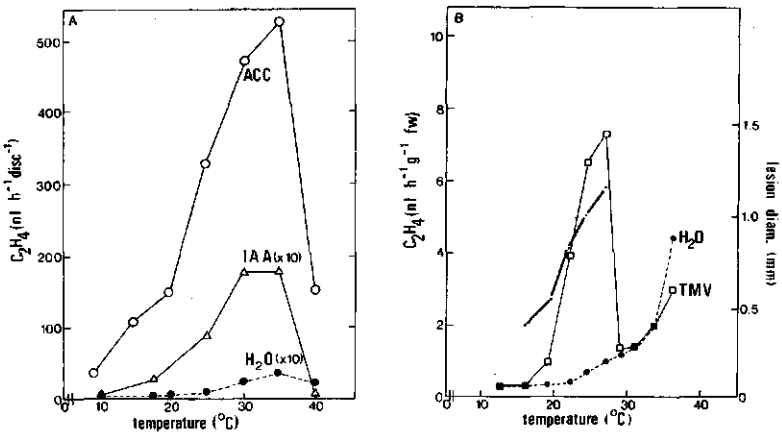


Fig. 1. Influence of temperature on ethylene production of non-infected or TMV-infected Samsun NN leaf discs. (A) Non-inoculated leaf discs were incubated in 1 ml water (●------●), 0.1 mM IAA (Δ——Δ), or 1 mM ACC (o——o). Ethylene production was measured over a 24 h incubation period. (B) Normal (●------●) or virus-stimulated (□——□) ethylene production was measured between 1 and 2 days after inoculation. The size of the developing lesions (·——·) was measured 3 days after inoculation.

for how long *N*-gene activity was required to induce local lesions, ethylene production, or both. TMV-inoculated Samsun NN leaf discs were incubated at 20°C either continuously (Fig. 2A) or for the first 48, 36, 30, 24, or 12 h, and then transferred to 30°C (Fig. 2B-F). Comparable leaf discs were incubated at 30°C continuously (Fig. 2G). As also confirmed in duplicate experiments, at least 24 h at 20°C were necessary for necrotic lesions to develop. No lesions appeared after 18 h incubation at 20°C (data not shown). The time of lesion appearance was not affected by the length of the incubation at 20°C and was always marked by an increase in ethylene production. In contrast, when temperature was maintained at 20°C for less than 24 h, neither lesions nor virus-stimulated ethylene were induced. Thus, both the lesion appearance and the associated increase in ethylene production between 36 and 48 h after inoculation can occur at 30°C (Fig. 2 C-E).

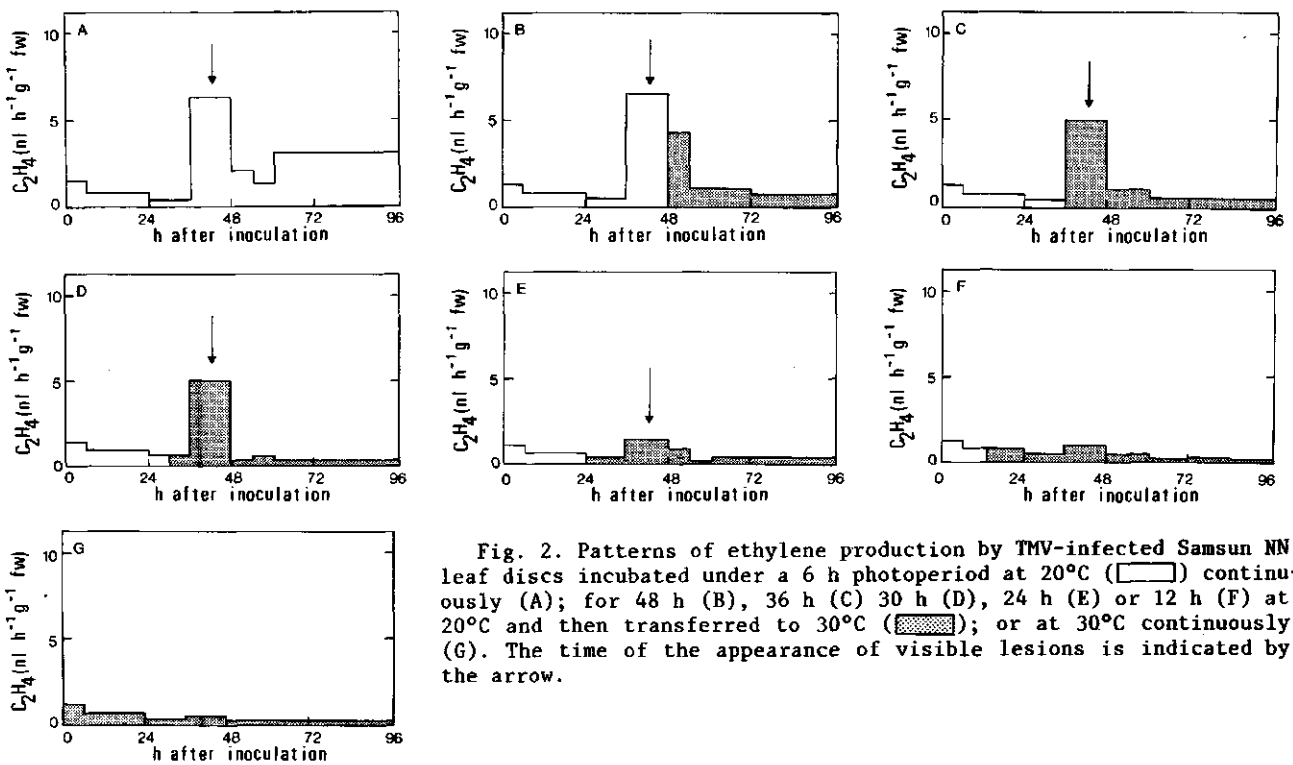


Fig. 2. Patterns of ethylene production by TMV-infected Samsun NN leaf discs incubated under a 6 h photoperiod at 20°C (□) continuously (A); for 48 h (B), 36 h (C) 30 h (D), 24 h (E) or 12 h (F) at 20°C and then transferred to 30°C (▨); or at 30°C continuously (G). The time of the appearance of visible lesions is indicated by the arrow.

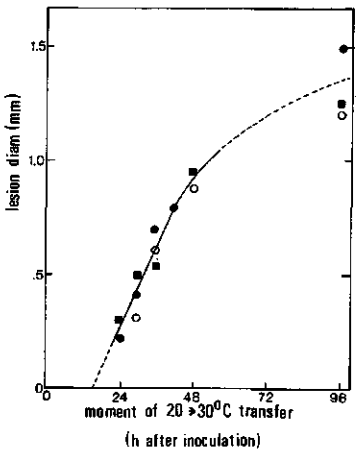


Fig. 3. Effect on the size of developing necrotic lesions of shifting the temperature from 20° to 30°C at successive intervals after inoculation of Samsun NN leaves with TMV. Lesion size was measured 4 d after inoculation. Data from 3 independent experiments (o,●,■).

The size of such lesions, measured 4 days after inoculation, strongly depended on the duration of the 20°C treatment. The longer the discs had remained at 20°C, the larger the lesions were (Fig. 3), indicative of the longer time that the *N* gene had been operative. Lesion size thus reflected the total area of tissue in which the hypersensitive reaction had been irreversibly initiated at the moment of the shift from 20 to 30°C. By extrapolating the curve from Fig. 3 to lesion size 0, one can estimate that irreversible initiation of the hypersensitive reaction starts between 15 and 18 h after inoculation.

To establish the minimum period of time during which the *N* gene must be active to irreversibly initiate local lesions or stimulate ethylene production, TMV-infected leaf discs were incubated at 30°C for 42 h, transferred to 20°C for 3, 6 or 9 h, and then transferred again to 30°C. Since the subsequent shift from 20 to 30° prevented further expression of the *N* gene, the necrotic area reflected the total amount of virus-infected tissue during the short 20°C incubation (Fig. 4). Local lesions became visible about 12 h after the shift from 30 to 20°C only when discs were incubated at 20°C for at least 6 h. Ethylene production was already significantly increased after a 3 h incubation at 20°C.

TMV-infected leaf discs were then incubated at 30°C and transferred to 20°C for 6 h at different times, starting 12 h after inoculation. The size of the resulting lesions reflected the spread of the virus during the 30° incubation prior to the shift to 20°C (Fig. 5). Virus spread was linear during the first 48 h and apparently started about 12 h after inoculation.

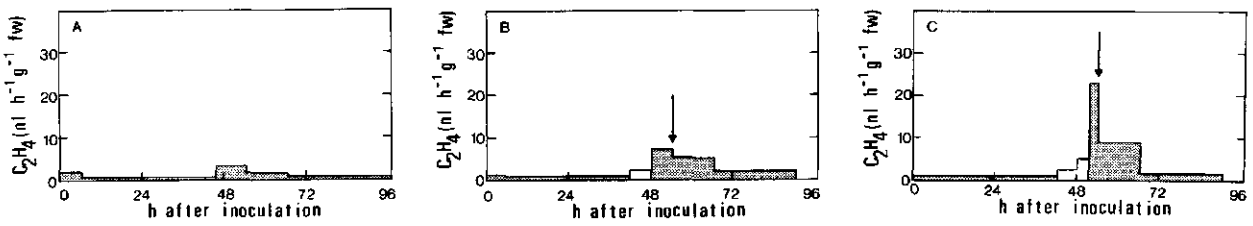


Fig. 4. Patterns of ethylene production by TMV-infected Samsun NN leaf discs kept at 30°C (▒) for 42 h, transferred to 20°C (□) for 3 h (A), 6 h (B) or 9 h (C), and then shifted again to 30°C. The time of lesion appearance is indicated by the arrow.

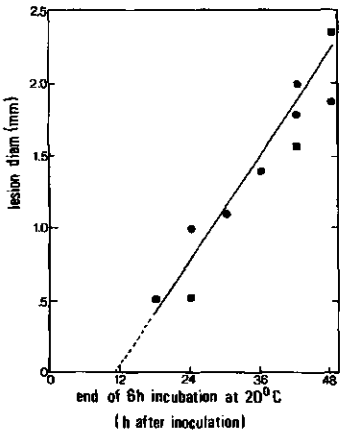


Fig. 5. Size of lesions developed on TMV-inoculated Samsun NN leaf discs incubated at 30°C and transferred to 20°C for 6 h at different times after inoculation. Lesion sizes were measured 3 days after inoculation. Data from 2 independent experiments (●,■).

Influence of light conditions

As shown in Table 1, the endogenous ethylene production of uninfected leaf discs was similar in continuous light and darkness; on the contrary, ACC-stimulated ethylene production was inhibited by light up to 93%, in agreement with previous results (5). Light conditions also influenced both virus-stimulated ethylene production and lesion development after TMV infection (Fig. 6A,B).

In darkness a sharp peak in ethylene production near the time of lesion appearance 2 days after inoculation was associated with slow lesion expansion. In contrast, in

Table 1 Endogenous and ACC-stimulated ethylene production in continuous light or darkness.

	C_2H_4 production (nl h^{-1} g^{-1} fresh wt)[a]	
Incubation	Light	Darkness
water	2.8	3.3
1 mM ACC	38	524

[a] Ethylene production by tobacco leaf discs was measured over a 20 h incubation period at 20°C.

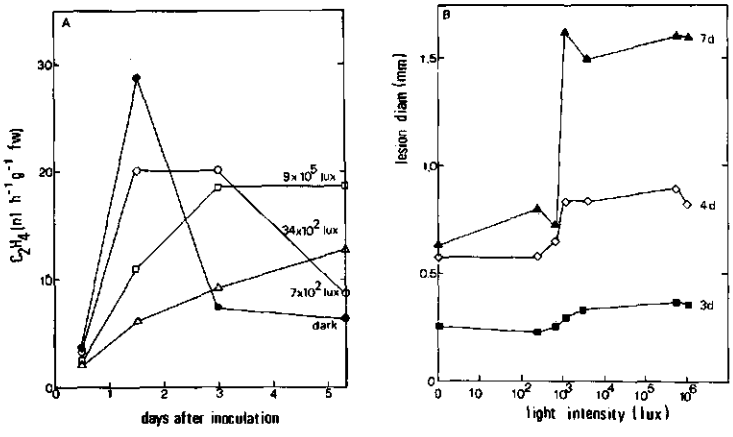


Fig. 6. Influence of continuous light or darkness on (A) virus-stimulated ethylene production and (B) lesion size. TMV-inoculated Samsun NN leaves were incubated at 20°C in (A) darkness (●——●), 700 lux (o——o), 3400 lux (Δ——Δ), or 900.000 lux (□——□). Ethylene production was measured daily. The size of the developing lesions was measured (B) after 3 days (■——■), 4 days (◇——◇), or 7 days (▲——▲).

continuous light no early peak in ethylene production was apparent, but ethylene production gradually increased from lesion appearance onwards (Fig. 6A).

A marked transition was apparent at 750 lux. Above this threshold, the early stimulation of ethylene production was abolished and lesions expanded for a longer time and far more rapidly than at lower light intensities (Fig. 6B).

When a hypersensitive reaction was induced in both Samsun NN and Samsun tobacco in response to TNV, again smaller lesions developed in darkness than in light (Table 2, expt. 1). The light effect thus appears to be general and not restricted to the combination of Samsun NN and TMV. A comparison of TMV lesion sizes on Samsun NN leaves with and without systemic acquired resistance demonstrated the light effect to be also evident in systemically resistant leaves (Table 2, expt. 2).

Whether the lower rate of lesion expansion in darkness resulted primarily from an increased localizing capacity of the host or a decreased rate of virus multiplication was investigated by determining the effect of light conditions on both virus multiplication and spread. To this end, TMV-inoculated Samsun NN leaves were incubated at 30°C in continuous light or darkness. After 3 days the temperature was shifted to 20°C and lesion diameters, reflecting the virus-infected tissue area, were measured 20 h later when necrosis was clearly established. Under these conditions, no effect of light conditions on lesion size was apparent (Table 3, expts. 1, 2), indicating that light did not affect virus spread in Samsun NN at 30°C.

However, in Samsun tobacco leaves systemically infected with TMV at 20°, the virus content was about 2 times higher in light than in darkness (Table 3, expts. 3,4). The effect of light could not be substituted by floating discs on 0.5 M glucose, indicating that TMV multiplication was not limited by lack of products of photosynthesis in darkness.

Table 2 Influence of light conditions on lesion size in TNV-inoculated Samsun NN and Samsun leaves (expt. 1) and in TMV-inoculated Samsun NN leaves with and without systemic resistance

Expt	Host	Virus	Lesion diam. (mm)[b] Light	Lesion diam. (mm)[b] Darkness	% of light[c]
1	Samsun NN	TNV	1.6 ± 0.7	0.9 ± 0.4	56***
	Samsun	TNV	1.5 ± 0.6	1.0 ± 0.2	67***
2	Samsun NN without systemic acquired resistance	TMV	1.7 ± 0.4	0.8 ± 0.3	47***
	Samsun NN with systemic acquired resistance	TMV	1.0 ± 0.4	0.6 ± 0.2	60***

[a] Leaves were incubated in continuous light or darkness at 20°C.
[b] Lesion size was determined 3 days after inoculation.
[c] Three asterisks indicate values significantly different at the 0.1% level.

Table 3 Influence of light conditions on virus spread in TMV-infected Samsun NN leaves kept at 20° or 30°C in continuous light or darkness for 3 days and then transferred to 20°C and 16 h photoperiod (expts 1 and 2), as well as on virus multiplication in systemically infected Samsun leaves kept at 20°C for 4 days (expts 3 and 4).

		Lesion diam. (mm)[a]		% of light[b]
		Light	Darkness	
Expt. 1	Samsun NN/TMV 20°C	1.4 ± 0.4	0.6 ± 0.2	43***
	Samsun NN/TMV 30°C	2.3 ± 0.5	2.2 ± 0.6	96
Expt. 2	Samsun NN/TMV 30°C	2.5 ± 0.5	2.5 ± 0.7	100
		Relative virus content[c]		% of light[b]
		Light	Darkness	
Expt. 3	Samsun/TMV 20°C	96 ± 56	43 ± 27	45***
Expt. 4	Samsun/TMV 20°C	46 ± 25	20 ± 15	43***

[a] Lesion diameter on Samsun NN leaves was measured 20 h after the transfer from 30 to 20°C.
[b] Three asterisks indicate values significantly different at the 0.1% level.
[c] Expressed as the mean number of lesions developing on 20 half leaves of N. glutinosa after inoculation with crude centrifuged extracts from dark- or light-incubated Samsun leaves.

Influence of leaf age

Non-infected discs from leaves at different stalk positions were incubated for 20 h at 20°C in light on either water or ACC. The endogenous ethylene production was only slightly influenced by leaf age, being about two times higher in the youngest leaves than in the oldest ones (Fig. 7 cf. ref. *1*). On the contrary, the capacity to convert ACC to ethylene decreased more strongly and progressively with increasing leaf age, the youngest leaves producing about 3 times more ethylene than the oldest ones.

When lesion expansion was followed from appearance to 10 days post inoculation, lesions on older leaves were found to be smaller only when measured rather late (e.g. 7 or 10 days after inoculation) (Fig. 8A). However, just after appearance, 2 days after inoculation, lesions on older leaves were consistantly somewhat larger than those on younger ones. During further lesion development, the situation reversed. Thus the rate of expansion of lesions on older leaves was very low, whereas the very small lesions on the younger leaves expanded more rapidly, overtaking the lesions on

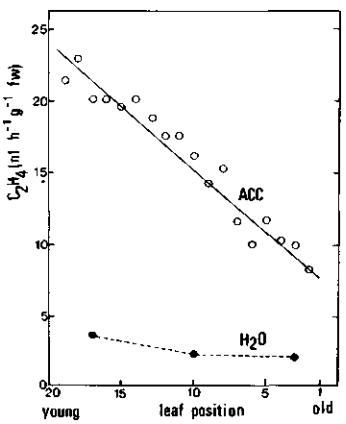
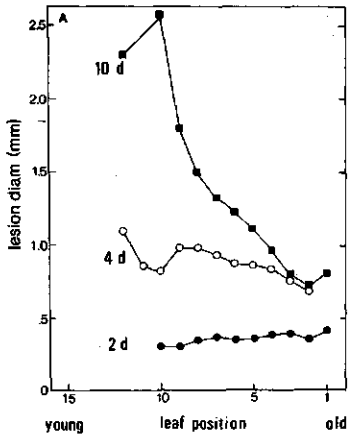


Fig. 7. Effect of leaf age on endogenous (●------●) and ACC-stimulated (o———o) ethylene production. Discs cut from leaves at different stalk positions from 14 weeks-old plants were incubated at 20°C, 16 h photoperiod, on either water or 1 mM ACC; ethylene production was measured over a 24 h period. Leaf 1 was the oldest leaf present on the plant.



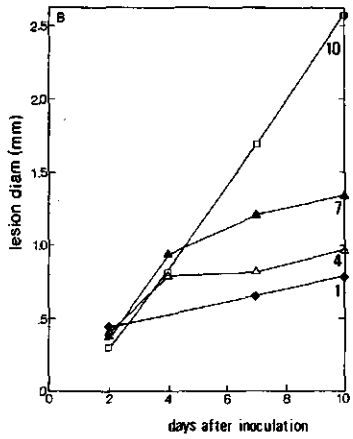


Fig. 8. Influence of leaf age on lesion expansion in vivo after inoculation of Samsun NN tobacco with TMV. Lesion size on different leaves was measured (A) 2 (●——●), 4 (o——o) and 10 days (■——■) after inoculation, or lesion development was studied (B) on leaves at stalk position 1 (◆——◆), 4 (Δ——Δ), 7 (▲——▲) or 10 (□——□).

the older leaves within 2 days (Fig. 8B).

Fig. 9 shows the patterns of basal and virus-induced ethylene production in young, expanding, in full-grown, and in old, senescing Samsun NN leaves. In leaves of all ages, ethylene production strongly increased 2 days after inoculation. However, whereas ethylene production rapidly subsided in old leaves, it remained elevated in young leaves for at least 5 days, and only gradually returned to control levels in leaves that were full-grown.

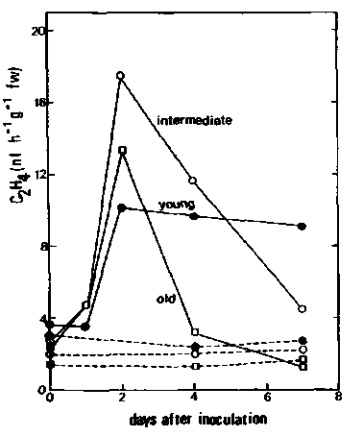


Fig. 9. Influence of leaf age on both basal (-----) and virus-stimulated (——) ethylene production. At specific times after inoculation of young (●) full-grown (o) or old (□) leaves with water or TMV, leaves were detached and discs were incubated at 20°C. Ethylene production was measured over a 24 h incubation period.

DISCUSSION

Temperature, light conditions and leaf age all affected endogenous and ACC-stimulated ethylene production in tobacco leaves. Ethylene production reached an optimum around 35°C, similar to the situation in apple (*2*) and bean leaves (*8*), and decreased with increasing leaf age. Since basal ethylene production is limited by the rate of ACC production (*22*), not only ACC conversion, but also ACC-synthase activity increased with increasing temperature, as confirmed by the temperature-dependent stimulation of ethylene production by IAA. By inference, ACC-synthase activity decreases slightly with leaf age, an observation in line with recent reports of Aharoni and Lieberman (*1*) and Hall *et al.* (*10*). Light only inhibited the conversion of ACC to ethylene (*5,9*). As demonstrated earlier (*5*), light suppresses the *de novo* synthesis of the ACC-converting enzyme.

Virus-stimulated ethylene production is primarily regulated at the level of ACC synthase. Only when lesions become visible, the capacity to convert the accumulated ACC is greatly increased, resulting in a burst of ethylene (*6*). It might be expected, therefore, that virus-stimulated ethylene production would be influenced by temperature, light conditions, and leaf age in the same way as demonstrated for ACC-stimulated ethylene production. Such a similarity is maintained only to a limited extent. Above 28°C the *N* gene is no longer expressed (*17*) and virus-stimulated ethylene production, being dependent on the symptom type (*7*), is abolished. Furthermore, virus-stimulated ethylene production is intimately connected with lesion development in that the increased ethylene production is localized in the cells immediately adjacent to the necrotic areas (*7*). Therefore, the rate of virus-stimulated ethylene production after lesion appearance reflects the amount of tissue involved in necrotization rather than the extent of stimulation *per se* (cf. *13*). This is clearly evident from the higher ethylene productions and enhanced lesion enlargement at higher light intensities and in younger leaves, as opposed to darkness and older leaves. Whether such late ethylene production could contribute to the resistance mechanism is difficult to assess. Calculating the ethylene production on a per cell bases could give an indication of the extent of stimulation. However, the effectiveness of this ethylene might greatly depend on the rate of lesion enlargement, the size of the zone of stimulated cells, and the sensitivity of the tissues to ethylene. Moreover, lesion size is a reflection of, on the one hand, the rate of virus multiplication and spread (*16*) and, on the other hand, the effectiveness of the operation of the resistance mechanism (*12*). Both may be affected in different ways by a change in one of the conditions imposed. Thus, the effectiveness of the resistance mechanism will break down (*11*) and multiplication of the virus may increase with increasing temperature, resulting in a progressive increase in final lesion size with temperature up to 28°C.

Various effects of light conditions on lesion development, connected with both increases and decreases in virus multiplication have been described (*12*). On the other hand, reports on the effect of leaf age are consistent in that final lesion size is progressively smaller on older leaves (*3,18,19,21*).

When lesions appeared at 20°C, their initial size consistently was somewhat larger in the oldest than in the younger leaves, but independent from light intensity. However, light intensity or leaf age greatly affected their subsequent rate of spread, high light intensity and young leaves sustaining continuing lesion growth. Under these latter conditions, the initial stimulation of ethylene production two days after inoculation was less than in darkness, or in older leaves, respectively. Because the amount of tissue involved in the reaction was still roughly similar at that stage, a higher amount of ethylene evolving reflected a greater stimulation. Enhanced lesion limitation under low light intensities and in old leaves was therefore associated with a far larger stimulation of ethylene production near the time of lesion appearance. This suggests that an early, high rise in ethylene production may be instrumental in reducing subsequent lesion spread. A similar conclusion was reached by Pritchard and Ross (*15*), and confirmed by Van Loon (*20*), on the basis of observations that treatment of uninfected tobacco leaves with ethylene enhances the virus-localizing mechanism. Likewise, the reduction in lesion enlargement after challange inoculation of leaves with systemic acquired resistance is associated with an increased capacity to convert

ACC to ethylene (*7*).

The observation that in Samsun tobacco at 20°C less virus was synthesized in darkness than in light, suggested that the smaller lesions in Samsun NN tobacco at 20°C in darkness might be the result of reduced virus multiplication due to a lack of photosynthetic substrates (cf. ref. *12*). However, glucose could not substitute for light and no difference in virus spread was observed in Samsun NN tobacco between darkness and light at 30°C, where the reactions in Samsun and Samsun NN are identical.

By treating TMV-infected Samsun NN plants alternately at 20 and 30°C, it was shown that realization of both virus-stimulated ethylene production and formation of necrotic local lesions can occur at 30°C, provided the *N* gene was activated at 20°C for at least 6 h. Thus the *N* gene is only required for the initiation of the hypersensitive reaction, not for its realization. In agreement with previous findings (*14,18*), the hypersensitive reaction was found to be initiated around 15 to 18 h after inoculation. Apparently the *N* gene is activated around this time and has to be active up to 24 h after inoculation to initiate the series of events that will culminate in a burst of ethylene and formation of necrotic local lesions 24 h later. So far, the action of the *N* gene has been elusive; however, a minimum period of activity of 6 h would allow transcription and translation to take place.

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Algemene diskussie

Dit proefschrift beschrijft de resultaten van een onderzoek naar de regulatie van de biosynthese en de funktie van het lokaal geproduceerde ethyleen tijdens de overgevoeligheidsreaktie van tabak op infektie met TMV.

Enkele belangrijke aspekten hiervan worden in deze diskussie nader belicht. Op de eerste plaats wordt de rol van methionine als ethyleenprekursor en de regulatie van deze biosyntheseweg in overgevoelig reagerende tabak besproken. Op de tweede plaats wordt ingegaan op de funktie van de lokale produktie van ethyleen bij de viruslokalisatie. Verder wordt aandacht besteed aan de mogelijke betrokkenheid van de ethyleenproduktie bij de realisatie van de verworven systemische resistentie. Tenslotte wordt de funktie van het *N*-gen bij de initiatie en realisatie van de overgevoeligheidsreaktie bediskussieerd.

De biosynthese van ethyleen

Methionine wordt algemeen beschouwd als een intermediair in de ethyleenvorming in hogere planten (Lieberman, 1979; Yang, 1980). In de hoofdstukken I en II is onderzocht of ook het ethyleen dat in tabaksbladeren geproduceerd wordt in de pathologische situatie van een overgevoeligheidsreaktie na infektie met TMV uit methionine afkomstig is. Enerzijds door gebruik te maken van AVG, een analogonremmer van de synthese van ethyleen uit methionine, en anderzijds door experimenten waarbij bladeren gemerkt werden met radioaktief methionine, is onderzocht in hoeverre methionine in ethyleen wordt omgezet.

Zowel de basale als de door het virus geïnduceerde ethyleenproduktie werden nagenoeg volledig geremd door AVG. Dit was een eerste aanwijzing voor een belangrijke rol van methionine als ethyleenprekursor. Na merking van bladeren met L-(U-^{14}C) methionine werd de specifieke radioaktiviteit van de endogene methioninepool vergeleken met die van het geproduceerde ethyleen. Als methionine de enige prekursor is mag verwacht worden dat, omdat 2 van de 5 C-atomen van methionine uiteindelijk in ethyleen terecht komen, de specifieke radioaktiviteit van ethyleen 40% zal bedragen van die van methionine.

In hoofdstuk I is uitvoerig ingegaan op de problemen die zich kunnen voordoen bij de interpretatie van zulke radioisotoopexperimenten. Na toediening van L-(U-^{14}C) methionine via de bladsteel bleef het grootste deel van de radioaktiviteit achter in de nerven. Ten gevolge van deze heterogene verdeling was de specifieke radioaktiviteit van de methionine pool in het bladmoes 10 tot 100 keer lager dan in de nerven. De stimulering van de ethyleenproduktie tijdens de overgevoeligheidsreaktie vindt hoofdzakelijk plaats in het bladmoes, waarin zich de nekrotische vlekken ontwikkelen. Omdat de specifieke radioaktiviteit van het methionine in het bladmoes erg laag was, ging de stimulering van de ethyleenproduktie gepaard met een sterke daling van de specifieke radioaktiviteit van het door het gehele blad geproduceerde ethyleen.

Voor een juiste kwantitatieve bepaling van een prekursor/produktrelatie bleek nader inzicht in de lokalisatie van de prekursorpool, van de radioaktieve tracer en van de plaats van produktvorming van essentieel belang. Een niet homogene verdeling kan optreden binnen een individu, een orgaan of weefsel, of zelfs binnen cellen.

Na toediening van ^{14}C-methionine door vacuuminfiltratie werd de radioaktiviteit veel homogener over het blad verdeeld. De specifieke radioaktiviteit van ethyleen geproduceerd door gezonde en overgevoelig op TMV reagerende tabaksbladeren was onder deze omstandigheden exact gelijk en redelijk in overeenstemming met die van de methioninepool. Nauwkeurige vergelijking van de specifieke radioaktiviteiten van de methioninepool en het geproduceerde ethyleen bleek door de snel afnemende specifieke radioaktiviteit van de methioninepool onmogelijk. Deze afname is het gevolg van enerzijds inbouw van methionine in eiwitten (niet meer dan circa 3% binnen de door ons gebruikte inkubatieduur van 4 uur) en anderzijds een snelle omzetting. Door na aminozuuranalyse van waterige extrakten de radioaktiviteit te bepalen in de verschillende frakties bleek dat reeds na korte tijd een groot aantal metabolieten gevormd was. In bladeren waarop zich zeer grote aantallen vlekken ontwikkelden bleef ondanks de lokaal zéér hoge ethyleenproduktie de endogene methioninekoncentratie tijdens de overgevoeligheidsreaktie konstant. Er moet dus een zeer efficiënte regulatie van de methionineproduktie bestaan die onder deze kondities de methioninekoncentratie binnen nauwe grenzen houdt. Door Hanson en Kende (1976) is aangetoond dat methionine via een cyclus gevormd wordt uit homocysteine tijdens de verwelking van de dagbloem (*Ipomoea tricolor*). Het is mogelijk dat ook na TMV infektie een zeer snelle aanmaak plaatsvindt.

In aanwezigheid van AVG was de specifieke radioaktiviteit van het, nog in zeer geringe mate, geproduceerde ethyleen onveranderd. Dit is een sterke aanwijzing dat ook het in aanwezigheid van AVG geproduceerde ethyleen afkomstig is uit methionine en dat de methionineweg dus de enige biosyntheseweg van ethyleen is in zowel gezonde als overgevoelig op TMV reagerende tabaksbladeren.

Door Kende en Baumgartner werd in 1974 gepostuleerd dat de beschikbaarheid van de prekursor van ethyleen een regulerende faktor zou zijn bij de ethyleenproduktie in verwelkende Ipomoea bloemen. Zij stelden zich voor dat de ethyleenprekursor, welke later inderdaad methionine bleek te zijn (Hanson & Kende, 1975), gekompartimentaliseerd is in de vacuole. Door veranderingen in de permeabiliteit van de tonoplast zou methionine geleidelijk naar buiten lekken en beschikbaar komen voor het in het cytoplasma gelokaliseerde ethyleen-producerende apparaat. In hoofdstuk III is aangetoond dat in overgevoelig reagerende tabak van een dergelijk mechanisme geen sprake kan zijn. Exogeen toegediend methionine werd onmiddellijk homogeen verdeeld over de totale methioninepool en was direkt beschikbaar voor omzetting in ethyleen. Recentelijk werd aangetoond dat in tabaksprotoplasten de methioninekoncentratie in het cytoplasma en de vacuole ongeveer gelijk is en dat transport vanuit de vacuole naar het cytoplasma waarschijnlijk niet beperkend is (Smith, 1981).

In gezonde en op infektie met TMV overgevoelig reagerende tabak bleek ethyleen via SAM en ACC uit methionine te worden gesynthetiseerd. Regulatie van de ethyleensynthese bleek hierbij plaats te vinden op zowel het niveau van de ACC produktie als op dat van de omzetting van ACC in ethyleen. De stimulering van de ethyleenproduktie vond zijn primaire oorzaak in een verhoging van de ACC produktie. In hoofdstuk IV

werden aanwijzingen verkregen die pleiten voor *de novo* synthese van het ACC synthase zoals bv. ook het geval is voor de door IAA gestimuleerde ACC produktie (Yu & Yang, 1979). Het bleek namelijk mogelijk om de stimulering van de ACC produktie tijdens de overgevoeligheidsreaktie te onderdrukken met remmers van RNA- en eiwitsynthese. De interpretatie van de verkregen resultaten is echter uitermate komplex. De RNA-of eiwitsynthese kan immers ook essentieel zijn voor een van de stappen die voorafgaan aan de stimulering van de ACC produktie. Ook is gezocht naar mogelijke oorzaken voor de verhoogde ACC produktie. Deze pogingen bleven tot dusver zonder enig resultaat. Door verschillende andere onderzoekers werd aangetoond dat ook in bv. rijpende vruchten (Boller *et al*, 1979; Yu *et al*, 1979; Hoffman & Yang, 1980), verwelkende bloemen (Buffer *et al*, 1980; Suttle & Kende, 1980) en als reaktie op verwonding (Boller & Kende, 1980; Kende & Boller, 1981; Konze & Kwiatkowski, 1981) een toename van de ACC produktie de primaire oorzaak is van de optredende stijging van de ethyleensynthese. Ook in deze systemen wordt momenteel nog steeds naarstig gezocht naar het mechanisme dat verantwoordelijk is voor de stimulering van de ACC-produktie. Voortzetting van het onderzoek naar de regulatie en de aard van het ACC synthase is van het allergrootste belang.

Wat is de funktie van het lokaal geproduceerde ethyleen tijdens de overgevoeligheidsreaktie?

Uit onderzoek bij verschillende waard/viruskombinaties bleek dat de stimulering van de ethyleenproduktie op identieke wijze wordt gereguleerd in alle tot nekrose en viruslokalisatie leidende infekties (hoofdstuk IV) en dus niet gerelateerd is aan de genetische konstitutie van de waardplant of aan de specificiteit van het virus.

Pritchard & Ross toonden in 1975 aan dat ethyleen, afhankelijk van het tijdstip van endogene produktie of exogene toediening zowel vlekvergroting als vlekverkleining tot gevolg kan hebben. De vroege piek in de ethyleenproduktie, ongeveer 2 dagen na inokulatie, zou betrokken zijn bij de viruslokalisatie. Deze hypothese was gebaseerd op de volgende waarnemingen: a) begassing van bladeren vóór inokulatie leidde tot signifikant kleinere vlekken (Ross & Pritchard, 1972), b) bij verlaging van de temperatuur van 32 naar 22°C 42 uur na inokulatie vond enerzijds enorme stimulering van de ethyleenproduktie plaats al voor de nekrose zichtbaar werd terwijl anderzijds de vlekken zich nauwelijks uitbreidden, en c) ethyleen is betrokken bij de regulatie van verschillende biochemische veranderingen die tijdens de overgevoeligheidsreaktie optreden en waarvan aangenomen mag worden dat ze betrokken zijn bij de viruslokalisatie (Pritchard & Ross, 1975; Van Loon, 1981). De latere ethyleenproduktie zou daarentegen betrokken zijn bij voortgaande uitbreiding van de vlekken. Dit bleek o.a. uit experimenten waarbij het geproduceerde ethyleen werd weggevangen, door verlaagde druk, dan wel werd geantagoneerd door toediening van CO_2, of waarbij na het verschijnen van de vlekken extra ethyleen werd toegediend. De vlekuitbreiding werd hierdoor respectievelijk negatief en positief beïnvloed.

Uit onderzoek van Van Loon (1977), waarbij bladeren werden aangeprikt met naalden die gedoopt waren in ethepton, bleek dat de resulterende lokale ethyleenproduktie verantwoordelijk gesteld kan worden voor nagenoeg alle biochemische en fysiologische veranderingen die ook bij een overgevoeligheidsreaktie optreden.

In dit onderzoek werd het belang van ethyleen bij de viruslokalisatie onderzocht door bestudering van de invloeden van temperatuur, lichtomstandigheden en bladleeftijd op enerzijds het patroon van ethyleenproduktie en anderzijds de vlekontwikkeling (Hoofdstuk VI). Direkte effecten op bv. de snelheid van virusvermenigvuldiging en virusverspreiding vormden een komplicerende faktor bij de interpretatie van de gevonden resultaten. Toch kon in deze experimenten de hypothese van Pritchard en Ross worden bevestigd. Bij inkubatie in kontinu donker of in oude bladeren bleven de vlekken signifikant kleiner dan in kontinu licht of in jonge bladeren. De betere viruslokalisatie was geassocieerd met een scherpe piek in ethyleenproduktie 2 dagen na inokulatie en een relatief lage produktie in de daaropvolgende dagen. Bij inkubatie in licht of in jonge bladeren daarentegen steeg de ethyleenproduktie slechts geleidelijk vanaf het verschijnen van de nekrotische vlekken.

Omdat het gevormde ethyleen slechts lokaal, direct om de zich ontwikkelende vlekken heen, geproduceerd wordt, zou in plaats van globale middeling over een geheel blad of een bladsponsje de ethyleenproduktie van uitsluitend de betrokken cellen nog duidelijker informatie verschaffen. Dit zou een nauwkeurige bepaling van het aantal vlekken en van de kinetiek van de vlekontwikkeling en verdere bestudering van de omvang van het weefsel in en romdom de nekrotische vlek, waarin de ethyleenproduktie gestimuleerd is, nodig maken. Door Legrand *et al.* (1976) is op soortgelijke wijze de stimulering van de enzymen fenylalanine ammonia-lyase (PAL) en kaneelzuur-4-hydroxylase, die betrokken zijn bij de aromatische biosynthese, op celbasis uitgedrukt. Hierbij bleek dat bij goede viruslokalisatie (kleine vlekken) de stimulering op celbasis het grootst was. Hiervooor zijn twee mogelijke verklaringen aan te voeren. De eerste mogelijkheid is, dat t.g.v. de tragere virusverspreiding de cellen meer tijd hebben om de enzymaktiviteiten te verhogen voordat ze afsterven. Een tweede mogelijkheid is dat de sterkere stimulering van de enzymaktiviteiten een sterkere afweer weerspiegelt en mede bepalend is voor de geringere uitbreiding van het virus. Door Van Loon (1981) is gesuggereerd dat de stimulering van PAL een direkt gevolg is van de lokale ethyleenproduktie.

Wanneer tijdens de overgevoeligheidsreaktie de ethyleenproduktie geremd wordt door AVG, dan wordt de vlekontwikkeling niet wezenlijk beïnvloed. Het is mogelijk dat ondanks de efficiëntie van de remming (méér dan 95%) tóch lokaal op de plaats van infektie een drempelwaarde wordt overschreden waardoor de van ethyleen afhankelijke latere processen in gang gezet worden. Indirekt zijn er aanwijzingen dat op de eerste plaats een ethyleengradiënt in het weefsel en niet de hoogte van de ethyleenproduktie als zodanig van belang is. Wellicht blijft ook in aanwezigheid van AVG een gradientsituatie bestaan. Ook kan na infektie een aantal processen parallel worden geaktiveerd waarvan de stimulering van de ethyleenproduktie er slechts één is die eventueel gemist kan worden. Een soortgelijke situatie is beschreven in rijpende vruchten (Lieberman, 1979) en verwelkende bloemen (Suttle & Kende, 1978; Veen & v.d. Geijn, 1978); hoewel zowel rijping als verwelking geïnitieerd worden door ethyleen treden bij onderdrukking van de ethyleenproduktie of -werking facetten van deze processen, zij het enigszins vertraagd, in een aantal gevallen tóch op. Toch wordt in deze gevallen algemeen ethyleen als de regulator van deze processen aangemerkt.

De verworven systemische resistentie

Op basis van overeenkomstige effekten van verschillende behandelingen op de vlekontwikkeling, suggereerden Pritchard & Ross (1975) dat het mechanisme van viruslokalisatie in bladeren met en zonder verworven resistentie identiek is. Het kleiner blijven van de vlekken in de systemisch resistente bladeren zou volgens deze auteurs het gevolg zijn van een snellere initiatie van het lokaliserend mechanisme. Zo treedt ook de stimulering van de ethyleenproduktie vroeger op. In hoofdstuk IV werd de biosynthese van ethyleen in systemisch resistente bladeren onderzocht. In deze bladeren blijkt de kapaciteit om ACC in ethyleen om te zetten te zijn verhoogd waardoor snellere omzetting van ACC in ethyleen plaatsvindt. Daarmee is de verhoogde ACC-omzettingskapaciteit wellicht rechtstreeks betrokken bij de realisatie van de systemische resistentie.

De vlekreduktie bij inkubatie in kontinu donker is met deze hypothese in overeenstemming. Ook in het donker is namelijk de kapaciteit om ACC in ethyleen om te zetten sterk gestimuleerd, zoals blijkt bij inkubatie van bladsponsjes in ACC-oplossingen (Hoofdstuk V). In jonge bladeren daarentegen ontwikkelen zich, ondanks een hogere ACC-omzettingskapaciteit, grotere lokale vlekken dan in oudere bladeren. Misschien wordt in de jonge bladeren de werking van het ethyleen geantagoneerd door relatief hoge koncentraties van auxinen en cytokininen (Wightman 1977), of is in deze bladeren het viruslokaliserend mechanisme nog niet volledig ontwikkeld zoals ook bij inokulatie van jonge planten dikwijls systemische nekrose optreedt (Dijkstra *et al.* 1977).

Uit onderzoek van Van Loon (1977) bleek dat ethyleen rechtstreeks verantwoordelijk gesteld kan worden voor de induktie van de systemische resistentie. Eveneens wordt de induktie van zgn. "pathogenesis related proteins" (PR's), die wellicht funktioneel betrokken zijn bij de realisatie van de verworven resistentie, aan ethyleen toegeschreven. Uit recent onderzoek (Van Loon, niet gepubliceerd) is gebleken dat na infiltratie van bladponsjes met AVG de induktie van PR's sterk onderdrukt werd, overigens zonder dat het aantal en de grootte van de vlekken op het oog verandering onderging.

Verder onderzoek naar de rol van ethyleen en de verhoogde ACC-omzettingskapaciteit bij respectievelijk de induktie en de realisatie van de systemische resistentie is noodzakelijk.

Wat is de funktie van het N-gen

Het vermogen van tabak om overgevoelig te reageren op virusinfektie is niet voorbehouden aan variëteiten die een zgn. *N*-gen bezitten. Ook kultivars die dit gen missen hebben het vermogen om met lokale vlekken te reageren en vertonen daarbij alle fysiologische en biochemische veranderingen die optreden tijdens de overgevoeligheidsreaktie van Samsun NN op TMV, bv. Samsun tabak na infektie met TNV of met de TMV-stammen 1952 D en Ni/2338 (van Loon, 1972).

Uit onderzoek van Mundry & Gierer (1958) bleek dat het vermogen van het virus om zich ongebreideld te vermenigvuldigen en zich systemisch door de gehele plant te verspreiden een specifieke virusfunktie is. Door een puntmutatie in het virus-RNA kan de symptoomexpressie van de waardplant veranderen van systemisch mozaïek naar lokale

nekrotische vlekken; terugmutatie *in vitro* is nooit gekonstateerd, zodat aangenomen moet worden dat er bij de overgang van systemisch mozaïek naar lokale vlekken sprake is van een verliesmutatie.

Het *N*-gen in Samsun NN tabak zorgt voor overgevoeligheid na infektie met enigerlei stam van TMV. Klaarblijkelijk is het *N*-gen verantwoordelijk voor "herkenning" van alle stammen van TMV, waarna initiatie van een aspecifieke overgevoeligheidsreaktie optreedt. Dit zou betekenen dat overgevoeligheid zou ontstaan door aktivering van een deel van het plantegenoom. Het is echter niet mogelijk gebleken om met behulp van remmers van de DNA-afhankelijke RNA-synthese de overgevoeligheidsreaktie te onderdrukken (Van Loon, 1981).

Door Samuel werd in 1931 aangetoond dat de fenotypische expressie van het *N*-gen temperatuurafhankelijk is. Boven 28°C worden ook tabaksvariëteiten die een *N*-gen bezitten systemisch door TMV geïnfekteerd. Uit experimenten waarbij op verschillende momenten de temperatuur verhoogd werd van 20 naar 30°C of omgekeerd verlaagd, bleek dat voor een irreversibele initiatie de aktiviteit van het *N*-gen slechts gedurende 6 uur nodig is en wel tussen 16 en 24 uur na inokulatie. Omdat ook bij 30° nog vorming van nekrotische vlekken en stimulering van de ethyleenproduktie kan optreden is het *N*-gen dus alleen betrokken bij de initiatie en niet bij de realisatie van de symptomen als zodanig (hoofdstuk VI).

Ook in tabaksvariëteiten die geen *N*-gen bezitten is de overgevoeligheidsreaktie temperatuurafhankelijk. Zo wordt bij 30°C Samsun tabak na inokulatie met TMV HR systemisch geïnfekteerd zonder dat karakteristieke symptomen optreden en verschijnen na infektie met TMV 1952D in plaats van kleine lokale vlekjes grote chlorotische vlekken (Van Loon, 1972). Dit betekent dat niet de expressie van het *N*-gen zelf temperatuurgevoelig behoeft te zijn, maar dat in ieder geval een van de daarop volgende stappen, onderdeel uitmakend van het aspecifieke deel van de overgevoeligheidsreaktie, temperatuurgevoelig is.

Tussen de irreversibele initiatie van de overgevoeligheidsreaktie (ongeveer 24 uur na inokulatie) en de vroegst detekteerbare verandering, nl. de stimulering van de ACC-produktie, bestaat nog een interval van ongeveer 12 uur. Wat er in deze periode gebeurt is tot dusver volstrekt onduidelijk. In hoofdstuk IV is aangetoond dat in deze fase van de overgevoeligheidsreaktie zowel RNA als eiwitsynthese plaatsvindt.

Zoals al eerder opgemerkt is, blijkt hier opnieuw dat verder onderzoek naar het mechanisme van de stimulering van de ACC-produktie een essentiële stap is in de opheldering van de initiatie van de overgevoeligheidsreaktie.

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Samenvatting

De vorming van lokale nekrotische vlekken tijdens de overgevoeligheidsreaktie van Samsun NN tabak (*Nicotiana tabacum* L.) op infektie met tabaksmozaiekvirus (TMV), gaat gepaard met een sterke toename van de ethyleenproduktie. Door middel van labelingsexperimenten, het gebruik van specifieke remmers en bepaling van koncentraties van mogelijke intermediairen werd de biosynthese van ethyleen *in vivo* bestudeerd in gezonde en in op TMV overgevoelig reagerende bladeren.

Bepaling van de rol van een bepaalde verbinding als voorloper in een biosyntheseweg kan erg gekompliceerd zijn. Door vergelijking van de specifieke radioaktiviteiten van enerzijds de endogene prekursorpool en anderzijds het betrokken produkt kan de omzetting van de prekursor in het produkt kwantitatief worden vastgesteld. Wanneer echter sprake is van een niet-homogene verdeling van de radioaktief gemerkte prekursor en/of produktvorming binnen een individu, een orgaan of weefsel, of zelfs binnen cellen, dan kan dit leiden tot verkeerde konklusies.

Aangenomen wordt dat methionine de algemene prekursor is van ethyleen in hogere planten. Methionine wordt via S-adenosylmethionine (SAM) en 1-aminocyclopropaan -1-carboxylzuur (ACC) in ethyleen omgezet. In dit onderzoek is nagegaan of methionine ook als voorloper van ethyleen fungeert in pathologische situaties zoals na virusinfektie. Om het belang van methionine als ethyleenprekursor in gezonde en overgevoelig op virusinfektie reagerende tabaksbladeren te onderzoeken werden bladeren gemerkt met L-(U-^{14}C)methionine. Na toediening van het radioaktieve methionine via de bladsteel bleef een aanzienlijk deel in de nerven achter. Het door virusinfektie geïnduceerde ethyleen werd daarentegen lokaal in en net rondom de zich ontwikkelende vlekken in het bladmoes geproduceerd. Onder deze omstandigheden kon de rol van methionine als ethyleenprekursor niet worden vastgesteld (Hoofdstuk I). Door bladeren via vacuuminfiltratie te merken kon een homogene verdeling van het gemerkt methionine worden bereikt. Uit deze experimenten werden door vergelijking van de specifieke radioaktiviteiten van de endogene methioninepool en het geproduceerde ethyleen sterke aanwijzingen verkregen dat methionine de enige ethyleenprekursor is in zowel gezonde als in overgevoelig op TMV-infektie reagerende tabaksbladeren.

Dit werd verder bevestigd door experimenten met aminoethoxyvinylglycine (AVG), een specifieke remmer van de omzetting van SAM in ACC, en door bepaling van endogene koncentraties van ACC. Zelfs de geringe hoeveelheid ethyleen die nog geproduceerd werd in aanwezigheid van AVG bleek uit methionine afkomstig te zijn (Hoofdstuk II).

Tijdens de overgevoeligheidsreaktie van Samsun NN op TMV-infektie bleven de endogene koncentraties van methionine en SAM gedurende minstens 4 dagen konstant. Hoewel exogeen toegediend methionine of SAM direkt beschikbaar was voor het ethyleenproducerend systeem, stimuleerden deze verbindingen de ethyleenproduktie niet. De ethyleenproduktie wordt dus niet gereguleerd op het niveau van de koncentratie of beschikbaarheid van methionine of SAM. Aangezien bij inkubatie van bladponsjes in oplossingen van ACC de ethyleenproduktie wel sterk toenam, bleek de produktie van ACC primair beperkend te zijn (Hoofdstuk III).

De scherpe piek in de ethyleenafgifte die optrad bij het verschijnen van de nekrotische vlekken werd voorafgegaan door een piek in de produktie van ACC, ongeveer 8 uur eerder. Ten gevolge van deze toename in de synthese van ACC vond ophoping van ACC plaats. Pas na het verschijnen van de nekrotische vlekken nam in de bladeren het vermogen om ACC in ethyleen om te zetten toe, waardoor het endogene ACC-gehalte snel afnam en sterke stijging van de ethyleenproduktie plaatsvond. Tijdens de overgevoeligheidsreaktie van tabak bleek de ethyleenproduktie dus te worden gereguleerd op het niveau van zowel de synthese van ACC als de omzetting van ACC in ethyleen (Hoofdstuk III).

Uit onderzoek bij genetisch verschillende waardplant/viruskombinaties bleek dat de stimulering van de ethyleenproduktie na virusinfektie niet afhankelijk was van de genetische konstitutie van de waardplant of van de eigenschappen van het virus, doch uitsluitend samenhing met het type symptomen dat zich ontwikkelde. In het geval van systemische mozaïeksymptomen vond geen stimulering van de ACC-produktie plaats, en dientengevolge ook geen toename in ethyleenproduktie (Hoofdstuk IV).

De toename van de ACC-produktie tijdens de overgevoeligheidsreaktie bleek afhankelijk van zowel RNA- als eiwitsynthese; *de novo* synthese van ACC-synthase lijkt daarom aannemelijk. Pogingen om een faktor te vinden die produktie van ACC induceert waren tot dusver tevergeefs. Ook kon de produktie van ACC niet worden gestimuleerd door lokale membraanbeschadiging of door extracten van bladeren waarop zich vlekken ontwikkelen (Hoofdstuk IV).

De omzetting van ACC in ethyleen werd geremd door licht via (een deel van) het fotosynthetisch apparaat. De sterke stimulering van de omzetting van ACC in ethyleen bij overbrenging van licht naar donker kon vrijwel volledig geremd worden door eiwitsyntheseremmers. Er blijkt dus sprake te zijn van *de novo* synthese van het betrokken enzym. De snelle afname van de ethyleenproduktie bij overplaatsing van donker naar licht kan echter niet zo eenvoudig worden verklaard. Een aktieve afbraak en/of inaktivatie kunnen hiervoor verantwoordelijk zijn (Hoofdstuk V).

Na een primaire infektie van overgevoelig reagerende planten werd de kapaciteit om ACC in ethyleen om te zetten systemisch door de gehele plant heen verhoogd. Na inokulatie van nog niet eerder geïnfekteerde bladeren van deze planten hoopte zich bij/voor het zichtbaar worden van lokale vlekken tijdens de overgevoeligheidsreaktie geen ACC op. Wellicht is de stimulering van de ACC-omzettingskapaciteit betrokken bij de realisatie van de verworven systemische resistentie.

De funktie van de door virusinfektie gestimuleerde ethyleenproduktie bij de viruslokalisatie werd verder onderzocht door de invloeden van temperatuur, lichtomstandigheden en bladleeftijd op zowel de ethyleenproduktie als de vlekontwikkeling te bestuderen (Hoofdstuk VI). Zowel de endogene als de door ACC gestimuleerde ethyleenproduktie in gezonde bladeren steeg met toenemende temperatuur tot 35°C, en daalde bij toenemende bladleeftijd. Licht remde alleen de omzetting van ACC in ethyleen. Het patroon van ethyleenproduktie tijdens een overgevoeligheidsreaktie werd door temperatuur, licht en bladleeftijd op vergelijkbare wijze beïnvloed. In oude bladeren was een betere viruslokalisatie geassocieerd met een scherpe piek in ethyleenproduktie op het moment van het verschijnen van de vlekken. In kontinu licht of in jonge bladeren daarentegen ontwikkelden zich grote vlekken. Hierbij steeg de ethyleenpro-

duktie geleidelijk vanaf het verschijnen van de vlekken. Een vroege piek in ethyleenproduktie bleek nauw gekoppeld te zijn met een sterke viruslokalisatie.

Expressie van het *N*-gen, dat in Samsun NN tabak verantwoordelijk is voor het overgevoelig reageren op enigerlei stam van TMV, treedt niet op boven 28°C. In experimenten waarbij de temperatuur op diverse tijdstippen veranderd werd van 20° naar 30°C of omgekeerd, werd aangetoond dat het *N*-gen alleen betrokken was bij de initiatie, en niet bij de realisatie van de overgevoeligheidsreaktie. Voor de realisatie van de ontwikkeling van lokale vlekken en de stimulering van de ethyleenproduktie moest het *N*-gen tussen 16 en 24 uur na inokulatie minimaal 6 uur aktief geweest zijn.

Summary

During the hypersensitive reaction of tobacco (*Nicotiana tabacum* L.) cv. Samsun NN to tobacco mosaic virus (TMV), the appearance of local lesions was accompanied by a large burst of ethylene. Biosynthesis of both basal and virus-stimulated ethylene production was investigated *in vivo* by labeling experiments, the use of specific inhibitors, and the determination of the concentration of the probable precursor and intermediates.

Determination by labeling of the role of a specific compound as a precursor in a particular biosynthetic pathway may be complicated. By comparing the specific radioactivities of, on the one hand, the endogenous precursor pool, and, on the other hand, the product involved, a quantitative estimate of precursor-product conversion can be obtained. However, this ratio is altered by unequal distribution of the labeled precursor and/or the product formation within an individual plant or animal, within a specific organ or tissue, or even within cells, leading to erroneous conclusions.

The main biosynthetic pathway of ethylene in plant tissues has been established as methionine → S-adenosylmethionine (SAM) → 1-aminocyclopropane-1-carboxylic acid (ACC) → ethylene. In this research we investigated the role of methionine as ethylene precursor in a pathological situation suchas a hypersensitive reaction. Non-infected and hypersensitively-reacting tobacco leaves were labeled with L-(U-^{14}C)methionine by petiolar uptake. Under these conditions the labeled methionine was retained mainly in the veins, whereas the virus-induced ethylene production was restricted to the immediate vicinity of the developing lesions in interveinal tissues, preventing the assessment of the importance of methionine as the ethylene precursor (Chapter I). This complication was avoided by labeling leaves by vacuum infiltration. A comparison of the specific radioactivities of the methionine pool and the ethylene produced by non-infected or hypersensitively-reacting leaves, was highly indicative of methionine being the only ethylene precursor in both cases.

By using aminoethoxyvinylglycine (AVG), a specific inhibitor of methionine-derived ethylene synthesis, and by determination of endogenous concentrations of ACC, methionine was further demonstrated to be the only precursor of ethylene in both non-infected and TMV-infected Samsun NN tobacco. Even the small amount of ethylene emanated in the presence of AVG was derived from methionine.

Endogenous concentrations of both methionine and SAM remained constant up to 4 days after inoculation. Furthermore, exogenously applied methionine or SAM did not increase ethylene production in non-infected leaf discs, although both precursors were directly available for ethylene production. In contrast, ethylene production was increased severalfold upon incubation of leaf discs in solutions of ACC. Thus, ethylene production in tobacco was not regulated at the level of the concentration or availability of either methionine or SAM, but was primarily limited at the level of ACC production (Chapter III).

The sharp peak in ethylene production near the time of lesion appearance was preceded by a strong rise in ACC production, peaking 8 h earlier. As a result, ACC accumulated in the tissue. Only after lesions had become macroscopically visible, the capacity of the leaf to convert ACC to ethylene increased severalfold, associated with a sharp decrease in ACC content and a large rise in ethylene evolution. Thus, virus-stimulated ethylene production during a hypersensitive reaction turned out to be regulated at the level of both the production of ACC and its conversion to ethylene (Chapter III).

Investigation of genetically different host/virus combinations revealed that an increased ethylene production after virus infection was determined neither by the genetic constitution of the host plant, nor by the properties of the infecting virus, but was related exclusively to the type of symptoms expressed. No rise in ACC production occurred in combinations leading to systemic mosaic symptoms.

The rise in ACC production in hypersensitively-reacting combinations depended on both RNA and protein synthesis, suggesting the ACC-synthase to be synthesized *de novo*. So far efforts to find an ACC-synthase-inducing factor have failed: the increase in ACC production could not be mimicked by local membrane damage, and no ACC-synthase-stimulating agent could be isolated from leaves with developing lesions.

Light inhibited the conversion of ACC to ethylene via (part of) the photosynthetic system. Inhibiting protein synthesis during the shift from light to darkness abolished the increase in ACC conversion, indicating that the enzyme is synthesized *de novo*. However, the rapid decrease upon a shift from darkness to light cannot be easily explained and may involve both active degradation and/or inactivation (Chapter V).

After primary infection of hypersensitively-responding plants the ACC-converting capacity was increased systemically within the plant. As no ACC accumulated upon challenge inoculation of systemically-resistant leaves, acquired resistance may be related to the increased capacity to convert ACC to ethylene (Chapter IV).

The involvement of virus-stimulated ethylene production in virus localization was further investigated by studying effects of temperature, light conditions, and leaf age on both ethylene production and lesion size (Chapter VI). In non-infected leaves, both endogenous and ACC-stimulated ethylene production increased with increasing temperature up to 35°C, and decreased with increasing leaf age. Light inhibited only the conversion of ACC to ethylene. Temperature, light and leaf age similarly affected the pattern of virus-stimulated ethylene production; enhanced localization of the virus in old leaves was associated with a sharp peak in ethylene production near the time of lesion appearance. In contrast, large lesions developed in continuous light or in young leaves, where virus-induced ethylene production increased only gradually from lesion appearance onwards. Hence, an early burst of ethylene and the virus localizing reaction are closely connected.

The expression of the *N* gene in Samsun NN tobacco, which confers hypersensitivity towards all strains of TMV, does not occur above 28°C. From experiments in which temperature was shifted from 20° to 30°C and back, the *N* gene was demonstrated to be involved only in the initiation, and not in the realization of the hypersensitive reaction. *N*-gene activity is required for at least 6 h between 16 and 24 h after inoculation for both stimulation of ethylene production and local lesion

Curriculum vitae

Adrianus Marinus Maria de Laat werd op 13 juli 1955 te Udenhout geboren. Hij bezocht het Cobbenhagencollege te Tilburg waar hij in 1972 zijn HBS-b diploma behaalde. In datzelfde jaar werd de studie Biologie aan de Landbouwhogeschool te Wageningen aangevangen. Het doctoraalexamen in de (cel)biologie werd in juni 1978 afgelegd, met celbiologie (immunologie) als verzwaard hoofdvak en moleculaire biologie als tweede hoofdvak.

In november 1978 werd een begin gemaakt met een promotie-onderzoek aan de afdeling Plantenfysiologie van de Landbouwhogeschool onder (bege)-leiding van dr.ir. L.C. van Loon en prof.dr. J. Bruinsma. Dit onderzoek werd mogelijk gemaakt door een driejarig promotie-assistentschap van de Stichting Biologisch Onderzoek in Nederland (BION), en gesubsidieerd door de Nederlandse Organisatie voor Zuiver Wetenschappelijk Onderzoek (ZWO).

Vanaf februari 1982 is hij als wetenschappelijk ambtenaar werkzaam bij de Stichting ITAL in Wageningen.

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Light inhibited the conversion of ACC to ethylene via (part of) the photosynthetic system. Inhibiting protein synthesis during the shift from light to darkness abolished the increase in ACC conversion, indicating that the enzyme is synthesized *de novo*. However, the rapid decrease upon a shift from darkness to light cannot be easily explained and may involve both active degradation and/or inactivation (Chapter V).

After primary infection of hypersensitively-responding plants the ACC-converting capacity was increased systemically within the plant. As no ACC accumulated upon challenge inoculation of systemically-resistant leaves, acquired resistance may be related to the increased capacity to convert ACC to ethylene (Chapter IV).

The involvement of virus-stimulated ethylene production in virus localization was further investigated by studying effects of temperature, light conditions, and leaf age on both ethylene production and lesion size (Chapter VI). In non-infected leaves, both endogenous and ACC-stimulated ethylene production increased with increasing temperature up to 35°C, and decreased with increasing leaf age. Light inhibited only the conversion of ACC to ethylene. Temperature, light and leaf age similarly affected the pattern of virus-stimulated ethylene production; enhanced localization of the virus in old leaves was associated with a sharp peak in ethylene production near the time of lesion appearance. In contrast, large lesions developed in continuous light or in young leaves, where virus-induced ethylene production increased only gradually from lesion appearance onwards. Hence, an early burst of ethylene and the virus localizing reaction are closely connected.

The expression of the *N* gene in Samsun NN tobacco, which confers hypersensitivity towards all strains of TMV, does not occur above 28°C. From experiments in which temperature was shifted from 20° to 30°C and back, the *N* gene was demonstrated to be involved only in the initiation, and not in the realization of the hypersensitive reaction. *N*-gene activity is required for at least 6 h between 16 and 24 h after inoculation for both stimulation of ethylene production and local lesion

Curriculum vitae

Adrianus Marinus Maria de Laat werd op 13 juli 1955 te Udenhout geboren. Hij bezocht het Cobbenhagencollege te Tilburg waar hij in 1972 zijn HBS-b diploma behaalde. In datzelfde jaar werd de studie Biologie aan de Landbouwhogeschool te Wageningen aangevangen. Het doctoraalexamen in de (cel)biologie werd in juni 1978 afgelegd, met celbiologie (immunologie) als verzwaard hoofdvak en moleculaire biologie als tweede hoofdvak.

In november 1978 werd een begin gemaakt met een promotie-onderzoek aan de afdeling Plantenfysiologie van de Landbouwhogeschool onder (bege)-leiding van dr.ir. L.C. van Loon en prof.dr. J. Bruinsma. Dit onderzoek werd mogelijk gemaakt door een driejarig promotie-assistentschap van de Stichting Biologisch Onderzoek in Nederland (BION), en gesubsidieerd door de Nederlandse Organisatie voor Zuiver Wetenschappelijk Onderzoek (ZWO).

Vanaf februari 1982 is hij als wetenschappelijk ambtenaar werkzaam bij de Stichting ITAL in Wageningen.

The sharp peak in ethylene production near the time of lesion appearance was preceded by a strong rise in ACC production, peaking 8 h earlier. As a result, ACC accumulated in the tissue. Only after lesions had become macroscopically visible, the capacity of the leaf to convert ACC to ethylene increased severalfold, associated with a sharp decrease in ACC content and a large rise in ethylene evolution. Thus, virus-stimulated ethylene production during a hypersensitive reaction turned out to be regulated at the level of both the production of ACC and its conversion to ethylene (Chapter III).

Investigation of genetically different host/virus combinations revealed that an increased ethylene production after virus infection was determined neither by the genetic constitution of the host plant, nor by the properties of the infecting virus, but was related exclusively to the type of symptoms expressed. No rise in ACC production occurred in combinations leading to systemic mosaic symptoms.

The rise in ACC production in hypersensitively-reacting combinations depended on both RNA and protein synthesis, suggesting the ACC-synthase to be synthesized *de novo*. So far efforts to find an ACC-synthase-inducing factor have failed: the increase in ACC production could not be mimicked by local membrane damage, and no ACC-synthase-stimulating agent could be isolated from leaves with developing lesions.

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