AGRITOPE

95-324-01P

Mr. Michael A. Lidsky
Deputy Director, BBEP, APHIS, USDA
4700 River Road
Unit 146
Riverdale, MD 20737

November 16, 1995

Dear Mr. Lidsky:

Agritope, Inc. is submitting a Petition for Determination of Nonregulated Status to the Animal and Plant Health Inspection Service (APHIS) regarding cherry tomatoes with a *S*-adenosylmethionine hydrolase gene (SAMase). This petition requests a determination from APHIS that the SAMase cherry tomato line 35-1-N and any progenies derived from crosses between line 35-1-N and traditional tomato varieties no longer be considered a regulated article under 7 CFR.340.

Enclosed please find two copies of our petition and a complete set of references as requested. If you have any questions and or comments regarding this petition, please feel free to contact either myself or Dr. Richard K. Bestwick at the numbers listed above. Thank you for your consideration of our petition.

Sincerely,

Matthew G Kramer

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Director of Product Development
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FR
11/17/95

95-324-01p

Petition for Determination of Nonregulated Status:

Cherry Tomatoes with a *S*-adenosylmethionine hydrolase Gene

The undersigned submits this petition of 7 CFR 340.6 to request that the Director, BBEP, make a determination that the article should not be regulated under 7 CFR 340.

Submitted by:

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Contains No Confidential Business Information

95-324-01p

PETITION FOR DETERMINATION OF NON-REGULATED STATUS

SUMMARY

Agritope, Inc. is submitting a Petition for Determination of Non-regulated Status to the USDA Animal and Plant Health Inspection Service (APHIS) for tomatoes that contain a gene that encodes an enzyme capable of degrading *S*-adenosylmethionine (SAM). Production of this enzyme, *S*-adenosylmethionine hydrolase (SAMase) in fruit alters the ethylene biosynthetic pathway and causes a modified fruit ripening phenotype. Agritope, Inc. requests a determination from APHIS that the SAMase containing tomato line 35-1-N and any progeny derived from hybrid crosses between this line and any other non-transformed tomato varieties, no longer be considered a regulated article under 7 CFR Part 340.

Using standard Agrobacterium binary vectors, Agritope scientists have introduced a SAMase encoding gene derived from E. coli bacteriophage T3 into the tomato genome. This results in transformed tomato plants that exhibit significantly reduced levels of *S*-adenosylmethionine (SAM), the substrate for conversion (through ACC synthase) to 1-aminocyclopropane-1-carboxylic acid (ACC) which is the first committed step in ethylene biosynthesis. Ethylene is an endogenous plant hormone known to play an important role in fruit ripening of climacteric fruits. Lack of a sufficient pool of SAM for conversion to ACC in fruit results in tomatoes with significantly reduced ethylene biosynthetic capabilities and a modified ripening phenotype. In the case of line 35-1-N the phenotype is characterized by fruit in which ripening on the vine is delayed while ripening off the vine is essentially suspended. However, in either case, tomato fruits expressing SAMase ripen normally when exposed to exogenous ethylene.

The tomato line for which Agritope is requesting this determination, line 35-1-N, contains a a version of the SAMase gene modified in the 5' region of the gene with a Kozak consensus sequence. This construct encodes a functional SAMase protein. Since SAM plays a central role in numerous biosynthetic pathways in plants, expression of SAMase is under the control of an organ specific (fruit) and temporally regulated (post-climacteric) promoter. The efficacy of this strategy is demonstrated by the fact that the organ specific and temporal expression pattern of ethylene biosynthesis precisely matches the SAMase expression kinetics (ethylene synthesis is inversely correlated to SAMase expression) and provides an explanation of the observed modified ripening phenotype.

Agritope, Inc. believes that the observed ripening phenotype has numerous commercial applications in the current fresh tomato production and distribution system. These include but are not limited to the following:

1. Reduction in producer losses through reduced harvest of immature and/or over mature fruit.
2. Improved production dynamics and reduced harvest frequency.
3. Reduced spoilage and loss through out the distribution system.
4. Enhanced fruit quality due to harvest of more physiologically mature fruit.

Tomato fruit expressing SAMase have been field tested since 1992 in the principal tomato growing regions of the United States. These tests were carried out under field release permits and/or notifications granted by APHIS (USDA permits Nos. 92-085-01, 93-49-01M, 93-050-01, 93-176-01N, 93-340-02N, 93-361-01-N, 940048-01N, 94-143-03-N, 94-353-01N, 95-121-03N, 95-121-04N, 95-121-03N) and further tests are currently being conducted in additional locations in Mexico (permission granted by Sanidad Vegetal, May 4, 1995) Data collected from these trials as well as from laboratory analyses and literature references presented in this petition demonstrate that SAMase expressing tomato line 35-1-N exhibits no plant pathogenic properties, is no more likely to become a weed than the non-transgenic parental variety, is unlikely to increase the weediness potential of any other cultivated plant or native wild species, does not damage or cause to be damaged processed agricultural commodities and finally is unlikely to harm other organisms that are beneficial to agriculture.

ABBREVIATIONS AND SCIENTIFIC TERMS

SAM - *S*-adenosylmethionine
SAMase - *S*-adenosylmethionine hydrolase
sam - native bacteriophage T3 gene encoding *S*-adenosylmethionine hydrolase
sam-k - modified *S*-adenosylmethionine hydrolase gene
ACC - 1-aminocyclopropane-1-carboxylic acid
ACC synthase - 1-aminocyclopropane-1-carboxylic acid synthase
kan^r - kanamycin resistance gene
nptII - aminoglycoside phosphotransferase 3' II
RPA - RNAse protection assay
ELISA - enzyme linked immunosorbent assay
nos - nopaline synthase
E8 - ethylene responsive gene promoter
bp - nucleotide base pair
HAREC- Hermiston Agricultural Research and Experiment Station

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Standard of Identity:

Identity of transformed line: Line 35-1-N is detailed in this petition.

Construct: pAG-5420

Recipient species: L. esculentum mill var. cerasiforme, (Dunal) Alef. cultivar Large Red Cherry

Table 1 Identity and source of DNA elements contained in pAG 5420:

DNA Element	Size (Kb)	Function
Right Border	1.8	Right border region of T-DNA from A. tumefaciens (An et al., 1985).
p*nos*	0.3	The untranslated promoter region of the nopaline synthase gene from A. tumefaciens controls expression of the *kan*^{r} gene (Depicker et al., 1985)
kan^r	1.02	The *kan*^{r} gene from transposon Tn5 (Beck et al., 1982) encodes the npt II protein which renders transformed cells resistant to the antibiotic kanamycin.
nos 3'	1.1	The untranslated 3' region of the nopaline synthase gene from A. tumefaciens controls expression of the *kan*^{r} gene (Depicker et al., 1985).
pE8	2.3	The modified E8 gene promoter from tomatoes which is used to drive the *sam-k* gene in a tissue specific, developmentally regulated manner as described by Good et al., 1994.
sam-k	0.51	The *S*-adenosylmethionine hydrolase gene modified to contain a consensus eukaryotic translation initiation site by altering the nucleotide sequence surrounding the *sam* ATG start codon (Good et al., 1994).
nos 3'	0.27	The untranslated 3' region of the nopaline synthase gene from A. tumefaciens controls expression of the *sam-k* gene (Good et al., 1994).
ori pBR322	1.54	E. coli origin of replication which ensures replication in E. coli (Sutcliffe et al., 1979).
lambda-*cos*	0.4	A lambda *cos* site to allow for the potential re-cloning and molecular characterization of the individual integration events. Together the Col EI origin of replication the lambda *cos* site provides the necessary elements to rescue or re-clone the insert from transgenic plants (An et al., 1988)
LB	0.88	The left border region of T-DNA from A. tumefaciens (An et al. 1985).

Line 35-1-N may also contain the following DNA elements from the backbone of plasmid pAG 5420:

DNA Element	Size (Kb)	Function
ori T	0.7	Initiates conjugation in E. coli when other essential elements exist in *trans* (Schmidhauser, et al, 1985; Pansegrau, et al, 1994)
*trf*A	1.5	A portion of an operon which functions in the stable replication of the plasmid in host bacteria. This element requires the remaining operon components as well as the cis acting element in order to be functional (Schmidhauser, et al, 1985; Pansegrau, et al, 1994)

Rationale for Development of SAMase Tomato

Agritope, Inc. has developed the SAMase tomato as means of improving the production and distribution economics of cherry, roma and fresh market tomatoes through reduced spoilage and losses in the field and in the packing, shipping, handling and distribution systems. The SAMase encoding gene was isolated from E. coli bacteriophage T3 and introduced into the tomato in combination with promoter technology which results in specific down regulation of ethylene produced only in ripening fruit. The result is a modified ripening phenotype which will provide production flexibility and help prevent losses due to immature and overripe fruit in the field, packing, shipping, handling and distribution systems. In addition, this technology will enable the production of a more physiologically mature fruit able to withstand the rigors of the current production and distribution system resulting in a product of higher overall quality.

Removal from Regulated Status

Agritope requests that USDA, APHIS based on data presented in this document, determine that SAMase tomatoes, defined as a tomato cultivar or progeny of a cultivar genetically engineered using the following binary vector: pAG 5420 containing the *sam-k* gene and its associated promoter and terminator, do not represent a plant pest risk, are not otherwise deleterious to the environment and are therefore not a regulated article.

SAMase tomatoes contain specific sequences introduced into the plant genome via binary vector pAG 5420 and the *sam-k* gene with its associated promoter and terminator regions (Good et al., 1994). The *nos* 5' promoter and the *nos* 3' frame shifted region and terminator and the right and left border regions are from Agrobacterium tumefaciens (Barker et al.,1983). The E8 promoter (Good et al., 1994) is from tomato and is not considered a plant pest according to 7 CFR 340.2. In addition, the *sam-k* gene is a modification of a gene isolated from E. coli bacteriophage T3 and is not considered a plant pest according to 7 CFR 340.2. The *kan*^{r} gene encoding nptII was isolated as a component of transposon Tn5 (Beck et al., 1982) and has been previously reviewed by U.S. regulatory agencies and determined to be safe (USDA, 1992; FDA, 1994).

Although A. tumefaciens is a regulated article, the SAMase tomato containing DNA sequences from this regulated article should not be classified as a plant pest risk nor as a regulated article under 7 CFR 340.2 for the following reasons:

1. Tomato is not a regulated article.
2. DNA sequences from regulated articles used to produce the SAMase tomato have been disarmed or are frame shifted to prevent translation and as such do not pose a plant pest risk.
3. DNA sequences introduced into the SAMase tomato from regulated articles do not cause the SAMase tomato to become a plant pest risk.
4. No new compounds have been measured in the SAMase tomato that cause an environmental hazard.

Petition to USDA APHIS

This petition is a request for USDA APHIS to determine that SAMase tomatoes, genetically engineered using the binary vectors described above (pAG 5420) and the *sam-k* gene with its associated promoter and terminator regions do not present a plant pest risk, are not deleterious to the environment and are therefore not a regulated article.

I. The Recipient Plant: Tomato

A. Description of Non-transformed Tomato Cultivar Large Red Cherry

Tomato line Large Red Cherry is an open pollinated cherry tomato line developed by Petoseed, Inc. and available in the Public Domain through various commercial outlets. It is characterized as an indeterminate small fruited cherry type variety with an average fruit size of 1.5 inches in diameter. Fruit ripen to a deep red approximately 70 days post transplanting and are borne in clusters on highly productive plants (American Hort. Soc., 1982).

B. The Origins of Tomato

Domesticated tomato is a member of the genus Lycopersicon, species esculentum (Rick, 1978). The genus is native to South America and the natural distribution ranges from northern Chile to southern Columbia, westward to the Pacific Ocean and eastward to the foothills of the Andes range (Esquinas-Alcazar, 1981). No wild Lycopersicon can be found outside Latin America with the exception of the very uniform Lycopersicon esculentum var cerasiforme (cherry tomato) (Esquinas-Alcazar, 1981). According to Rick (1983), the wild source of cultivated tomato must certainly have been Lycopersicon esculentum var cerasiforme , which previously had migrated from the Andean center of origin of the genus through northern South America, across the Panamanian Isthmus to Central America and Southern Mexico.

While it is known that the cultivated tomato originated in the New World, the exact site of domestication is uncertain. Most evidence however points to Mexico (Taylor, 1986). It is believed that the tomato had reached an advanced stage of domestication before its introduction to Europe since the first varieties cultivated in Europe appear to have been large fruited types in contrast to the wild species which have relatively small fruit. Early reports describe a variety of shapes and colors (Rick 1978).

Tomato has been grown for food for hundreds of years and continues to be a popular commodity both for commercial production and also in home gardens. That tomato is one of the most popular fruits consumed is evidenced by the fact that in the United States alone, per capita consumption is approximately 25.5 kg/year (Rick 1978).

C. Taxonomy, Genus Lycopersicon

The genus Lycopersicon is a member of the Solanaceae family. This family is one of the most important serving mankind and contains many essential fruits and vegetables. Within the family Solanaceae, Lycopersicon is a member of the subfamily Solanoidae, tribe Solaneae. Within the genus Lycopersicon , there are nine species. Of these, there are seven closely related species known as the "esculentum complex"(L. esculentum, L. pimpinellifolium, L. cheesmanii, L. chmielewskii, L. hirsutum, L. parvifolium and L. pennelli). In addition there exist two additional species (L. peruvianum and L. chilense) known as the "peruvianum complex". All Lycopersicon species have the same number of chromosomes (2n=2x=24) and chromosome morphology (Rick, 1976).

D. Genetics of the Tomato

Tomato is a simple diploid species with twelve pairs of highly differentiated chromosomes. The genome size of tomato is estimated at 7.1×10^8 bp per haploid genome (Galbraith et al., 1983). Cultivated tomato is self fertile and for all practical purposes, under commercial and home garden cultivation it is exclusively self pollinating (Rick, 1979; Taylor, 1986). Cross pollination normally requires human intervention and in general is only carried out in variety development and/or hybrid seed production programs. Inter-specific hybrids generally occur only as a result of new variety development efforts and are carried out via hand pollination. To this end, tomato serves as a classical example of the use of interspecific hybridization to improve the characteristics of the

cultivated species. Wild species represent an excellent germplasm pool for new genetic material aimed at addressing the problems of disease resistance, insect resistance and tolerance to environmental stresses such as drought, poor soil conditions and cool temperatures (Rick et al, 1987). While the efficacy of such wide crosses in variety improvement programs is indisputable, the natural occurrence of such crosses appears to be very limited.
The closest genetic relatives to the cultivated tomato (outside of the genus Lycopersicon) are found in the genus Solanum . Hybrids have been obtained between S. lycopersicoides and L. esculentum and gene transfer has been accomplished between the two with limited success (Stevens et al., 1986; DeVerna et al. ,1990). Such hybrids however were only possible using specialized laboratory techniques. Hybrids have also been obtained between S. rickii and L. esculentum using a sesquidipoloid bridging species (DeVerna et al., 1990) but no other member of the genus including S. nigrum, a common weed in commercial tomato fields, have produced any viable hybrids with cultivated tomato (Taylor, 1986). Table 2 summarizes the occurrence of inter-specific hybrids with L. esculentum.

Table 2. Occurrence of Inter-Specific hybrids with L. esculentum (Stevens et al., 1986, DeVerna et al., 1990)

Genus	Species	Naturally Occurring Hybrids	Hybrid Requires Human Intervention
Lycopersicon	cheesmanii	yes (rare)	Facilitated
Lycopersicon	chmielewskii	yes (rare)	Facilitated
Lycopersicon	hirsutum	yes (rare)	Facilitated
Lycopersicon	parvifolium	yes (rare)	Facilitated
Lycopersicon	pimpinellifolium	yes (rare)	Facilitated
Lycopersicon	pennelli	yes (rare)	Facilitated
Lycopersicon	peruvianum	no	yes, necessary
Lycopersicon	chilense	no	yes, necessary
Solanum	lycopersicoides	no	yes, necessary, offspring usually sterile
Solanum	nigrum	no	never successful

E. Tomato as a Crop

Practically speaking, all cultivated forms of tomato belong to the species L. esculentum. As a crop, tomatoes are grown commercially wherever environmental conditions permit the production of an economically viable yield. In the United States, the principal fresh market tomato growing regions are Florida and California which produce approximately 85,000 acres out of a total annual acreage of more than 130,000 acres. Fresh tomatoes are available in the United States year-round although the greatest supply is from June through October. Fresh tomatoes are consumed in many forms. They are eaten whole, sliced or diced and used cooked as an ingredient in many prepared foods. Due to the versatility of the tomato and its ability to be consumed in so many various forms, tomato has become one of the most widely consumed vegetable crops with worldwide consumption estimated at upwards of 50 million metric tons (Tigchelaar, 1986).

F. Weediness Potential

A "weed" or "weed pest" is defined by the USDA as a "plant that grows persistently in locations where it is unwanted" (USDA APHIS 1991), or is a "plant with harmful or objectionable characteristics, which grow where they are not wanted, usually in places where it is desired that something else should grow" (Muenscher, 1980). Based on these definitions, the cultivated tomato is not, nor is it likely to become a weed or weed pest. While it might appear that by using a variety of plant breeding techniques, plant varieties which are continually improved with respect to

productivity, tolerance to environmental stress and pest resistance would be more persistent and more likely to become weedy, plant breeders have a long history of incorporating these traits into crops without enhancing weediness (USDA APHIS 1991).

It follows then that the same plant breeding practices employed in the development of conventionally improved cultivars should be sufficient to insure that changes to the genome using molecular biological techniques result in no additional weediness potential in target crops. In fact, since molecular methods are so specific in terms of the addition of genetic material, it would be expected that the weediness potential of new cultivars developed using these methods may actually be less.

In the case of cultivated tomato, we are dealing with a perennial which is grown almost exclusively as an annual crop in the United States. It is highly inbred and not normally persistent without human intervention. According to Holm et al., (1991) L. esculentum is not a serious or principal weed anywhere in the world and is a common weed only in Taiwan. While it is true that tomato volunteers are not uncommon, they are easily controlled using normal production practices. Tomato has been grown extensively for commercial and home use for many years in the United States and throughout the world without any reports of serious weed problems (Holm, 1991; Muenscher, 1980) and is not classified as a noxious weed by the Federal Government (7 CFR Part 360).

G. Potential for Outcrossing

1. Wild relatives

Since tomato itself is not considered a weed pest the only possible weediness problems would be the result of outcrossing to weed pest relatives. The only wild species sexually compatible with cultivated tomato are members of the "esculentum complex" (Rick, 1976) whose normal geographic range is limited to South America and these are not considered weed pests. In addition, only through specific controlled crosses is hybridization to these relatives possible (Stevens, et al., 1986, DeVerna et al., 1990). Solanum nigrum, is the only major weed pest related to tomato and is sexually incompatible with Lycopersicon species (see Table 2). Because tomato has no weed pest relatives, there is no possibility of a cross between a SAMase tomato and a wild pest relative that would be capable of creating or enhancing the competitiveness of a weed pest.

2. Cultivated tomato

In tomato, genes can move via pollination from one individual to another within the species L. esculentum. When field grown, tomato is predominantly self pollinated due to its self compatibility and floral structure. Rates of cross pollination on the order of 4% have been reported (Rick, 1978), but present commercial varieties have a floral structure (i.e., inserted stigma) which creates a crop which is exclusively inbreeding (Taylor, 1986). As a result of this and the ability to maintain pure seed stocks using standard practices including selection and isolation, here is little if any potential for outcrossing of SAMase tomatoes to commercial stocks.

II. Description of the donor for SAMase: Coliphage T3

T3 is a bacteriophage that infects B, K, and C strains of Escherichia coli. T3 is in the T7 group of coliphages. T3 is commonly found in sewage and can be isolated from the human intestinal tract (Furuse et al., 1983). T3 is neither a human pathogen or a plant pest (42 CFR 72; 7 CFR 340). Since it is a coliphage, it has undoubtedly been associated with humans and other vertebrates for eons. Coliform bacteria as well as bacteriophages are also present in low levels in drinking water so that it can be reasonably concluded that humans regularly consume bacteriophages that contain sam genes (Goyal et al., 1980). When a bacteriophage infection of the intestinal E. coli occurs, T3 proteins are present in the infected bacteria. Thus, we conclude that both the sam gene and the

SAMase enzyme are commonly found within the intestinal microflora of humans and other vertebrate animals.

SAMase is the first bacteriophage T3 protein produced upon phage infection of E. coli. SAMase inhibits the host bacterium's restriction endonuclease system both by binding the type 1 restriction endonuclease and hydrolyzing SAM, an essential co-enzyme. This protects the remaining phage DNA from host mediated degradation (Studier and Movva, 1976; Spoerel et al., 1979). The coliphage BA14 (Mertens and Hausmann, 1982) and klebsiellaphage K11 (Dietz et al., 1985) also contain SAMase coding genes, though each is poorly characterized.

III. The Transformation System

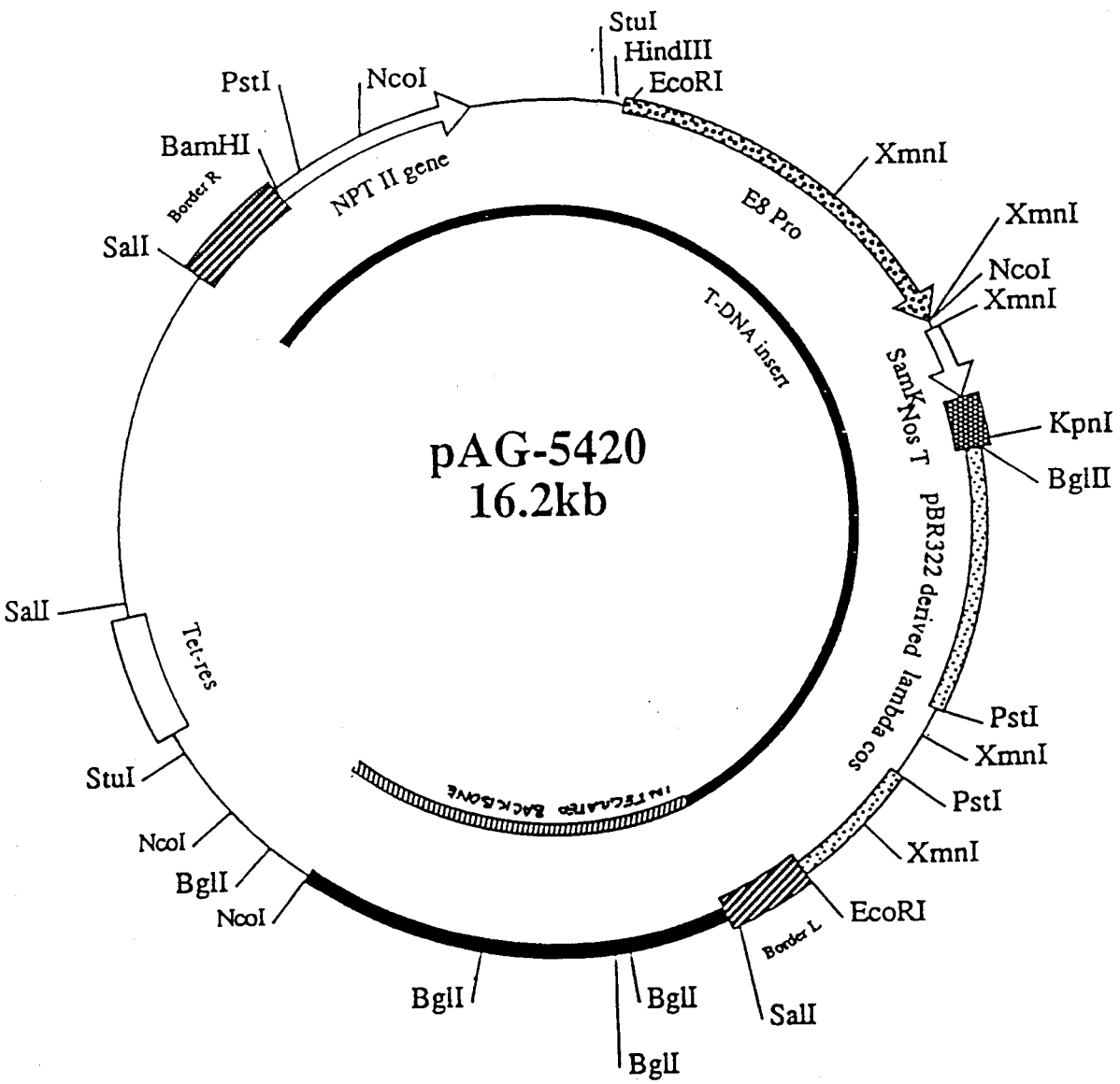
The SAMase tomato line 35-1-N contains a gene derived from E. coli bacteriophage T3 which encodes a functional SAMase protein (Good et al., 1994). The SAMase encoding transgene is derived from a previously reported M13 clone (Hughes et al., 1987) modified to contain a consensus eukaryotic translation initiation site by altering the nucleotide sequence surrounding the *sam* ATG start codon (Good et al., 1994) and fused to a modified E8 promoter (Good et al., 1994) isolated from tomato and a *nos 3'* termination sequence from Agrobacterium tumefaciens. The resulting gene was named *sam-k* and was utilized in plasmid pAG 5420. Plasmid pAG 5420 was used in the transformation of the Large Red Cherry parental line and also contains a selectable marker gene *kan*^{r} (Beck et al., 1982). The *kan*^{r} gene was isolated from transposon Tn5 and is under transcriptional control of a *nos* (Depicker et al., 1985) promoter and *nos 3'* (Barker et al.,1983) termination sequence.

A. Agrobacterium mediated transformation system

The vector system used to transfer pAG 5420 to the Large Red Cherry parental line is based upon the Ti plasmid from Agrobacterium tumefaciens. This system utilizes a "disarmed" Ti plasmid (i.e., all genes responsible for the crown gall disease have been removed from the T-DNA) binary (all genes to be transferred are on one plasmid, the *vir* genes encoding the functions necessary for transfer are on another) vector system (Hoekema et al., 1983; An et al., 1988). In the case of line 35-1-N, Agrobacterium strain EHA 101 (Hood et al., 1986) was used to transform the plant. This strain, EHA 101; does not contain any DNA sequences responsible for plant pathogenesis (Hood et al., 1986) and it has been demonstrated with this strain that the *vir* genes on the second plasmid are not transferred to the plant and therefore the transferred DNA sequences will not be remobilized within the plant.

Following transformation, Agrobacterium are killed with the antibiotic carbenicillin or cefotaxine (Fillatti et al., 1987) so no subsequent transformation or infection can occur. After transformation, the plants themselves are grown to fruit, analyzed and selected transformants used for seed production and advancement in the product development process.

Figure 1. Plasmid Map of pAG 5420



Plasmid map of pAG 5420. The binary vector pGA 482 (An et al., 1985), which contains the *kan*^{r} gene fused to the *nos* promoter for selection in plants, and the *sam-k* gene with its associated promoter and terminator were used to produce the pAG 5420 plasmid. pAG 5420 is composed of a 2.3 Kb E8: *sam-k* chimera inserted into the multiple cloning site of pGA 482 (Good et al., 1994). The complete DNA sequence of the T-DNA region of pAG 5420 is shown in Appendix 1. A map of binary vector pAG 5420 is shown in Figure 1 . A description of the DNA elements in pAG 5420 is also shown in Appendix 2.

IV. Gene Sequences from Organisms Considered Regulated Articles

The SAMase tomato line 35-1-N has been considered a regulated article because it contains DNA sequences from A. tumefaciens, an organism considered to be a plant pest under 7 CFR 340.2. Specifically, these sequences are as follows:

Left and Right Borders: These sequences represent the left and right border regions of the T-DNA from A. tumefaciens (Barker et al., 1983). The border sequences function in the transfer of gene sequences from the Ti plasmid to the tomato genome. These border regions represent the only necessary *cis*-acting elements in the T-DNA for insertion into the plant genome (Klee and Rogers, 1989). The other necessary elements act in *trans* through the use of the binary vector system. Border regions do not confer virulence or pathogenicity on the part of A. tumefaciens (Nester et al., 1984; Zambryski, 1988).

nos Promoter: The *nos* promoter present in line 35-1-N has the untranslated 5' region of the nopaline synthase gene from A. tumefaciens (Depicker et al., 1985). It functions in line 35-1-N in the expression of the *kan*^{r} selectable marker gene. As used in line 35-1-N this sequence is no longer a regulated article since it is not associated with the nopaline synthase gene and therefore does not function to create or enhance either virulence or pathogenicity.

nos Termination Sequences: The nopaline synthase 3' termination sequences from A. tumefaciens function in the expression of the *kan*^{r} and *sam-k* genes respectively. In the case of the *kan*^{r} gene, in order to enhance expression and aid in the transformation and selection process, approximately 0.8 Kb of the nos 3' coding region (*frame shifted to prevent transcription, therefore considered to be non-coding*) in addition to 0.27 Kb containing the polyadenylation signal was used to insure the proper expression and processing of the *kan*^{r} gene product in plants (An et al., 1985). In the case of the *sam-k* gene, the 0.27 Kb polyadenylation signal was used to assist in the control of expression of the *sam-k* gene. These sequences as used in the production of SAMase tomatoes no longer function as regulated sequences since they are either not associated with any nopaline synthase coding regions (i.e., *sam-k*) or the coding region has been frame shifted to eliminate the possibility of transcription (i.e., *kan*^{r}). This conclusion is supported by data collected in field trials (Appendix 5) where no plant pest symptoms have been observed. We therefore conclude that SAMase tomatoes containing these sequences do not represent a plant pest risk.

V. Description of SAMase Tomato Line 35-1-N

A. Derivation of SAMase Tomato Line 35-1-N

Line 35-1-N is derived from a homozygous R_1 progeny selection from an original R_0 transformant 35-1, obtained by transformation of the variety Large Red Cherry with binary vector pAG 5420. Line 35-1-N may be used either as a commercial variety itself or as source of the *sam-k* gene in a breeding program for the development of additional large fruited, roma, cherry and fresh market tomato varieties with modified ripening phenotypes.

B. Genetic characterization of Line 35-1-N

1. Segregation analysis

The initial transformant 35-1 was selected as part of a group of several primary transformants screened in the greenhouse for a modified ripening phenotype and the presence of a single T-DNA insert of the *sam-k* gene using a germination assay on kanamycin containing media. Results of the germination assay fit the expected chi-square value for a single Mendelian locus as shown in Table 3 (for methods, see App. 3).

Table 3. Segregation analysis of progeny of pAG 5420 R_0 transformant 35-1

Line	Resistant	Sensitive	X^2
35-1	43	17	0.35 *

*Chi square goodness of fit test for hypothesis of 3:1 segregation ratio.
X^2=3.84 for a 3:1 ratio @ p=0.05 df=1

2. Southern analysis:

Southern analysis of line 35-1-N and Large Red Cherry controls was used to demonstrate Mendelian inheritance, homozygosity in progeny (to facilitate selection), to confirm the earlier germination results which indicated the presence of a single T-DNA insertion within the genome of plant 35-1 and to demonstrate stability of the insert over multiple generations in progeny of line 35-1-N. Results of these analysis are shown in Figures 2, 3 and 4 respectively. Genomic DNA was isolated from leaf material of 35-1 generations R_1 and R_2. The genomic DNA was digested with various restriction enzymes as indicated in the respective figures and separated electrophoretically for transfer to a nylon membrane (complete methods of molecular biology techniques are found in Appendix 4). After transfer, the membranes were probed with either a biotin labeled DNA probe or random primed labeled ^{32}P DNA probe specific for the *sam-k* gene alone or in combination with the ACC Oxidase gene. The ACC Oxidase gene was used as an internal standard in the initial Southern blot analyses to allow for the quantitative comparison of *sam-k* band intensities between heterozygous and homozygous progeny of line 35-1. Scanning densitometry data is found in Appendix 7.

The results of the R_1 Southern analysis (Fig.2) support the initial segreagation data indicating integration of the T-DNA at a single genetic locus. If this was not the case, multiple banding patterns would be observed between the R_1 progeny of line 35-1. The presence of two *sam-k* bands with a HindIII digest does however suggest more than one copy of the *sam-k* gene at this locus. Additional Southern analysis with other restriction enzymes (Fig. 3) resulted in a single band when probed with *sam-k*. This would indicate that the second copy of the T-DNA is incomplete. Integration of multiple copies of the T-DNA at a single locus is a common occurence and has been previously reported in the literature (Jorgensen, 1987).

3. Mendelian Inheritance:

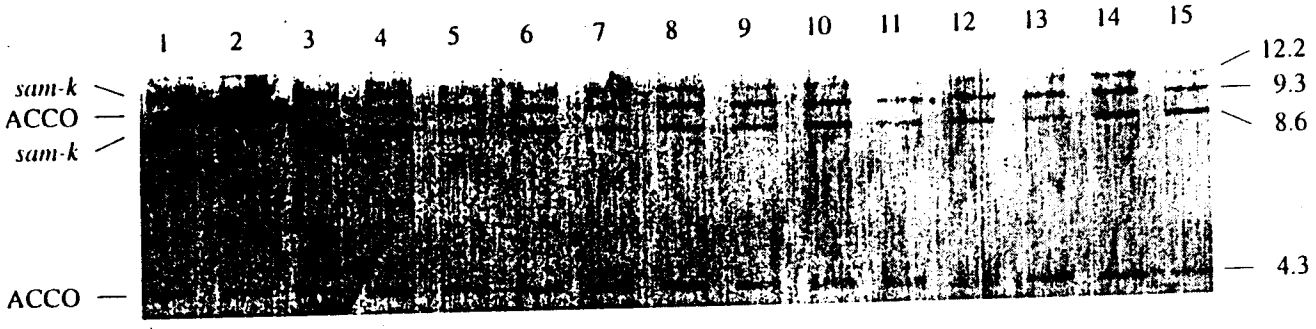
A number of R_1 progeny of R_0 plant 35-1 which germinated on kanamycin containing media as part of the segregation analysis (Table 3) were selected at random and planted to soil for further analysis and selection. A set of 15 of these progeny were chosen for Southern analysis to determine if it was possible to detect homozygous individuals from the population and to determine if the segregation observed would be indicative of a single genetic locus. Figure 2 represents the results of this analysis. Genomic DNA from progeny of line 35-1 were digested with restriction enzyme HindIII, electrophoresed and probed with a biotinylated probe which was a combination of *sam-k* and ACC oxidase gene probes. The ACC oxidase bands represent an internal control which was used for comparison of band intensities. Band intensities were quantitated using scanning densitometry and comparisons were made between the *sam-k* bands and the ACC oxidase bands. It was assumed that the ACC Oxidase bands would remain relatively constant across samples while *sam-k* bands would vary in intensity based upon gene dosage (i.e. homozygous individuals would have *sam-k* band intensities double that of heterozygous individuals). Based upon this analysis, it was determined that lanes 1, 2, 4, 6, 8 and 15 contained homozygous individuals while lanes 3, 5, 7, 9, 10, 11, 12, 13 and 14 contained heterozygous individuals. These results give a ratio of approximately 2:1 heterozygous to homozygous individuals. This is the expected result if the T-DNA is inserted at a single locus within the genome of line 35-1-N. Based upon these results, R_2 plant 35-1-N (lane 14) was one of four R_1 progeny selected for advancement. Continued monitoring of the modified ripening phenotype resulted in progeny of line 35-1-N being one of

two selections subsequently sent to the field in the summer of 1993 for further phenotypic characterization and detailed horticultural evaluation. Line 35-1-N was selected from the field in 1993 based upon phenotypic analysis demonstrating modified ripening and horticultural evaluation comparing field performance to the non-transgenic variety Large Red Cherry (see appendix 5, field trial reports). Seed from self fertilization of line 35-1-N have subsequently been planted for analysis, seed increase, and hybrid seed production in Hermiston, OR, Naples, FL., Indio CA and Baja California Mexico.

To confirm the initial segregation analysis which indicated insertion of the pAG 5420 T-DNA at a single locus within the genome of line 35-1-N, Southern blot analysis was carried out using a *sam-k* probe in combination with specific restriction enzymes. Genomic DNA of line 35-1-N was digested with BamHI, BglII and a combination of the BamHI/BglII. BamHI and BglII represent unique restriction enzyme sites within the T-DNA of pAG 5420. Single bands of unknown size would be predicted from digests with these enzymes if *one complete copy* of the T-DNA was inserted at one locus within the genome. A single band of 5098 bp would be predicted for a digest which uses a combination of these two enzymes. Results shown in Figure 3 support the conclusion of *one complete copy* of the T-DNA at a single Mendelian locus. Additional restriction digests idicate that a portion of a second copy of the T-DNA is present at this locus as well (see Fig. 2).

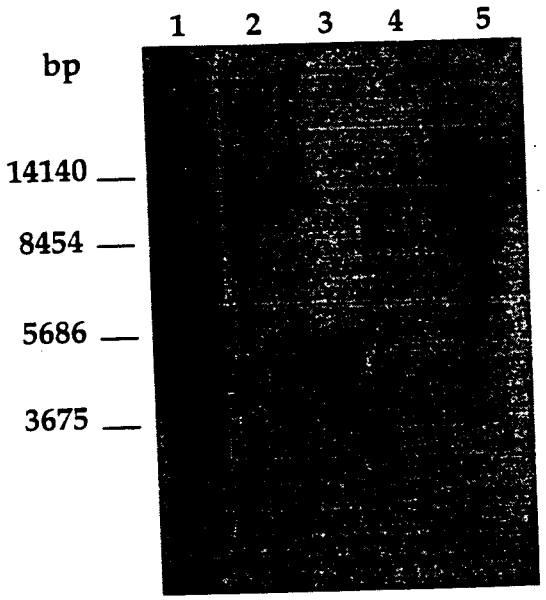
To confirm the stability of the genetic insert over multiple generations, Southern analysis of R_3 and R_5 progeny as well as non-transgenic Large Red Cherry controls were compared and used to establish the presence of a stable T-DNA insertion within the genome of line 35-1-N over the course of three subsequent generations. Results of this analysis are shown in Figure 4. Individual progeny of two generations show identical banding patterns indicating that the insert is stable over generations. Since EcoRI is not a unique site within the T-DNA, the two bands observed are expected.

Figure 2. Southern blot analysis of R_1 progeny of plant 35-1 demonstrating Mendelian inheritance and homozygous individuals for selection



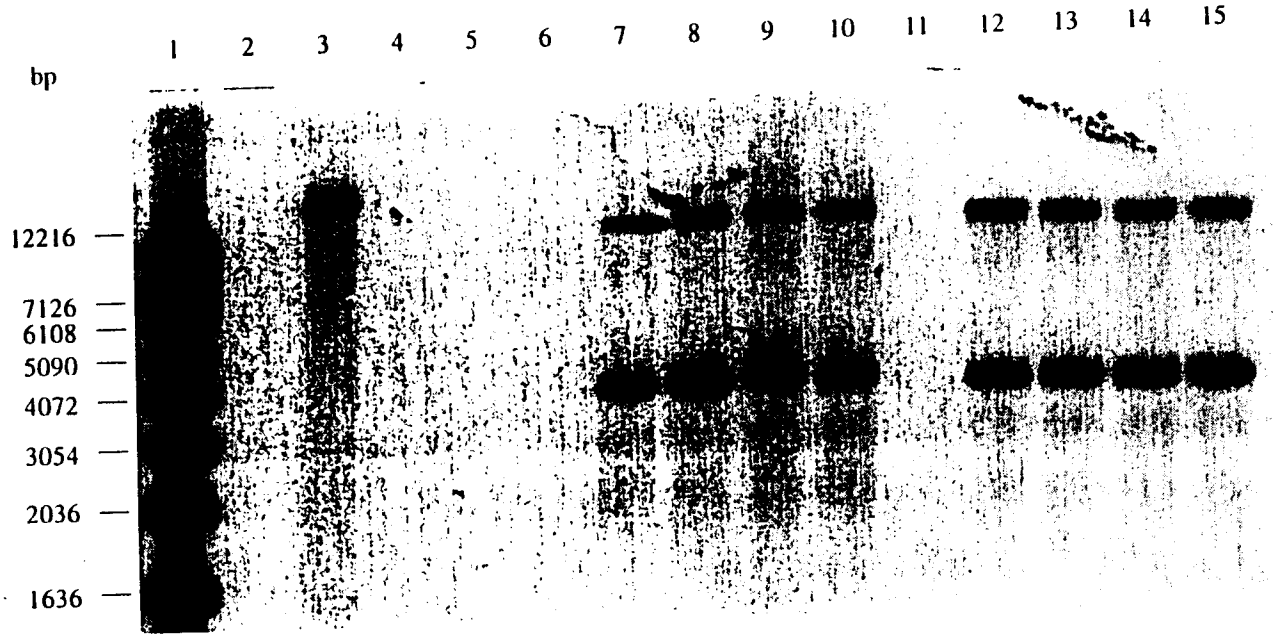
Southern blot analysis of R_1 progeny of line 35-1-N digested with restriction enzyme HindIII. Each of the lanes (1–15) represents 15 µg of genomic DNA isolated from individual R_1 progeny (A–O, respectively) of line 35-1. The digests were carried out per manufacturers instructions and the electrophoresis, and Southern blot analysis was carried out as described in the Appendix 4. The resultant Southern blot was probed with a biotinylated *sam-k* and ACC oxidase combination probe. The final blot was scanned and the band intensities quantitated (Appendix 7). Results were used to determine homozygous individuals for selection and advancement. R_1 progeny 35-1-N (lane 14) was one of several progeny chosen for advancement.

Figure 3. Southern Analysis of R3 progeny of line 35-1-N confirming the presence of a single T-DNA insertion.



Southern blot analysis of line 35-1-N digested with BglII (lane 1), BamHI (lane 2), a combination of BglII and BamHI (lane 3) tomato variety Large Red Cherry control DNA digested with BamHI (lane 4) blank lane and pAG 5420 positive control DNA (lane 5). Each of the lanes represents 10 μg of genomic DNA with the exception of the positive control (lane 5) which contains 100 pg of plasmid DNA. The digests were carried out as per manufacturers instructions and the electrophoresis, Southern blot analysis was carried out as described in the Appendix 4. The resultant Southern blot was probed with a ^{32}P labeled *sam-k* gene fragment isolated from pAG 5420.

Figure 4. Southern blot analysis of R3 and R5 progeny of line 35-1-N confirming stable integration of the inserted DNA over multiple generations



Southern blot analysis of R3 and R5 progeny of line 35-1-N digested with restriction enzyme EcoRI to demonstrate stability over multiple generations. Molecular weight standards (lane 1), blank (lane 2) pAG 5420 positive control (lane 3) blank (lane 4), Large Red Cherry Control (lane 5) blank (lane 6) Four R3 individuals of line 35-1-N (lane 7–10) blank (lane 11) and four R5 individuals of line 35-1-N (lane 11–15). Each of the lanes represents 15 µg of genomic DNA with the exception of the positive control (lane 3) which contains 100 pg of plasmid DNA. The digests were carried out as per manufacturers instructions and the electrophoresis, and Southern blot analysis was carried out as described in the Appendix 4. The resultant Southern blot was probed with a ^{32}P labeled *sam-k* gene fragment isolated from pAG 5420.

C. Analysis of inserted DNA from outside the border regions:

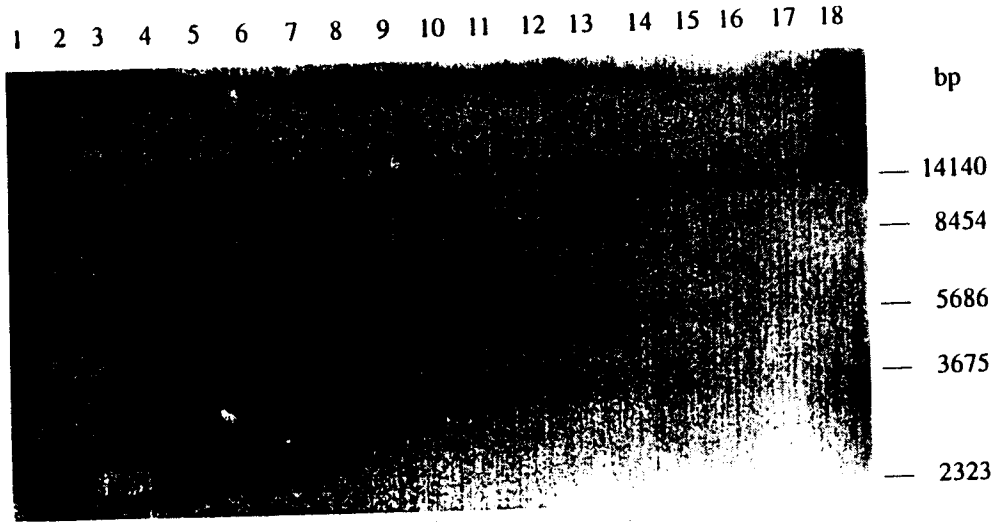
In addition to the transfer of T-DNA from within the borders, an additional region of pAG 5420 backbone DNA was transferred to the genome of line 35-1-N. This is a relatively frequent occurrence when using A. tumefaciens to transform plant cells (Martineau et al., 1993). Southern analysis has identified the pAG 5420 backbone DNA as extending from the left border of pAG 5420 to no more than 2.6 Kb into the plasmid backbone. (see Table 4 and Appendix 6 for complete detail). This DNA fragment contains a portion of the pAG 5420 plasmid DNA elements listed in Table 4. None of the DNA or DNA elements present on this fragment are derived from or function as plant pests, nor does this fragment contain any additional antibiotic resistance marker genes (see Figure 5). The oriT element functions in trans with other host bacterial DNA elements in E. coli and is responsible for the initiation of conjugation between host bacteria. The *trfA* element is part of a larger operon which is regulated by *cis* acting elements in the host bacteria and functions in the stable replication of the plasmid within the host (Schmidhauser, et al, 1985; Pansegrau, et al, 1994). These elements are non-functional in plants since they are not in the presence of the cis acting elements nor the remaining components of the operons. In addition, it would not be expected that the *trfA* gene product is present since the gene is not in frame with any gene promoter capable of regulating its expression in eukaryotes. As a result of these analyses we conclude that the presence this fragment of pAG 5420 backbone DNA in line 35-1-N does not represent a plant pest issue. The complete Southern analysis and sequence of this DNA fragment are found in Appendix 6.

Table 4. DNA elements of integrated backbone DNA from pAG 5420.

DNA Element	Size (Kb)	Function
ori T	0.7	Initiates conjugation in E. coli when other essential elements exist in *trans* (Schmidhauser, et al, 1985; Pansegrau, et al, 1994)
trfA	1.5	A portion of an operon which functions in the stable replication of the plasmid in host bacteria. This element requires the remaining operon components as well as the cis acting element in order to be functional (Schmidhauser, et al, 1985; Pansegrau, et al, 1994)

Figure 5 illustrates Southern analysis of line 35-1-N which indicates that the tetracycline antibiotic resistance gene from pAG 5420 has not been transferred to the genome of line 35-1-N.

Figure 5. Southern analysis of line 35-1-N for the presence of the *tet*^{r} antibiotic resistance gene



Southern blot analysis of R3 progeny of line 35-1-N digested with restriction enzyme EcoRI and probed with a 484bp SalI/StuI *tet*^{r} gene fragment isolated from pAG 5420. Lanes 1–15 contain 15 µg of genomic DNA from individual R3 progeny of line 35-1-N. Lane 16 is blank, lane 17 contains 15 µg of genomic DNA isolated from Large Red Cherry control and lane 18 contains 100 pg of pAG 5420 positive control. The digests were carried out as per manufacturers instructions and the electrophoresis and Southern blot analysis was carried out as described in the Appendix 4.

D. Field Tests of SAMase Tomatoes

Agritope, Inc has carried out ten field trials of SAMase tomatoes in processing types, large fruited fresh market types, roma type and cherry tomato type cultivars in Florida, California, Oregon and Mexico. These trials have been conducted to produce seed and plant material for genetic and molecular analysis, demonstrate and characterize the modified ripening phenotypes produced, generate horticultural performance data relative to non transgenic controls and to produce seed of selected lines for advancement in variety development programs. These tests were carried out between 1992 and the present. Appendix 5 contains the field trial reports submitted to the USDA for each of the field tests completed.

Table 6. Agritope, Inc. Field Trials of SAMase Tomatoes

Permit #	Site	Trial Status
92-085-01	Hermiston, OR	Completed
93-050-01	Hermiston, OR	Completed
93-361-01N	Naples, FL	Completed
93-048-01N	Davis, CA	Completed
94-143-03N	Hermiston, OR	Completed
93-361-01N	Naples, FL	Completed
94-353-01N	Indio, CA	Completed
95-121-04N	Hermiston. OR	In Progress
95-121-03N	Davis, CA	In Progress
SARH	Vizcaino, Baja CA, Mexico	In Progress

1. Horticultural Evaluations

Cooperative researchers at the Oregon State University Hermiston Agricultural Research Experiment Station (HAREC) conducted detailed horticultural evaluations on line 35-1-N in both the 1992 and 1993 field seasons. The parameters evaluated included vigor (plant growth and development), plant height, leaf area, dry weight, fruit set, pH, soluble solids, titratable acidity, average fruit weight and yield. Disease susceptibility and the presence of crown gall was also monitored during the course of the trials. No evidence of Crown Gall disease was observed in either the transgenic or control plots. This is further supported by post harvest monitoring of all SAMase field trial locations which has demonstrated that the use of normal field clean up techniques (i.e. disking of desiccated vines post harvest) has resulted in an absence of volunteer SAMase tomatoes the following season. We therefore conclude that the SAMase tomatoes have no advantage over the parental Large Red Cherry line in terms of its ability to survive in the environment.

Table 6a. Summary of Horticultural Evaluations of Tomato Line 35-1-N

Genotype	Plant Height (cm)	Leaf Area (cm^2)	Dry Weight (g)
Large Red Cherry Ctrl.	106.0 a	40890 a	372.4 a
35-1-N	69.7 b	17844 b	170.4 b

Values reported represent the means of five replications. Means followed by different letters are significantly different at p=0.05 using Duncan's Multiple Range Test. Complete data is found in Appendix 3 field trial reports.
Leaf area and dry weight are of 2 plants/plot.

Table 6b. Summary of Fruit Quality Evaluations of Tomato Line 35-1-N

Genotype	pH	Soluble Solids (brix)	Titratable Acidity (meq)	Average Fruit Weight (g)
Large Red Cherry Ctrl.	4.29 a	6.93 a	157.5 a	19.3 a
35-1-N	4.35 a	6.75 a	134.5 a	17.7 a

Values reported represent the means of five replications. Means followed by different letters are significantly different at p=0.05 using Duncan's Multiple Range Test. Complete data is found in Appendix 3 field trial reports.

Table 6c. Summary of Fruit Development Evaluations of Tomato Line 35-1-N

Heat units required from transplant to:

	Fruit Set	Mat. Green	Breaker	Pink	Red
Genotype					
Large Red Cherry Ctrl.	707 a	1398 a	1531 a	1629 a	1675 a
35-1-N	756 a	1419 a	1709 b	1824 b	1901 b

Means followed by different letters are significantly different at p=0.05 using Duncan's Multiple Range Test.
Heat units based upon a 45 F base, 90 F maximum

2. Yield Characteristics

As part of the ongoing horticultural evaluation and phenotype characterization carried out at various field test locations, yield characteristics have been evaluated by quantitative harvest at specific developmental stages. At HAREC in the summer of 1993, three consecutive harvests were carried out on specific dates. Fruit which had reached at least the breaker stage by the harvest date were harvested. Harvested fruit were then sorted by color, counted and weighed. As expected, yield results were effected by a genotype by harvest date interaction. To resolve this interaction, data was sorted by both variables for analysis. Results indicate that total yield of line 35-1-N was negatively affected and the distribution of fruit maturity at harvest was significantly impacted. Relative to control Large Red Cherry lines, line 35-1-N yielded the majority of its fruit at the breaker to pink stage at each harvest. The modified ripening phenotype significantly slowed the rate at which the fruit on line 35-1-N ripened from breaker to full red. This phenotype is the intended technical effect. Results of yield measurements for line 35-1-N are found in Table 7. Complete yield data can be found in field trial reports in Appendix 5.

Table 7. Summary of Yield Measurements in Tomato Line 35-1-N

Harvest 1

Color Stage

	Breaker	Pink	Red	Total
Genotype	kg/plot	kg/plot	kg/plot	kg/plot
Large Red Cherry Ctrl.	0.25	0.48	2.70 a	3.45 a
35-1-N	0.11	0.15	0.34 b	0.62 b

Means followed by different letters are significantly different at p=0.05 using Duncan's Multiple Range Test.

Harvest 2

	Color Stage			
	Breaker	Pink	Red	Total
Genotype	kg/plot	kg/plot	kg/plot	kg/plot
Large Red Cherry Ctrl.	0.58	0.92	3.01 a	4.52 a
35-1-N	0.40	0.58	0.34 b	1.32 b

Means followed by different letters are significantly different at p=0.05 using Duncan's Multiple Range Test.

Harvest 3

	Color Stage			
	Breaker	Pink	Red	Total
Genotype	kg/plot	kg/plot	kg/plot	kg/plot
Large Red Cherry Ctrl.	0.32	1.92	5.26 a	7.63 a
35-1-N	0.85	2.80	0.83 b	4.56 b

Means followed by different letters are significantly different at p=0.05 using Duncan's Multiple Range Test.

Since 35-1-N, compared to Large Red Cherry, is lower yielding, produces smaller fruit and is slower to ripen, it is less likely to be a weed or plant pest.

3. Disease and Pest Characteristics

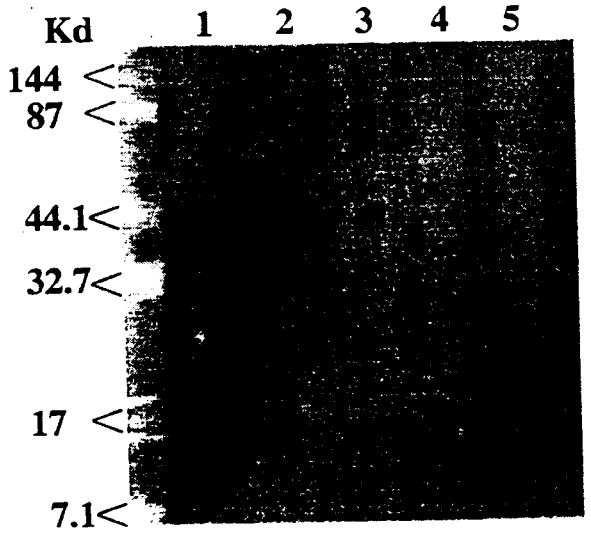
During the course of all of the field trials listed in Table 5, cooperating researchers and company personnel monitored each of the field trials for disease and pest susceptibility of line 35-1-N and controls. Plots were evaluated for overall vigor and evidence of fruit or plant tissue damage due to bacterial, fungal and/or viral diseases. In addition, plants were monitored for their susceptibility to insect pests. Detailed insect counts or disease incidence data were not collected from either experimental or control plots from these trials. However, cooperating reseachers and company personnel did note any presence of disease and/or insect pest symptoms. When symptoms did occur, standard commercial treatment measures were employed. Specific control measures taken are detailed in the chronological log of the field trial reports found in Appendix 5. No significant difference was observed between transgenic and control plants in their response to any disease and/or insect pest pressure in these trials. No evidence of crown gall was noted in any of the field trials conducted.

VI. Environmental Consequences of Introduction of SAMase Tomatoes

A. SAMase Tomatoes

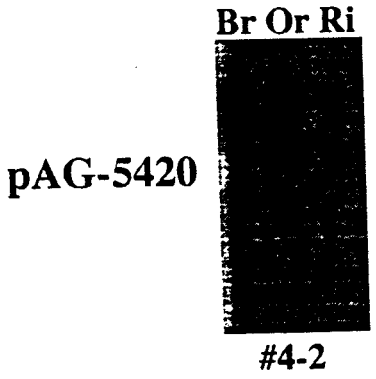
SAMase tomato line 35-1-N contains a gene which expresses an enzyme responsible for degradation of *S*-adenosylmethionine (SAM) specifically in fruit during the ripening process. This results in an absence of 1-aminocyclopropane-1-carboxylic acid (ACC), the penultimate step in ethylene formation. By expressing this enzyme in a fruit specific and developmentally regulated fashion; (Fig. 6a and 6b) fruit of SAMase line 35-1-N produce substantially less ethylene than non transgenic fruit resulting in a modified ripening phenotype (Fig. 7). The resulting phenotype does not create a tomato with increased weediness potential nor does this phenotype result in a tomato more likely to become a plant pest. Analysis of disease susceptibility, seed germination tests, agronomic traits, and observations related to the lack of any Crown Gall formation lead us to conclude that SAMase tomatoes do not represent a plant pest as described in 7 CFR.340.

Figure 6a. Western blot analysis of line 35-1-N demonstrating tissue specific expression of the *sam-k* gene.



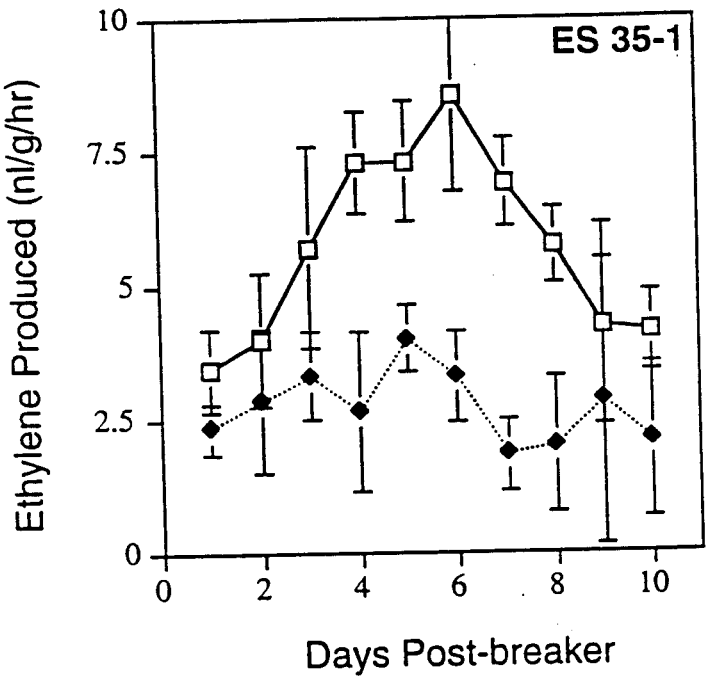
Western blot analysis demonstrates tissue specific expression of the *sam-k* gene in tomato line 35-1-N. A 20ng sample of purified SAMase (lane 1), and 9ug samples of protein extracted from leaf (lane 3), stem (lane 4), and flower (lane 5) were resolved on an SDS-PAGE gel and blotted to a nylon membrane as described in Appendix 4. The resulting blot was probed with a monoclonal antibody specific to the SAMase protein and visualized using chemiluminescence as per manufacturer's instructions.

Figure 6b. RNAse protection analysis of fruit of line 35-1-N demonstrating developmental regulation of *sam-k* gene expression.



RNAse protection assays were used to demonstrate the pattern of *sam-k* gene expression in ripening tomato fruit. Tomato fruit were picked at the breaker stage (Br) and allowed to ripen to the orange (Or) or red (Ri) stage. Between 0.7 and 1.0 µg of poly A+ mRNA was used in each reaction. The 510 bp protected probe was resolved on a denaturing polyacrylamide gel and visualized by autoradiography.

Figure 7. Expression Profile of Ethylene in Ripening Transgenic Fruit of Line 35-1-N



Ethylene produced from transgenic tomatoes of pAG 5420 transformed line 35-1. The graph represents ethylene produced by fruit of R_0 transformant 35-1 (diamonds) and Large Red Cherry controls (squares). The values for transgenic fruit represent the average of three fruit from the individual R_0 plant. The values for the controls represent the average of six fruit from two different plants. Error bars represent one standard deviation of the data.

B. npt II

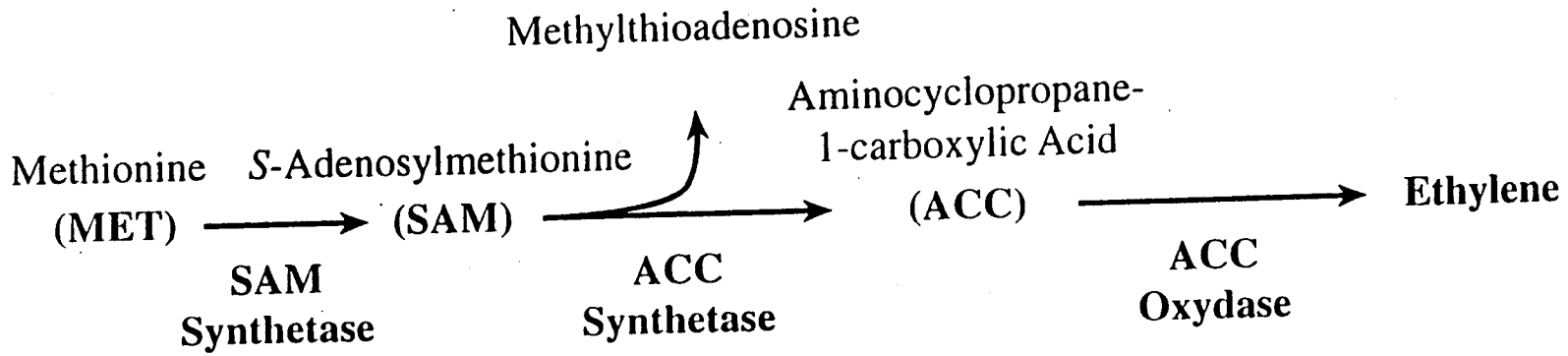
The *kan*^{r} gene and its product the npt II protein have been previously reviewed by US regulatory agencies and determined to be safe (USDA, 1992; FDA, 1994).

C. *S*-adenosylmethionine hydrolase

S-adenosylmethionine (SAM) is a ubiquitous nucleotide used in many activities in all cells (for reviews see Salvatore et al., 1977 and Usdin et al., 1979). SAM acts as a co-factor in a variety of reactions and as a methyl group donor in a specific variety of reactions and as a methyl group donor in specific transmethylation reactions. Among these reactions are the biosynthesis of biotin, rare nucleotides, 5'-methylthioadenosine (MTA), polyamines and the production of the plant hormone ethylene. SAM also acts a methyl donor during modifications of proteins, lipids, polysaccharides and nucleic acids. SAMase hydrolyzes SAM to homoserine and 5'-methylthioadenosine (Gold et al., 1964). In the course of ethylene biosynthesis, 1-Aminocyclopropane-1-carboxylic Acid (ACC), the immediate precursor to ethylene, is produced from SAM by the enzyme ACC synthase. As the pool of SAM is depleted by the action of SAMase, neither ACC nor ethylene are produced. A diagram of the ethylene biosynthesis pathway is shown in Figure 8.

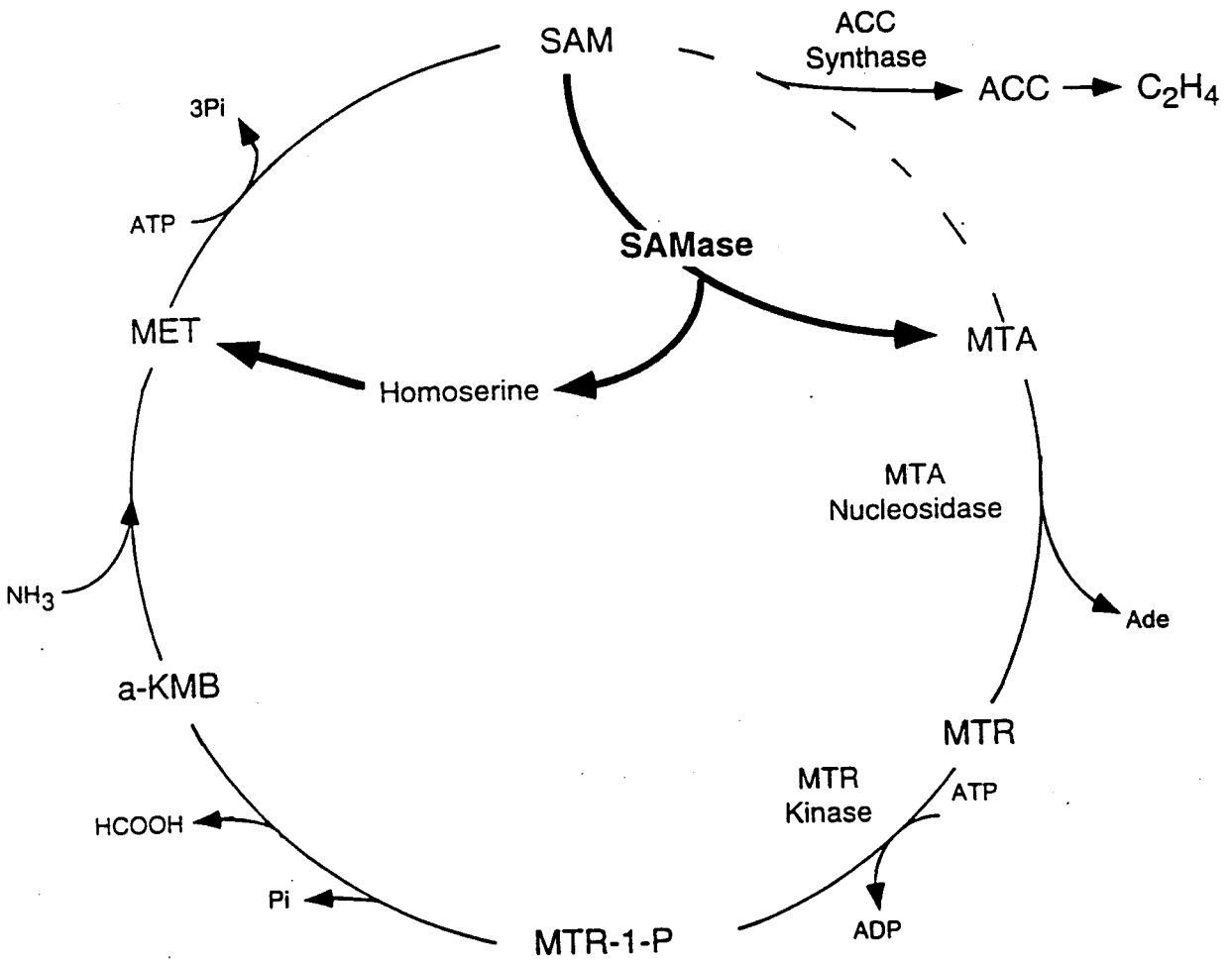
The *sam-k* gene is derived from an E. coli bacteriophage T3 gene which encodes a functional *S*-adenosylmethionine hydrolase (SAMase) protein (Hughes et al., 1987). The SAMase protein catalyzes the conversion of *S*-adenosylmethionine (SAM) to methylthioadenosine (MTA). Figure 6 above demonstrates fruit specific, developmentally regulated expression of the *sam-k* gene in tomato. The effect on the methionine recycling pathway of SAMase expression is illustrated in Figure 9. Expression of the SAMase protein in fruit does not represent an environmental exposure to a novel protein as SAMase occurs naturally in the environment. (Furuse et al., 1983). The enzymatic degradation of SAM is carried out by a number of naturally occurring enzymes in plants, among them ACC synthase. The tissue specific and developmentally regulated expression system employed to express SAMase in tomato line 35-1-N insures that the only impact on the plant of SAM hydrolysis is the down regulation of ethylene production in ripening fruit through the reduction of the pool of SAM available in the ripening fruit for conversion to ACC and then ethylene.

Figure 8. Ethylene Biosynthetic Pathway



Ethylene biosynthetic pathway in plants. Ethylene synthesis is an offshoot of the methionine recycling pathway where *S*-adenosylmethionine (SAM) is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by the enzyme ACC synthase. ACC is subsequently oxidized to ethylene by the enzyme ACC oxidase (Kende, 1993).

Figure 9. Methionine Recycling Pathway



The methionine recycling pathway in plants. The bold line shows the effect of *sam-k* expression on the pathway where the reaction products re-enter the cycle (Good et al., 1994). Abbreviations: MET, methionine; SAM, *S*-adenosylmethionine; ACC, 1-aminocyclopropane-1-carboxylic acid; MTA, 5'-methylthioadenosine; MTR, 5-methylthioribose; Add, adenine; KMB, a-ketomethylthiobutyric acid.

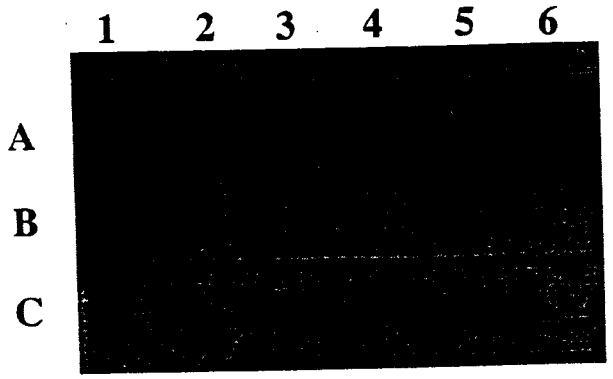
The E8: *sam-k* chimera present in line 35-1-N expresses a functional *S*-adenosylmethionine hydrolase (SAMase) protein in a fruit specific, developmentally regulated manner (Fig.6). Transgenic fruit of line 35-1-N express the SAMase protein in breaker to light red fruit and expression is attenuated in fruit that is fully ripe. The fact that E8: *sam-k* gene expression follows the normal pattern of ethylene expression means that the SAMase protein is a transient species and final concentrations in the ripe fruit are minimal (Fig. 7). As a result of these data we conclude that there is no increased risk to the environment from the gene product SAMase.

D. Safety of the gene products in the environment

Use of the SAMase tomato will not alter the weediness potential of tomato or any related species. While the *sam-k* gene encodes a functional SAMase protein, it is not a novel protein in the environment (Furuse et al., 1983), is not homologous with any known toxins and/or allergens as listed in the University of Geneva reference database, nor is it a novel enzymatic activity in plants. SAM degrading enzymes (i.e. ACC synthase) carry out a number of important metabolic functions in plants.

The SAMase protein sequence is presented in Appendix 7 and is not homologous to any of the known protein toxins listed in the University of Geneva reference database. Glycosylation and subsequent increase in the antigenic capacity of SAMase will not occur because SAMase does not contain the necessary sequence information needed for transport of the protein to the subcellular locations where glycosylation takes place. Furthermore, we have determined that the SAMase protein is rapidly inactivated by incubation under simulated gastric conditions (Fig. 10).

Figure 10. Results of SAMase Protein Digestibility Studies



Inactivation of SAMase by simulated gastric fluid. Digestibility studies of the SAMase protein were carried out using a SAMase fusion protein produced as described in the manufacturers instructions and purified by affinity column chromatography (Appendix 4). Simulated gastric fluid (0.32% pepsin, 0.2% NaCL pH 1.2) either without (**A**) or with (**B**) pepsin was prepared to 25ng/ml of SAMase with the fusion protein and incubated for 0 min. (lane 1), 5 min. (lane 2), 10 min. (lane 3), 20 min. (lane 4), 30 min. (lane 5) or 60 min. (lane 6) at 37° C. (**C**).Non-transgenic tomato fruit control prepared to 25 ug/ml of total protein in simulated gastric fluid without pepsin.

The npt II protein and the gene have been previously reviewed by US regulatory agencies and determined to be safe (USDA, 1992; FDA, 1994).

E. Effects on Agriculture and Cultivation Practices

SAMase tomatoes have the potential to improve the economics of production and distribution in today's fresh market tomato industry in the following ways: First, this technology will provide additional flexibility to the producer in terms of the time frame during which the crop will mature and be harvestable as well as the impact of outside environmental influences upon the rate of crop maturation and necessity of harvest. Second, it may be possible to reduce the number of harvests needed to get an equivalent economic yield from a production field and thus reduce labor needs and costs. Third. losses in the production, handling and distribution systems currently experienced due to immature and over ripe product will be mitigated.

Using a fruit specific developmentally regulated SAM degradation strategy as a means to reduce ethylene biosynthesis in plants has a number of distinct advantages. The fruit specific nature of gene expression targets only the SAM found in fruit and diverted to ACC for ethylene production (Figure 9). This means that normal ethylene biosynthetic processes as well as other SAM dependent reactions in the remainder of the plant proceed uninhibited. The developmental regulation of the *sam-k* gene expression allows for the initial climacteric burst of ethylene necessary in climacteric fruit like tomato for initiation of the ripening process prior to down regulation of ethylene. The result is a fruit of higher quality due to a lack of immaturity in the crop. The fact that *sam-k* gene expression follows the normal pattern of expression of ethylene during the ripening process means that the SAMase protein is essentially transient and final concentrations in the ripe fruit are minimal (Figure 7).

The use of an enzyme that degrades SAM also allows for the selection of broad range of modified ripening phenotypes which can be predictably determined by the level of SAMase protein expressed. This results in an array of precise and predictable phenotypes which may be adapted for use in a variety of applications within the production and distribution system depending upon the problem to be addressed. It is possible to use exogenous ethylene to ripen these fruit or if necessary they may be left on the vine for an extended period to ripen on their own. The lack of an immediate ripening response due to environmental effects such as increased temperature will further benefit producers.

We believe that together, all of these benefits will allow for a substantial increase in production flexibility, help to reduce production and handling related losses and produce a higher quality in the crop as a whole with the concomitant cost savings in labor and distribution costs.
All SAMase tomato field trials have been carried out using standard tomato cultivation practices with no adverse effects on the environment or surrounding commercial crops observed(see Appendix 5; field trial reports). It is expected that there will be no adverse effects on the standard tomato cultivation practices of non transgenic crops as a result of the de-regulation of the SAMase tomato.

F. Human and Animal Exposure

In general tomatoes are marketed in two distinct ways, either as a fresh or processed product . We do not anticipate that SAMase tomatoes will result in any change in the way both humans and animals are currently exposed to tomatoes. Tomato line 35-1-N is a Cherry type tomato intended to be consumed fresh. The presence of a SAMase protein will not result in any modification to the food product as it is currently consumed. The SAMase protein occurs naturally in the human digestive tract, it is not toxic and will not affect allergenicity of the fresh or processed tomato product. The exposure of livestock animals to SAMase tomatoes is not expected to be any different than current exposures and there is no reason to believe that SAMase tomatoes will result in any increased use of tomato as an animal feed. Safety of SAMase tomatoes as human food is

the subject of an ongoing consultation process between Agritope, Inc. and the Federal Food and Drug Administration.

G. Unintended Effects on Non-Target Organisms

SAMase tomato line 35-1-N is one of numerous SAMase tomato lines that has been tested in tomato producing areas throughout the United States. These tomatoes have shown no deleterious effects towards the environment or any non target organisms found within the production areas (Appendix 5, field trial reports). This is an expected result since SAMase occurs naturally in the environment, it is not toxic and will not affect allergenicity of the fresh tomatoes of which it is a constituent. Exposure to organisms such as local wildlife (i.e., deer, rodents and birds etc.) would be casual and in the absence of toxicity on the part of SAMase tomatoes there would be no deleterious effects expected. This is borne out by observations at field trial sites. In addition, as stated by Rick (1976), tomato flowers are not normally attractive to bees or other beneficial insects in a field production setting. The main insect species which feed on tomato plants and fruit in a field production setting are limited to those insect pests most commonly encountered as production problems. Table 9 lists the major species of tomato insect pests in the major production regions of North America (U.C. Davis Cooperative Extension Pub. 3274). None of these species is listed as an endangered species (50 CFR 17.11, 50 CFR 17.12) nor would SAMase tomatoes be expected to be toxic to these organisms.

Table 8. Major Species of Tomato Insect Pests

Genus	Species	Common Name
Agrotis	*ipsilon*	Cutworm
Epitrix	*hirtipennis*	Flea Beetle
Frankliniella	*sp.*	Thrips
Manduca	*sp.*	Hornworm
Liriomyza	*sativae*	Leafminer
Aculops	*lycopersici*	Tomato russet mite
Myzus	*persicae*	Green peach aphid
Heliothus	*zea*	Tomato fruitworm
Bemisia	*tabaci*	Sweetpotato whitefly
Heliothus	*virescens*	Tobacco budworm
Spodoptera	*exigua*	Beet armyworm
Keiferia	*lycopersicella*	Tomato pinworm

Line 35-1-N also contains the *kan*^{r} gene which encodes the npt II protein. The human and environmental safety of both the protein and the gene have been previously reviewed by USDA APHIS and FDA and been determined to be safe **(USDA, 1992; FDA, 1994)**.

H. Expression specificity of pAG 5420 in transgenic tomato

Stable insertion of the *sam-k* transgene into the tomato genome results in the production of functional SAMase protein in a fruit specific and temporally regulated manner as shown in Figure 6. Using this expression system, SAMase is induced at the onset of ripening and/or by the treatment of the fruit with exogenous ethylene. In addition to the fruit specific and temporally regulated expression, the expression of SAMase in transgenic fruit using this system is transient. The expression pattern of SAMase in this system, closely mirrors the pattern of ethylene expression normally found in ripening fruit (Good et al., 1994; Figure 7).

I. Compositional Analysis

Composition of fresh tomato fruit can vary considerably due the effects and interactions of environmental conditions, horticultural practices and genetic background. We have carried out a compositional analysis of various tomato lines transformed with pAG 5420 to determine if tomato

fruit expressing the *sam-k* gene differ from the Large Red Cherry non transgenic parental background only in the presence of the npt II and SAMase proteins and that in all other respects, the SAMase tomatoes differ from traditionally bred tomatoes only for the intended technical effects.

We have evaluated pH, soluble solids and titratable acidity of fruit from line 35-1-N and compared this data to values obtained from non transgenic Large Red Cherry parental lines. Fruit were grown at HAREC during the summer of 1993, allowed to ripen on the vine, hand harvested and approximately 20 fruit per line were combined for analysis. Fruit were chopped in a Waring blender, screened to remove the pulp and then vacuum filtered to produce the juice that was analyzed. Soluble solids were measured by refractometer, pH was measured with a pH meter and titratable acidity was measured by titration with 0.1N NaOH. There were no significant differences in the parameters measured.

We have also measured a set of nutritional components in the SAMase tomatoes and controls. Table 5 summarizes a selected set of the components measured and the values obtained. Values obtained for the vitamin and amino acid analysis fall well within the range normally reported for fresh tomatoes (Davies et al., 1981; Souci et al., 1986 and Souci et al., 1989).

Table 9. Nutritional Components in pAG 5420 SAMase Tomatoes and Large Red Cherry Control lines.

Constituent	Normal Range (Davies et al., 1981; Souci et al., 1986; Souci et al., 1989)	Measured Range SAMase Tomatoes	Measured Range Large Red Cherry Controls
Vitamin A (IU)	192 - 3833	1922 - 2131	2649 - 2727
Vitamin C (mg/100g)	8.4 - 59.0	24.64 - 27.05	17.53 - 23.37
Selected soluble amino acids (mg/100g)			
Methionine	0.5 - 11.0	2.20 - 2.39	1.75 - 2.33
Threonine	6.5 - 42.0	26.0 - 36.4	9.23 - 25.0

J. Tomatine Content

Tomatine is a steroidal glycoalkaloid found naturally in a number of solanaceous plants (Roddick, 1974). In cultivated tomato, it is found in the leaves, stems and immature green fruit. Normally, tomatine levels are highest in young fruit and decline to barely detectable levels at physiological maturity. In normal ripening genotypes, levels ranging between 0.3-0.6 mg/100g fresh weight have been reported in ripe fruit while in ripening mutant genotypes such as *nor* and *rin*, levels of 0.9-1.2 mg/100g fresh weight have been reported (Eltayeb and Roddick, 1984). Toxic levels of tomatine have been determined by studies in mice, which indicate an LD_{50} for tomatine of 500 mg/kg body weight (Grierson and Kader, 1986). As such, levels found in tomatoes; even in young green fruit, do not represent a food safety hazard. Nevertheless, in order to insure that SAMase tomatoes have tomatine levels which are substantially unchanged from the non transgenic Large Red Cherry parental genotype we have measured tomatine content of a representative set of ripe fruit from line 35-1-N and non transgenic Large Red Cherry. As expected, the tomatine content of all samples of SAMase tomatoes was non detectable, just as in the non transgenic Large Red Cherry samples. Table 10 summarizes the results of the tomatine analysis.

Table 10. Tomatine Content of Representative Ripe Fruit From SAMase Tomatoes and Large Red Cherry Control Toamtoes (mg/100g fresh weight)

Normal Range (mg/100g)	SAMase Tomatoes	Large Red Cherry Control
0.3-0.6	n.d.	n.d.

n.d.=not detectable, (LOQ = 0.5mg/100g)

K. Weediness Potential

The introduction of an additional SAMase activity to tomato cultivars should not result in an increase in the "weediness" potential of the cultivar. A general consensus of the traits common to many weeds has been put forth by Baker (1974) and include: 1) germination requirement fulfilled in many environments; 2) discontinuous germination and great longevity of seed; 3) rapid growth through vegetative phase to flowering; 4) continuous seed production for as long as growing conditions permit; 5) self-compatibility but not completely autogamous and apomictic; 6) when cross pollinated, unspecialized visitors or wind pollinated; 7) high seed output in favorable environment and some seed production in a wide range of environments; 8) adaptation for short and long distance dispersal; 9) if perennial, vegetative production or regeneration from fragments and brittleness (so as to be not easily removed from the ground) and 10) ability to compete

interspecifically by special means (i.e. rosette formation and presence of allelochemicals). While not all weeds share all of these traits, tomato possesses few of these characteristics which make plants successful as weeds. It is an annual crop which is considered to be highly domesticated and not persistent in the environment without the aid of direct human intervention (USDA, 1992). The non transgenic Large Red Cherry cultivar which has been used as a genetic background for line 35-1-N, is not considered to be a weed and the modified ripening phenotype exhibited in the engineered line 35-1-N resulting from an additional SAMase activity has not imparted any new "weedy " characteristics. No changes in seed germination, seed production, disease and pest resistances and plant growth characteristics that would confer weed characteristics have been noted in any of the greenhouse or field trials comparing line 35-1-N to Large Red Cherry or other non transgenic tomato varieties.

L. Potential for Outcrossing

1. Outcrossing with wild species

As previously described in section II part F, hybridization between Lycopersicon esculentum and wild Lycopersicon species is possible, although normally only through specific controlled crosses is hybridization to the wild relatives possible. As a result, there is little probability of a naturally occurring cross between line 35-1-N and a wild relative in the United States.

2. Outcrossing to cultivated tomato

As previously described in section II part F, when field grown, tomato is predominantly self pollinated due to its self compatibility and floral structure. In addition, present commercial varieties have a floral structure which creates a crop which is exclusively inbreeding (Taylor, 1986). As a result of these facts and the ability to maintain pure seed stocks using standard practices including selection and isolation, there is little if any potential for outcrossing of SAMase tomatoes to commercial stocks. The only instances in which line 35-1-N will be crossed to commercially cultivated tomatoes, will be in the context of a traditional variety improvement program directed at developing improved hybrid and open pollinated cultivars.

3. Transfer of genetic information to organisms to which it cannot interbreed

USDA has previously stated in an interpretive ruling on a petition by Calgene, Inc. (57FR, No.202 p. 47608-47616, Oct. 19, 1992) "There is no published evidence for the existence of any mechanism, other than sexual crossing" by which genes can be transferred from a plant to other organisms. Evidence presented in the FR Notice suggest that, based on a limited homology of the DNA in question transfer from plants to microbes may have occurred in evolutionary time over a period of many millennia. However, even if such transfer were to occur, transfer of the *sam-k* or *kan*^{r} gene to microbes would not pose a plant pest risk. Neither of the genes involved is derived from a plant pest and there is no evidence to suggest that transfer of these genes to microbes would result in the creation of a plant pest.

VII. Statement of Grounds Unfavorable

No negative aspects have been determined for SAMase tomatoes

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APPENDICES

1. Complete DNA Sequence of T-DNA Region of pAG 5420
2. A description of the DNA Elements Found in pAG 5420
3. Kanamycin Germination Assay Methods
4. Molecular Biology Methods used in the Characterization of Line 35-1-N
5. Field Trial Reports, SAMase Tomatoes
6. DNA Sequence of the Integrated Backbone DNA from pAG 5420
7. Complete Protein Sequence of *S*-adenosylmethionine hydrolase
8. Scanning Densitometry of *sam-k* and ACC Oxidase Band intensities in R_1 Progeny of Line 35-1
9. Chromatographs of Tomatine Measurements in Large Red Cherry Control and Transgenic pAG 5420 Transformed Tomatoes

APPENDIX 1

Complete DNA Sequence of T-DNA Region of pAG 5420

pAG .20 T-DNA seq
Monday, November 13, 1995 12:52 PM

```
          10        20        30        40        50        60        70
          *  *      *  *      *  *      *  *      *  *      *  *      *  *
GCCTTTCCTC AATCGCTCTT CGTTCGTCTG GAAGGCAGTA CACCTTGATA GGTGGGCTGC CCTTCCTGGT

          80        90       100       110       120       130       140
          *  *      *  *      *  *      *  *      *  *      *  *      *  *
TGGCTTGGTT TCATCAGCCA TCCGCTTGCC CTCATCTGT- TACGGCGGTA GCGGCCAGCC ATGCAGAGCA

         150       160       170       180       190       200       210
          *  *      *  *      *  *      *  *      *  *      *  *      *  *
GGATTCCCGT TGAGCACCGC CAGGTGCGAT AAGGGACAGT GAAGAAGGA- ACACGCTCGC GGGTGGGCCT

         220       230       240       250       260       270       280
          *  *      *  *      *  *      *  *      *  *      *  *      *  *
ACTTCACCTA TCCTGCGGCC TGACGCCGTT GGATACACCA AGGAAAGTCT ACACGAACCC TTTGGCAAAA

         290       300       310       320       330       340       350
          *  *      *  *      *  *      *  *      *  *      *  *      *  *
TCCTGTATAT CGTGCGAAAA AGGATGGATA TACCGAAAAA ATCGCTATAA TGACCCCGAA GCAGG-TTAT

         360       370       380       390       400       410       420
          *  *      *  *      *  *      *  *      *  *      *  *      *  *
GCAGCGGAAA AGATCCGTCG ACGATCGTCA ACGTTCACTT CTAAAGAAAC AGCGCCACTC AGCTTCCTCA

         430       440       450       460       470       480       490
          *  *      *  *      *  *      *  *      *  *      *  *      *  *
GCGGCTTTAT CCAGCGATTT CCTATTATGT CGG-AAGTTC T-AAGATCGA CAGCCTGTCA CGGTTAAGCG

         500       510       520       530       540       550       560
          *  *      *  *      *  *      *  *      *  *      *  *      *  *
AGAAATGAAT AAGAAGGCTG ATAATTCGGA TCTCTGCGAG GGAGATGATA TTTGATCACA GGCAGCAACG

         570       580       590       600       610       620       630
          *  *      *  *      *  *      *  *      *  *      *  *      *  *
CTCTGTCATC GTTACAATCA ACATGCTACC CTCCGCGTGA TCATCCATAT TTCAAACCCG GCAGTTAGTT

         640       650       660       670       680       690       700
          *  *      *  *      *  *      *  *      *  *      *  *      *  *
GCCGTTCTTC CGATAGCATC GGTAACATGA GCAAAGTCTG CCGCCTTACA ACGGCTCTCC CCGTGACGCG

         710       720       730       740       750       760       770
          *  *      *  *      *  *      *  *      *  *      *  *      *  *
TCCCGGACTG ATGGGCTGCC TGTATCGAGT GGTGATTTTG TGCCGAGCTG CCGGTCGGGG AGCTGTTGGC
```

pAG 1220 T-DNA seq
Monday, November 13, 1995 12:52 PM

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          780        790        800        810        820        830        840
          *  *       *  *       *  *       *  *       *  *       *  *       *  *
TGGCTGGTGG CAG-ATATAT TGTGGTGTAA ACAAATTGAC GCTTAGACAA CTTAATAACA CATTGCGGAC

          850        860        870        880        890        900        910
          *  *       *  *       *  *       *  *       *  *       *  *       *  *
GTTTTTAATG TACTGAATTC TTGAAGACGA AAGGGCCTCG TGATACGCCT ATTTTTATAG GTTAATGTCA

          920        930        940        950        960        970        980
          *  *       *  *       *  *       *  *       *  *       *  *       *  *
TGATAATAAT GGTTTCTTAG ACGTCAGGTG GCACTTTTCG GGGAAATGTG CGCGGAACCC CTATTTGTTT

          990       1000       1010       1020       1030       1040       1050
          *  *       *  *       *  *       *  *       *  *       *  *       *  *
ATTTTTCTAA ATACATTCAA ATATGTATCC GCTCATGAGA CAATAACCCT GATAAATGCT TCAATAATAT

         1060       1070       1080       1090       1100       1110       1120
          *  *       *  *       *  *       *  *       *  *       *  *       *  *
TGAAAAAGGA AGAGTATGAG TATTCAACAT TTCCGTGTCG CCCTTATTCC CTTTTTTGCG GCATTTTGCC

         1130       1140       1150       1160       1170       1180       1190
          *  *       *  *       *  *       *  *       *  *       *  *       *  *
TTCCTGTTTT TGCTCACCCA GAAACGCTGG TGAAAGTAAA AGATGCTGAA GATCAGTTGG GTGCACGAGT

         1200       1210       1220       1230       1240       1250       1260
          *  *       *  *       *  *       *  *       *  *       *  *       *  *
GGGTTACATC GAACTGGATC TCAACAGCGG TAAGATCCTT GAGAGTTTTC GCCCGAAGAA CGTTTTCCAA

         1270       1280       1290       1300       1310       1320       1330
          *  *       *  *       *  *       *  *       *  *       *  *       *  *
TGATGAGCAC TTTTAAAGTT CTGCTATGTG GCGCGGTATT ATCCCGTGTT GACGCCGGGC AAGAGCAACT

         1340       1350       1360       1370       1380       1390       1400
          *  *       *  *       *  *       *  *       *  *       *  *       *  *
CGGTCGCCGC ATACACTATT CTCAGAATGA CTTGGTTG-G TACTCACCAG TCACAGAAAA GCATCTTACG

         1410       1420       1430       1440       1450       1460       1470
          *  *       *  *       *  *       *  *       *  *       *  *       *  *
GATGGCATGA CAGTAAGAGA ATTATGCAGT GCTGCCATAA CCATGAGTGA TAACACTGCG GCCAACTTAC

         1480       1490       1500       1510       1520       1530       1540
          *  *       *  *       *  *       *  *       *  *       *  *       *  *
```

pAG 20 T-DNA seq
Monday, November 13, 1995 12:52 PM

TTCTGACAAC GATCGGAGGA CCGAAGGAGC TAACCGCTTT TTTGCACAAC ATGGGGGATC ATGTAACTCG

1550 1560 1570 1580 1590 1600 1610

CCTTGATCGT TGGGAACCGG AGCTGAATGA AGCCATACCA AACGACGAGC GTGACACCAC GATGCCTGCA

1620 1630 1640 1650 1660 1670 1680

GGGGGGGGGG GGGGGGGGAC ATGAGGTTNC CCCGTATTCA GTGTCGCTGA TTTGTATTGT CTGAAGTTGT

1690 1700 1710 1720 1730 1740 1750

TTTTACGTTA AGTTGATGCA GATCAATTAA TACGATACCT GCGTCATAAT TGATTATTTG ACGTGGTTTG

1760 1770 1780 1790 1800 1810 1820

ATGGCCTCCA CGCACGTTGT GATATGTAGA TGATAATCAT TATCACTTTA CGGGTCCTTT CCGGTGATCC

1830 1840 1850 1860 1870 1880 1890

GACAGGTTAC GGGGCGGCGA CCTCGCGGGT TTTCGCTATT TATGAAAATT TTCCGGTTTA AGGCGTTTCC

1900 1910 1920 1930 1940 1950 1960

GTTCTTCTTC GTCATAACTT AATGTTTTTA TTTAAAATAC CCTCTGAAAA GAAAGGAAAC GACAGGTGCT

1970 1980 1990 2000 2010 2020 2030

GAAAGCGAGC TTTTTGGCCT CTGTCGTTTC CTTTCTCTGT TTTTGTCCGT GGAATGAACA ATGGAACCCC

2040 2050 2060 2070 2080 2090 2100

CCCCCCCCCC CCCCTGCAGC AATGGCAACA ACGTTGCGCA AACTATTAAC TGGCGAACTA CTTACTCTAG

2110 2120 2130 2140 2150 2160 2170

CTTCCCGGCA ACAATTAATA GACTGGATGG AGGCGGATAA AGTTGCAGGA CCACTTCTGC GCTCGGCCCT

2180 2190 2200 2210 2220 2230 2240

TCCGGCTGGC TGGTTTATTG CTGATAAATC TGGAGCCGGT GAGCGTGGGT CTCGCGGTAT CATTGCAGCA

2250 2260 2270 2280 2290 2300 2310

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                                                                  *   *   *   *   *   *   *   *   *   *   *   *   *   *
CTGGGGCCAG ATGGTAAGCC CTCCCGTATC GTAGTTATCT ACACGACGGG GAGTCAGGCA ACTATGGATG

      2320        2330        2340        2350        2360        2370        2380
*   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
AACGAAATAG ACAGATCGCT GAGATAGGTG CCTCACTGAT TAAGCATTGG TAACTGTCAG ACCAAGTTTA

      2390        2400        2410        2420        2430        2440        2450
*   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
CTCATATATA CTTTAGATTG ATTTAAAACT TCATTTTTAA TTTAAAAGGA TCTAGGTGAA GATCCTTTTT

      2460        2470        2480        2490        2500        2510        2520
*   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
GATAATCTCA TGACCAAAAT CCCTTAACGT GAGTTTTCGT TCCACTGAGC GTCGAGCCCC GTAGAAAAGA

      2530        2540        2550        2560        2570        2580        2590
*   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
TCAAAGGATC TTCTTGAGAT CCTTTTTTTC TGCGCGTAAT CTGCTGCTTG CAAACAAAAA A---MMCGCT

      2600        2610        2620        2630        2640        2650        2660
*   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
ACCAGCGGTG GTTTGTTTGC CGGATCAAGA GCTACCAACT CTTTTTCCGA AGGTAACTGG CTTCAGCAGA

      2670        2680        2690        2700        2710        2720        2730
*   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
GCGCAGATAC CAAATACTGT CCTTCTAGTG TAGCCGTAGT TAGGCCACCA CTTCAAGAAC TCTGTAGCAC

      2740        2750        2760        2770        2780        2790        2800
*   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
CGCCTACATA CCTCGCTCTG CTAATCCTGT TACCAGTGGC TGCTGCCAGT GGCGATAAGT CGTGTCTTAC

      2810        2820        2830        2840        2850        2860        2870
*   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
CGGGTTGGAC TCAAGACGAT AGTTACCGGA TAAGGCGCAG CGGTCGGGCT GAACGGGGGG TTCGTGCACA

      2880        2890        2900        2910        2920        2930        2940
*   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
CAGCCCAGCT TGGAGCGAAC GACCTACACC GAACTGAGAT ACCTACAGCG TGAGCTATGA GAAAGCGCCA

      2950        2960        2970        2980        2990        3000        3010
*   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
CGCTTCCCGA AGGGAGAAAG GCGGACAGGT ATCCGGTAAG CGGCAGGGTC GGAACAGGAG AGCGCACGAG
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      3020       3030       3040       3050       3060       3070       3080
       *  *       *  *       *  *       *  *       *  *       *  *       *  *
GGAGCTTCCA GGGGGAAACG CCTGGTATCT TTATAGTCCT GTCGGGTTTC GCCACCTCTG ACTTGAGCGT

      3090       3100       3110       3120       3130       3140       3150
       *  *       *  *       *  *       *  *       *  *       *  *       *  *
CGATTTTTGT GATGCTCGTC AGGGGGGCGG AGCCTATGGA AAAACGCCAG CAACGCGGCC TTTTTACGGT

      3160       3170       3180       3190       3200       3210       3220
       *  *       *  *       *  *       *  *       *  *       *  *       *  *
TCCTGGCCTT TTGCTGGCCT TTTGCTCACA TGTTCTTTCC TGCGTTATCC CCTGATTCTG TGGATAACCG

      3230       3240       3250       3260       3270       3280       3290
       *  *       *  *       *  *       *  *       *  *       *  *       *  *
TATTACCGCC TTTGAGTGAG CTGATACCGC TCGCCGCAGC CGAACGACCG AGCGAV---- GTCAGTGAGC

      3300       3310       3320       3330       3340       3350       3360
       *  *       *  *       *  *       *  *       *  *       *  *       *  *
GAGGAAGCGG AAGAGCGCCT GATGCGGTAT TTTCTCCTTA CGCATCTGTG CGGTATTTCA CACCGCATAT

      3370       3380       3390       3400       3410       3420       3430
       *  *       *  *       *  *       *  *       *  *       *  *       *  *
GGTGCACTCT CAGTACAATC TGCTCTGATG CCGCATAGTT AAGCCAGTAT ACACTCCGCT ATCGCTACGT

      3440       3450       3460       3470       3480       3490       3500
       *  *       *  *       *  *       *  *       *  *       *  *       *  *
GACTGGGTCA TGGCTGCGCC C-GACACCCG CCAACACCCG CTGACGCGCC CTGACGGGCT TGTCTGCTCC

      3510       3520       3530       3540       3550       3560       3570
       *  *       *  *       *  *       *  *       *  *       *  *       *  *
CGGCATCCGC TTACAGACAA GCTGTGACCG TCTCCGGGAG CTGCATGTGT CAGAGGTTTT CACCGTCATC

      3580       3590       3600       3610       3620       3630       3640
       *  *       *  *       *  *       *  *       *  *       *  *       *  *
ACCGAAACGC GCGAGGCAGC AGATCTATCG ATGGTACCGT TAACGAGCTC TAGCCCGATC TAGTAACATA

      3650       3660       3670       3680       3690       3700       3710
       *  *       *  *       *  *       *  *       *  *       *  *       *  *
GATGACACCG CGCGCGATAA TTTATCCTAG TTTGCGCGCT ATATTTTGTT TTCTATCGCG TATTAAATGT

      3720       3730       3740       3750       3760       3770       3780
       *  *       *  *       *  *       *  *       *  *       *  *       *  *
ATAATTGCGG GACTCTAATC ATAAAAACCC ATCTCATAAA TAACGTCATG CATTACATGT TAATTATTAC
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      3790       3800       3810       3820       3830       3840       3850
       *  *       *  *       *  *       *  *       *  *       *  *       *  *
ATGCTTAACG TAATTCAACA GAAATTATAT GATAATCATC GCAAGACCGG CAACAGGATT CAATCTTAAG

      3860       3870       3880       3890       3900       3910       3920
       *  *       *  *       *  *       *  *       *  *       *  *       *  *
AAACTTTATT GCCAAATGTT TGAACGATCG GGGAAATTCG TTGTACTTGC CAGCGGCGAC CGAACTCATC

      3930       3940       3950       3960       3970       3980       3990
       *  *       *  *       *  *       *  *       *  *       *  *       *  *
AATAGTGAAG CAGCCTTGCA GCGGTGCGCC TTTAGGAACC TCTTGGAAAC TACCCGGCAG ACGTTCAGCC

      4000       4010       4020       4030       4040       4050       4060
       *  *       *  *       *  *       *  *       *  *       *  *       *  *
TTATACCCGT CGATACCTTT AGCGTACACC AGACCAGCCG TGTGAGTCTG TGATTTGTAT ACCAGTACGC

      4070       4080       4090       4100       4110       4120       4130
       *  *       *  *       *  *       *  *       *  *       *  *       *  *
AATCTTGCTC CCACTCATTA GAAGCTAGGC GTGCAACATT GAGTGGCTGC GCTTTGTCCT TGTAGCGTAC

      4140       4150       4160       4170       4180       4190       4200
       *  *       *  *       *  *       *  *       *  *       *  *       *  *
ACGAACAGTT TTTTCCTCAG TTGGTGCGCT TGAGATTGCC TCACGATAGC ACCCGGTCAA ATCGGTTGAC

      4210       4220       4230       4240       4250       4260       4270
       *  *       *  *       *  *       *  *       *  *       *  *       *  *
TCAACGGAGC CATAAAGACC CGGTGCGGCA CGTAAAGTGC TTACCATGTG GCGGTGTCTG CTCATATTCA

      4280       4290       4300       4310       4320       4330       4340
       *  *       *  *       *  *       *  *       *  *       *  *       *  *
CCTCATCGCA GAGGTTAGAA CGGAAAGCGG AAACCAGTAC ATAGAAGACG TTCGCAGGCT CTTTAGTGAA

      4350       4360       4370       4380       4390       4400       4410
       *  *       *  *       *  *       *  *       *  *       *  *       *  *
AACCATGGTC TTTTGCACTG TGAATGATTA GAATAATTTC TAAAAATCTC AATATGAGGA TGCCATATTT

      4420       4430       4440       4450       4460       4470       4480
       *  *       *  *       *  *       *  *       *  *       *  *       *  *
ATAATAGAAT AAAATAAAAT GTGAACAAAG AAAGAGATAA AGTAGTTCAC TTTTTGAAAT CTAAGAGAGA

      4490       4500       4510       4520       4530       4540       4550
       *  *       *  *       *  *       *  *       *  *       *  *       *  *
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AATGGGAACA AGAAAGAGAC AAAAGTAGTT TCAAACAAAC TTCTCTTCTA AGTTTAGTCC CTTTTTAAAA

      4560       4570       4580       4590       4600       4610       4620
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
TATGAAACCC AATACGTCTG ATTAAGAATA GAAAAATATC AAATTTTCAA TATAATTTAT ACTAATCGTT

      4630       4640       4650       4660       4670       4680       4690
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
TTGAATTTTT CATACTGATA TAGTGTACGT TTCATCATAA CAACCAAAAC GTTGTTGTTT CACAACAATA

      4700       4710       4720       4730       4740       4750       4760
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
ATATAGTAGT AGTTAATTTA TTATTTAGTA ATAAGTGGTC CTAAAAATTA GATAAATATT ACTATGATAA

      4770       4780       4790       4800       4810       4820       4830
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
TATAAAAATA TTTGAGTCAG TCCTAAAAAA TTATTTAGTA TTCATACATG AATCAAACTA ATTAGTTAAG

      4840       4850       4860       4870       4880       4890       4900
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
TGTCAACAAT TGGACAAGTG GCATGGAGGT TGTAAAAGAA TGACATAAGC CAACTGCTAT TTTTATCCAA

      4910       4920       4930       4940       4950       4960       4970
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
AAAAAAGAAG ACAACTTGAC AACTACATTT CTTTTATTTT TATAAATTTA CTAATATCTT CTATGCAAAA

      4980       4990       5000       5010       5020       5030       5040
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
TTATTCGGTG CCTTTCTAAA CTTTAAGGTT TTTATTTGAT GTACACCTAA ATTATATTTT ATTTTAATCA

      5050       5060       5070       5080       5090       5100       5110
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
CTTCACTGAA CTTGTTTATT CCTTCATCAT ATACACCTAC TCCTATTATG ACTACAAGTT GGCAAAAGTA

      5120       5130       5140       5150       5160       5170       5180
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
ATGATATGAA TTTCTACTTA AATAAATAAT AGTCACCTAG ATAAATTAAT TTAACAAAAG ATAAATATCA

      5190       5200       5210       5220       5230       5240       5250
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
AACCTTCTCA CCTAAAATTT TGAGCAAAAC TTCTCACTAA AACTTGTGGA CTAAACCCGA AAATCTTCAG

      5260       5270       5280       5290       5300       5310       5320
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                                                                  *   *   *   *   *   *   *   *   *   *   *   *   *   *
AAAATTAATA TTTAGTACTG GAAAAGTCAG ATTAAATGTC TGCACAAGAC TTTCTATTGT TGGGAATAAA

      5330        5340        5350        5360        5370        5380        5390
*   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
ACAAATTAAT ATTGGATTAA AATAGTTGAA ATATTTAGGT AAAATGCTAC ATGTCATTTA TTCATTGGAT

      5400        5410        5420        5430        5440        5450        5460
*   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
ATTATTTCTT AAAATTTAAA ATTCATTATT TAAAAGTTAT TTTTGAATAA GGGCCGATTT CGTGAAATTC

      5470        5480        5490        5500        5510        5520        5530
*   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
CTTTCTAGAC GTAAAGTTGA TCTATTAAAT TTTAAATTTA TCTTAAATTC TTACAAAGTA AGTATTAATC

      5540        5550        5560        5570        5580        5590        5600
*   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
TTTGTTTCCT TTACTATTCA TTTACATTTT GTCCTATATT TCGTTTAAAA TATGTCATAT ATTAAAAAAA

      5610        5620        5630        5640        5650        5660        5670
*   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
ATTAAAAATT TTACTTTCTT TTTTTACGTT ATAGCTATAT GACGTGACAA AAAATCAACC TTTCACATGC

      5680        5690        5700        5710        5720        5730        5740
*   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
GCCTAGGTAG ACTTCAAGTT AAAAGGGGAT AATGGATACT TTGCCTATCT TTTACCATAT ATTTTAANAT

      5750        5760        5770        5780        5790        5800        5810
*   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
CCTTAATTAT TAAGTTTTCC AATATCTCTC ACCATTCATT TTCTCCTATC ATATATTTTA GGAGTCCTTA

      5820        5830        5840        5850        5860        5870        5880
*   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
ATAATTAAGT TTACTAATAA ACTTTATTAT ATATTATAGG ACTCCTCAAT TATTAGTTCT CTTTATGTCT

      5890        5900        5910        5920        5930        5940        5950
*   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
CTCATCGTAC ATTTTCCTCT TGTCTTATTT GTTAGGACAC TTGAAATTTT CAAAATATAT TTTGCTTTTA

      5960        5970        5980        5990        6000        6010        6020
*   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
ATATATGAAG TTGTGTTTGA TTGTAGTTTT TGTAAATATA TTTAATTTTT TTGAATTTTT ATTTTCTAAA
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      6030       6040       6050       6060       6070       6080       6090
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
AGAAACATAA AATTTAAAAG ATTTAAAAGT ATCATTAAAC TATTAGAAAT AATATATCTA TGTTGTTAAA

      6100       6110       6120       6130       6140       6150       6160
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
AATGATGGTT CTTAATTAAC TGTTTTATTA TAAAATATCA GATAATTCGT TTTATTTACG CAAAAGTTAA

      6170       6180       6190       6200       6210       6220       6230
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
GTGAAGTAAC GAAATTATAA ATCCCATAGA ATATTGTGTA TATACTTGGC ACATGATGAT TGTAACATCC

      6240       6250       6260       6270       6280       6290       6300
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
TTAATTATTA TTAATTCATC GAACCTATTA TTTCTTCATT GTCTATGTAC ATTATCCTTA ATAATTCCAC

      6310       6320       6330       6340       6350       6360       6370
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
TTCAGATTTA TTAGBTCTTT GGTTATTGGT TTAAGTTTAT TTTACAACCA AGTGAATTGA ATTTGTCCTC

      6380       6390       6400       6410       6420       6430       6440
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
CATTAATATT TATTGGATTA AAAAATAAAT AAATTTGCTC TTATTTGTAG AAAGATTTAG ACTTTTAAAA

      6450       6460       6470       6480       6490       6500       6510
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
TATTACGTTT TCTGACTCTT TTCTTATCAA AATTGGACTC TCTCACTTCC ACAAAACTTA ATTACGTGAA

      6520       6530       6540       6550       6560       6570       6580
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
CAATATCATT AGGGATGTCA AAAATGAATT CAAACAAAGA TAACCGCCTA GAATTTTAAG GGTTTGGGCT

      6590       6600       6610       6620       6630       6640       6650
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
CAGAATAATT TGAATTACGT TCAATCTCAA CCAATTAAAG CTTCATCAAC GCAAGACATG CGCACGACCG

      6660       6670       6680       6690       6700       6710       6720
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
TCTGACAGGA GAGGAATTTC CGACGAGCAC AGAAAGGACT TGCTCTTGGA CGTAGGCCTA TTTCTCAGGC

      6730       6740       6750       6760       6770       6780       6790
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
ACATGTATCA AGTGTTCGGA CGTGGGTTTT CGATGGTGTA TCAGCCGCCG CCAACTGGGA GATGAGGAGG
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      6800       6810       6820       6830       6840       6850       6860
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
CTTTCTTGGG GGGCAGTCAG CAGTTCATTT CACAAGACAG AGGAACTTGT AAGGAGATGC ACTGATTTAT

      6870       6880       6890       6900       6910       6920       6930
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
CTTGGCGCAA ACCAGCAGGA CGAATTAGTG GGAATAGCCC GCGAATATCT AAGTTATGCC TGTCGGCATG

      6940       6950       6960       6970       6980       6990       7000
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
AGCAGAAACT TCCAATTCGA AACAGTTTGG AGAGGTTGTT TTTGGGCATA CCTTTTGTTA GTCAGCCTCT

      7010       7020       7030       7040       7050       7060       7070
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
CGATTGCTCA TCGTCATTAC ACAGTACCGA AGTTTGATCG ATCTAGTAAC ATAGATGACA CCGCGCGCGA

      7080       7090       7100       7110       7120       7130       7140
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
TAATTTATCC TAGTTTGCGC GCTATATTTT GTTTTCTATC GCGTATTAAA TGTATAATTG CGGGACTCTA

      7150       7160       7170       7180       7190       7200       7210
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
ATCATAAAAA CCCATCTCAT AAATAACGTC ATGCATTACA TGTTAATTAT TACATGCTTA ACGTAATTCA

      7220       7230       7240       7250       7260       7270       7280
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
ACAGAAATTA TATGATAATC ATCGCAAGAC CGGCAACAGG ATTCAATCTT AAGAAACTTT ATTGCCAAAT

      7290       7300       7310       7320       7330       7340       7350
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
GTTTGAACGA TCTGCTTCGA CGCACTCCTT CTTTACTCCA CCATCTCGTC CTTATTGAAA ACGTGGGTAG

      7360       7370       7380       7390       7400       7410       7420
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
CACCAAAACG AATCAAGTCG CTGGAACTGA AGTTACCAAT CACGCTGGAT GATTTGCCAG TTGGATTAAT

      7430       7440       7450       7460       7470       7480       7490
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
CTTGCCTTTC CCCGCATGAA TAATATTGAT GAATGCATGC GTGAGGGGTA GTTCGATGTT GGCAATAGCT

      7500       7510       7520       7530       7540       7550       7560
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
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GCAATTGCCG CGACATCCTC CAACGAGCAT AATTCTTCAG AAAAATAGCG ATGTTCCATG TTGTCAGGGC

      7570       7580       7590       7600       7610       7620       7630
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
ATGCATGATG CACGTTATGA GGTGACGGTG CTAGGCAGTA TTCCCTCAAA GTTTCATAGT CAGTATCATA

      7640       7650       7660       7670       7680       7690       7700
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
TTCATCATTG CATTCCTGCA AGAGAGAATT GAGACGCAAT CCACACGCTG CGGCAACCTT CCGGCGTTCG

      7710       7720       7730       7740       7750       7760       7770
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
TGGTCTATTT GCTCTTGGAC GTTGCAAACG TAAGTGTTGG ATCGGGGTGG GCGAAGAACT CCAGCATGAG

      7780       7790       7800       7810       7820       7830       7840
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
ATCCCCGCGC TGGAGGATCA TCCAGCCGGC GTCCCGGAAA ACGATTCCGA AGCCCAACCT TTCATAGAAG

      7850       7860       7870       7880       7890       7900       7910
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
GCGGCGGTGG AATCGAAATC TCGTGATGGC AGGTTGGGCG TCGCTTGGTC GGTCATTTCG AACCCCAGAG

      7920       7930       7940       7950       7960       7970       7980
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
TCCCGCTCAG AAGAACTCGT CAAGAAGGCG ATAGAAGGCG ATGCGCTGCG AATCGGGAGC GGCGATACCG

      7990       8000       8010       8020       8030       8040       8050
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
TAAAGCACGA GGAAGCGGTC AGCCCATTCG CCGCCAAGCT CTTCAGCAAT ATCACGGGTA GCCAACGCTA

      8060       8070       8080       8090       8100       8110       8120
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
TGTCCTGATA GCGGTCCGCC ACACCCAGCC GGCCACAGTC GATGAATCCA GAAAAGCGGC CATTTTCCAC

      8130       8140       8150       8160       8170       8180       8190
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
CATGATATTC GGCAAGCAGG CATCGCCATG GGTCACGACG AGATCCTCGC CGTCGGGCAT GCGCGCCTTG

      8200       8210       8220       8230       8240       8250       8260
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
AGCCTGGCGA ACAGTTCGGC TGGCGCGAGC CCCTGATGCT CTTCGTCCAG ATCATCCTGA TCGACAAGAC

      8270       8280       8290       8300       8310       8320       8330
```

pAG .20 T-DNA seq
Monday, November 13, 1995 12:52 PM

```
                                                                      *    *    *    *    *    *    *    *    *    *    *    *    *    *
CGGCTTCCAT CCGAGTACGT GCTCGCTCGA TGCGATGTTT CGCTTGGTGG TCGAATGGGC AGGTAGCCGG

      8340       8350       8360       8370       8380       8390       8400
 *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
ATCAAGCGTA TGCAGCCGCC GCATTGCATC AGCCATGATG GATACTTTCT CGGCAGGAGC AAGGTGAGAT

      8410       8420       8430       8440       8450       8460       8470
 *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
GACAGGAGAT CCTGCCCCGG CACTTCGCCC AATAGCAGCC AGTCCCTTCC CGCTTCAGTG ACAACGTCGA

      8480       8490       8500       8510       8520       8530       8540
 *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
GCACAGCTGC GCAAGGAACG CCCGTCGTGG CCAGCCACGA TAGCCGCGCT GCCTCGTCCT GCAGTTCATT

      8550       8560       8570       8580       8590       8600       8610
 *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
CAGGGCACCG GACAGGTCGG TCTTGACAAA AAGAACCGGG CGCCCCTGCG CTGACAGCCG GAACACGGCG

      8620       8630       8640       8650       8660       8670       8680
 *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
GCATCAGAGC AGCCGATTGT CTGTTGTGCC CAGTCATAGC CGAATAGCCT CTCCACCCAA GCGGCCGGAG

      8690       8700       8710       8720       8730       8740       8750
 *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
AACCTGCCCG GATCCGGGCG GAAATAGGTA AAGAAGTTGC GGATAAGGTA ATTGCCATTG CAGATTATTT

      8760       8770       8780       8790       8800       8810       8820
 *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
GGATTGAGAG TGAATATGAG ACTCTAATTG GATACCGAGG GGAATTTATG GAACGTCAGT GGAGCATTTT

      8830       8840       8850       8860       8870       8880       8890
 *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
TGACAAGAAA TATTTGCTAG CTGATAGTGA CCTTAGGCGA CTTTTGAACG CGCAATAATG GTTTCTGACG

      8900       8910       8920       8930       8940       8950       8960
 *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
TATGTGCTTA GCTCATTAAA CTCCAGAAAC CCGCGGCTGA GTGGCTCCTT CAACGTTGCG GTTCTGTCAG

      8970       8980       8990       9000       9010       9020       9030
 *    *    *    *    *    *    *    *    *    *    *    *    *    *    *    *
TTCCAAACGT AAAACGGCTT GTCCCGCGTC ATCGGCGGGG GTCATAACGT GACTCCCTTA ATTCTCCGCT
```

pAC 120 T-DNA seq
Monday, November 13, 1995 12:52 PM

```
      9040       9050       9060       9070       9080       9090       9100
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
CATGATCAGA TTGTCGTTTC CCGCCTTCAG TTTAAACTAT CAGTGTTTGA CAGGATATAT TGGCGGGTAA

      9110       9120       9130       9140       9150       9160       9170
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
ACCTAAGAGA AAAGAGCGTT TATTAGAATA ATCGGATATT TAAAAGGGCG TGAAAAGGTT TATCCGTTCG

      9180       9190       9200       9210       9220       9230       9240
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
TCCATTTGTA TGTGCATGCC AACCACAGGG TTCCCCTCGG GAGTGCTTGG CATTCCGTGC GATAATGACT

      9250       9260       9270       9280       9290       9300       9310
      *  *       *  *       *  *       *  *       *  *       *  *       *  *
TCTGTTCAAC CACCCAAACG TCGGAAAGCC TGACGACGGA GCAGCATTCC AAAAAGATCC CTTGGCTCGT

      9320       9330       9340       9350
      *  *       *  *       *  *       *  *
CTGGGTCGGC TAGAAGGTCG AGTGGGCTGC TGTGGCTTGA TC
```

pAG-5420 T-DNA seq Enzyme Cutters
Tuesday, November 14, 1995 9:50 AM

Sequence Range: 1 to 9352

Enzyme	#Cuts	Positions
BamH1	1	8690
Bgl2	1	3591

APPENDIX 2

A Description of the DNA Elements Found in pAG 5420

DNA Element	Size (Kb)	Function
Right Border	1.8	Right border region of T-DNA from A. tumefaciens (An et al., 1985).
p*nos*	0.3	The untranslated promoter region of the nopaline synthase gene from A. tumefaciens controls expression of the *kan*^{r} gene (Depicker et al., 1985)
kan^r	1.02	The *kan*^{r} gene from transposon Tn5 (Beck et al., 1982) encodes the npt II protein which renders transformed cells resistant to the antibiotic kanamycin.
nos 3'	1.1	The untranslated 3' region of the nopaline synthase gene from A. tumefaciens controls expression of the *kan*^{r} gene (Depicker et al., 1985).
pE8	2.3	The modified E8 gene promoter from tomatoes which is used to drive the sam-k gene in a tissue specific, developmentally regulated manner as described by Good et al., 1994.
sam-k	0.51	The S-adenosylmethionine hydrolase gene modified to contain a consensus eukaryotic translation initiation site by altering the nucleotide sequence surrounding the *sam* ATG start codon (Good et al., 1994).
nos 3'	0.27	The untranslated 3' region of the nopaline synthase gene from A. tumefaciens controls expression of the *sam-k* gene (Good et al., 1994).
ori pBR322	1.54	E. coli origin of replication which ensures replication in E. coli (Sutcliffe et al., 1979).
lambda-*cos*	0.4	A lambda *cos* site to allow for the potential re-cloning and molecular characterization of the individual integration events. Together the Col EI origin of replication the lambda *cos* site provides the necessary elements to rescue or re-clone the insert from transgenic plants (An et al., 1988)
LB	0.88	The left border region of T-DNA from A. tumefaciens (An et al, 1985).

Line 35-1-N may also contain the following DNA elements from the backbone of plasmid pAG 5420:

DNA Element	Size (Kb)	Function
ori T	0.7	Initiates conjugation in E. coli when other essential elements exist in *trans* (Schmidhauser, et al, 1985; Pansegrau, et al, 1994)
*trf*A	1.5	A portion of an operon which functions in the stable replication of the plasmid in host bacteria. This element requires the remaining operon components as well as the cis acting element in order to be functional (Schmidhauser, et al, 1985; Pansegrau, et al, 1994)

APPENDIX 3

Kanamycin Germination Assay Methods

KANAMYCIN GERMINATION ASSAY FOR TOMATO

TISSUE CULTURE METHOD

MATERIALS:

1. Seeds
   For Ro plants, 200+ seeds for germination
   For R1 plants, 30+ seeds for germination

2. 50% bleach/ 1% Tween 20 solution, made fresh daily

3. Sterilized seed strainer

4. Sterile forceps

5. Laminar flow hood

6. Appropriate media prepared in petri dishes.*

7. Sterile distilled water

8. 50 ml conical tubes

9. Petri dishes (17mm x 100mm)

10. Waste container for hood use

11. Parafilm

12. Plant growth chamber

METHODS:

1. Prepare media the day before plating of seeds.

Media is comprised of:

1X MS Salts
30 g/L Sucrose
100 mg/l Inositol
1X Gamborg's B5 Vitamins
0.8% Bacto Agar
100 mg/L Kanamycin (added post-autoclaving)

25 mls media per 17mm X 100mm petri dish.
Store in cold room until ready for use.
Shelf-life is 3 weeks.

2. Count out desired amount of seeds and transfer to a 50 ml conical tube, add approx. 30 mls of 50% bleach/1% Tween solution to the tube, cap and mix gently for 15 minutes.

3. Note: the rest of the procedure is carried out in a laminar flow hood.
Pour the contents of the conical tube through the seed strainer held over the waste container.
Gently pour sterile water into the strainer to rinse off the bleach/tween solution.
Usually a liter of water is sufficient to rinse away excess bleach/tween.

4. Using forceps, transfer individual seeds from the strainer and place them on the surface of the media of a petri dish. Plate between 10-15 seeds per plate, evenly spaced apart from each other. Seal each plate with a strip of parafilm. Label each plate with the contents and date of plating. Place completed petri dishes in a plant growth chamber.

5. After approx. 7 days, germination should be complete. After 14 days from plating, scoring of the individual seedlings can be done. Kanamycin resistant seedlings will have developed good lateral root growth off of the main root system and the foliage will be green. Kanamycin sensitive seedlings will have a single diminutive tap root and the foliage will be small and in many instances purplish in color.

6. For Ro seedling experiments, compare the total # of Kan resistant seedlings to the total # of Kan sensitive seedlings. See if it fits the expected Chi-square.

7. For R1 seedling experiments, also compare the # Kan resistant to # Kan sensitive. Non-segregating homozygous lines will be 100% Kan resistant.

APPENDIX 4

Molecular Biology Methods Used in the Characterization of Line 35-1-N

Standard Operating Procedures

ARD-0000	Reagent List
ARD-0001	CTAB Method of Isolating DNA (Tomato)
ARD-0003	Restriction Digestion of Plant Genomic DNA
ARD-0004	Agarose Gel Electrophoresis for Southern Transfer
ARD-0005	Southern Transfer by Capillary Blotting
ARD-0006	Isotopic DNA Probe Labeling and Southern Blot Hybridization
ARD-0007	Southern Blot Washing & Autoradiography
ARD-0008	Protein Concentration Determination of Plant Lysates
ARD-0009	Ribonuclease Protection Assay
ARD-0010	Ethylene Analysis
ARD-0011	Preparation of Single Stranded RNA Probe
ARD-0013	Preparationof SAMase RNA
ARD-0014	Total RNA Preparation (Tomato)
ARD-0015	Double Stranded DNA Sequencing
ARD-0017	Western Blotting Procedure
(New England Biolabs)	Protein Fusion and Purification System

LIST OF REAGENTS

- **5M TRIS pH 8.0**

  302.75 g. TRIZMA / 500mls

  Start dissolving tris in approx. 250-300 mls of distilled water, then begin pH determination with 12M HCl (it may take up to 100-160 mls of HCl).

- **1M TRIS pH 8.0**

  60.55 g. TRIZMA / 500 mls

  Start dissolving tris in approx. 300 mls of distilled water, then begin pH determination with 12M HCl (it may take up to 50 mls of HCl).

- **5M NaCl**

  146.1 g. NaCl / 500 mls

- **0.5M EDTA pH 8.0**

  93.05 g. Na2EDTA / 500 mls

  Need to add 10N NaOH to bring to pH 8.0.

- **CTAB EXTRACTION BUFFER**

  Makes 500 mls of extraction buffer:

  10 g. CTAB (Hexadecyltrimethyl-ammonium bromide)
  140 mls of 5M NaCl
  1.0 ml of 2-mercaptoethanol
  20 mls of 0.5M EDTA
  50 mls of 1.0M TRIS

  Q.S. to 500 mls with distilled water.

- **7.5M NH4OAc**

289.05 g. / 500 mls

- **Tris-EDTA Solution**

  0.4 mls of 0.5M EDTA
  10 mls of 1M Tris pH 8.0

  Q.S. to 1000 mls with distilled water

- **10X Loading Buffer**

  0.1 g. Bromphenol blue
  0.1 g. Xylene cyanol
  10.0 g. Ficoll 400

  Q.S. to 40 mls with distilled water

- **1X TAE Electrophoresis Buffer**

  Make a 50X TAE Concentrate:

  242 g. Trizma
  57.1 ml Glacial Acetic Acid
  37.2 g. Na2EDTAx2H2O

  pH to approx. 8.5, Q.S. to one litre.

  Dilute 1:50 to make a working solution of 1X TAE buffer

- **Ethidium bromide Solution (10 mg/ml)**

  Dissolve 1 g. of EtBr into 100 mls of Distilled water

  Note: Ethidium bromide is a carcinogen, always wear gloves when handling!

- **Depurination Solution**

  Note: Add concentrated acid to water! Never add water directly to a concentrated acid or violent reactions may occur!

  Add 83.2 mls of Concentrated HCl to approx. 2000 mls of distilled water

  Q.S. to 4000 mls with distilled water

- **Denaturation Solution**

  80 g. of NaOH
  350 g. of NaCl

  Q.S. to 4000 mls with distilled water

- **20X SSC**

  701.2 g. NaCl
  352.8 g. Na3-Citrate

  Q.S. to 1000 mls with distilled water.

- **10 % SDS**

  10 g. Sodium doceyl sulfate

  Q.S. to 100 mls with distilled water.

- **0.1X SSC 0.1% SDS**

  5mls of 20X SSC
  10mls of 10% SDS

  Q.S. to 1000 mls with distilled water.

- **0.5N NaOH**

  20 g. of NaOH

  Q.S. to 1000 mls with distilled water.

CTAB METHOD OF ISOLATING DNA FROM TOMATO

I. PURPOSE

To isolate plant nucleic acid from plant material.

II. MATERIALS AND EQUIPMENT

1) Mortar and Pestle (per Sample)
2) Liquid Nitrogen
3) CTAB Extraction Buffer
4) Chloroform
5) Isopropanol
6) Tris-EDTA Solution
7) 70 % Ethanol
8) Water bath
9) Microfuge Tubes
10) Micropipettor and tips
11) 50 ml Polypropylene screw cap conical tubes
12) Trays
13) 10 ml Pipettes and Dispenser
14) Test tube rack
15) Table top centrifuge
16) Transfer Pipettes

III. PROCEDURE

A. Preheat a water bath to 60°C.
B. Collect 0.5 - 1.0 grams of fresh tissue from each sample to be extracted.
C. Grind each sample in a separate mortar with liquid Nitrogen. Label each mortar with the sample identification code.
D. Add 10 mls of **CTAB Extraction Buffer** to each mortar.
E. Place the mortars in a room temperature water bath to thaw the sample/buffer mixture.
F. Once thawed, pour sample/buffer mixture into an appropriately labeled 50 ml polypropylene centrifuge tube and place vertically in a rack in the 60°C. water bath. Let sample(s) incubate for 30 minutes. Invert tubes every 10 minutes.
G. After incubation, add 10 mls of chloroform to tube; cap, mix and vent tube. Spin tube(s) in the Beckman centrifuge for ten minutes at 1600 x g (*e.g.*, J2-21 with JS7.5 @ 3,000 rpm, or TJ6 @ 2500 rpm).
H. After centrifugation, remove top layer of liquid with a transfer pipette and place in a clean 50 ml centrifuge tube. Add 0.67 volumes of isopropanol to tube; cap and mix tube. Spin tube(s) in the Beckman centrifuge, (refer to Step G) for ten minutes.

I. After centrifugation, carefully decant supernatant from tube(s); the DNA should be visible as a whitish pellet at the bottom of the centrifuge tube.

J. Wash pellet with 70 % Ethanol to remove salts. Resuspend DNA pellet in 0.1-1.0 ml of **Tris-EDTA.**

K. Add 2 µl RNAse Plus (5 Prime -> 3 Prime) and incubate at 37°C for at least 15 minutes.

L. Add equal volume Phenol:$CHCl_3$ to sample, mix well and spin through a light phase lock gel.

M. Add 1/10 vol. NaOAc + 2.5 vol 100% EtOH. Precipitate 10 minutes, spin for 10 minutes at room temperature.

N. Decant, wash twice, dry and resuspend in 400 µl TE. Store at 4°C. (DNA can be reprecipitated with 0.5 volumes of **7.5M NH_3OAc** and 3.0 volumes of 100% Ethanol)

RESTRICTION DIGESTION OF PLANT GENOMIC DNA

I. PURPOSE

Restriction endonuclease cleavage of DNA into discrete fragments is one of the most basic procedures in molecular biology. Restriction endonucleases recognize short sequences of DNA called recognition sites and under favorable conditions will cleave double-stranded DNA within or adjacent to the recognition site. The DNA molecule is such a large and variable structure that when digested completely with a restriction endonuclease, it produces fragments of DNA of every size. When this assortment of fragments is electrophoresed through an agarose gel, the fragments migrate due to their negative charge and are distributed by molecular weight. The fragments appear as a homogenous smear under UV illumination.

II. MATERIALS AND EQUIPMENT

1) DNAase free water
2) Restriction endonuclease(s)
3) 10X buffer per manufacturer's instructions.
4) 10X loading buffer
5) Microcentrifuge tubes
6) Micropipettor and tips
7) Ultramicropipettor and tips
8) Microcentrifuge tube rack
9) 37°C. Water bath
10) Ice bath

III. PROCEDURE

A. A restriction digestion contains 4+1 components:
   - DNA
   - Water
   - Buffer
   - Enzyme

B. After digestion is complete add loading buffer.

C. The following recipe is used as an example of the restriction digestion:

10 µg DNA(1.3 mg/ml)	7.7 µl
H_2O	24.3 µl
10X buffer	4.0 µl
Enzyme (20 µ/µl)	4.0 µl*
Total volume	40.0 µl

Record specific reaction conditions. They should be maintained at 37°C for a minimum of 30 minutes.

After the digestion is complete, add:
10X loading buffer (1/9 vol.) 4.4 µl

Final volume loaded onto gel: 44.4 µl

* In this example the enzyme:DNA ratio is 8 units/ng DNA which is sufficient for complete digest in most situations. The volume of added enzyme should never exceed 10% of the total reaction volume. The 50% glycerol in the enzyme storage buffer may interfere with the reaction of the concentration is greater than 5% of the reaction mixture.

D. Calculate the amount of DNA and water for each sample:

Example: A_{260} = 1.333 10 µg/1.333 = 7.5 µl (amount of DNA)
Water = 24.1 - DNA 24.1 µl-7.5 µl = 16.6 µl (amount of water)

E. Do this for each sample.

F. Label a 0.6 ml microcentrifuge tube for each sample. Take 10X buffer out of -20°C. freezer to thaw at room temperature. Obtain restriction enzyme from freezer and place it in an ice bath. Add corresponding DNA and water to the tubes. Add 10X buffer and first dose of enzyme to tubes, cap tubes and place in a 37°C. water bath incubator for at least 1 hour.

G. After incubation, add 6.6 µl of **10X Loading Buffer** to each tube. Samples are now ready to load onto an agarose gel.

AGAROSE GEL ELECTROPHORESIS FOR SOUTHERN TRANSFER

I. PURPOSE

Agarose gel electrophoresis is a highly effective method for separating and identifying DNA fragments by molecular size. The protocol can be divided into three stages (1) a gel is prepared with an agarose concentration appropriate for the size of DNA fragments to be separated; (2) the DNA samples are loaded into the sample wells of the gel and run at a voltage for a time period that will achieve optimal separation; and (3) the gel is stained with Ethidium Bromide for direct visualization of DNA under UV light. DNA, being negatively charged, migrates towards the positive end of the gel electrophoresis chamber through the agarose gel. The pores inherent in the agarose gel act as a molecular sieve, allowing the smaller DNA fragments to migrate faster throughout the gel and the larger fragments migrate much slower. A logarithmic distribution of DNA fragments by molecular size is the eventual result.

II. MATERIALS AND EQUIPMENT

1) LE Agarose (FMC, Seakem LE)
2) 1X Trizma-Borate-EDTA Buffer (TBE)
3) IBI Large gel electrophoresis chamber
4) Power supply with electrode leads
5) Microwave
6) Gel Boiling Bottles (Pyrex)
7) IBI Large gel casting tray
8) IBI gel casting comb
9) Time Tape
10) Micropipettor and tips
11) UV Camera and illuminator
12) Ethidium bromide Solution
13) Trays
14) Gel transfer device

III. PROCEDURE

A. Preparation of samples:

Samples for agarose gel electrophoresis are prepared at the end of restriction digestion when 6X stop buffer is added to the reaction tubes and are subsequently stored in the refrigerator until needed. Samples are then organized by gel well location and a plan is drawn up to correspond to the samples being loaded. The plan

must contain a lane reserved for a negative control (untransformed plant). Optionally, size markers with discrete fragments of known size shall span and flank the entire range of DNA loci being tested.

B. Preparation of agarose gels:
1. Add 350 mls of **1X TBE Buffer** to a gel boiling bottle. Repeat process for each gel to be made.
2. Add 2.45 g. of LE Agarose to each gel boiling bottle with 1X TAE. Loosely fit cap onto bottle.
3. Microwaving each boiling bottle separately, bring the agarose-buffer solution to a boil to effectively incorporate the agarose into the buffer solution. Usually 5 minutes on the HIGH setting will suffice.
4. Remove boiling bottle from microwave with pot holders and allow to cool to ≈40-50°C.

C. Preparation of gel casting tray:
1. Using a clean and dry gel casting tray, attach strips of time tape along the open ends of each tray.
2. Place the gel casting comb into the casting tray slot. Place completed casting tray on a level undisturbed surface.

D. Pouring of the agarose gel:
1. Once the gel solution has sufficiently cooled to 42°C., it is ready to be poured into the gel casting tray. Pouring a gel that is too hot can warp and damage the gel casting tray.
2. Slowly pour the agarose solution into the gel casting tray, avoiding the generation of large bubbles. Once gel has been poured, it must be left undisturbed for 1-2 hours to gel sufficiently.
3. After the gel has set, carefully remove gel casting comb by pulling up on the comb slowly. Next, remove time tape from the gel ends. The gel is now ready for transfer to the gel electrophoresis apparatus.

E. Setting up the gel electrophoresis apparatus:
1. Level the IBI gel electrophoresis chamber and add 2500 mls of 1X TAE buffer to the interior of the chamber. Gently "burp" the chamber to remove trapped air pockets.
2. Place the gel tray into the gel electrophoresis chamber with the wells at the "black" or anodal end of the chamber.

F. Loading samples into the gel:
1. Add samples into wells according to plan drawn up earlier.
2. The volume of sample loaded into each well is typically 40 µl. Using a micropipettor, draw up the first sample. It is important to fill the well with the sample. Place the pipette tip containing the sample in the first designated well of the gel at a 60-90 degree

angle. Slowly dispense the sample into the well being careful to contain the sample contents only to the desired well. Repeat for each sample.

3. When all the samples are loaded, close the lid of the gel chamber. Do not disturb gel chambers once samples have been loaded.

G. Running the gel electrophoresis:

1. Attach electrode leads to gel chamber leads and the power supply. Attach black leads to black terminals and red leads to red terminals. Turn on power supply and set for 40 Volts overnight.

H. Terminating the gel electrophoresis:

1. Turn off the power supply and disconnect leads from respective terminals.
2. Carefully remove gel casting tray containing the gel from the gel chamber. Caution: gels are quite slippery and will slide completely off of gel casting tray without much effort; always carry gels level and with a hand for support on the open ends of the gel casting tray.
3. Gently slide gel off of gel casting tray into a adequately sized tray containing the used running buffer (TBE) with 0.5 µg/ml Ethidium bromide. Allow gel to remain for 15-30 minutes in this solution with gentle agitation.

I. Viewing, photographing and cutting the gel:

1. After the gel has been adequately stained, direct visualization of the DNA can be accomplished with UV illumination.
2. Carefully move gel from stain solution (wear gloves) onto gel transfer device. Transport gel to UV camera area.
3. Carefully slide gel off of gel transfer device onto the face of the UV camera/illuminator. Important: Always use appropriate eye protection when using UV camera.
4. Turn UV light source on.
5. Evaluate gel: At this point, one can evaluate individual samples for completeness of digestion with restriction endonucleases. A complete digestion will appear as a homogenous smear with uniform width throughout the gel lane. Partial digestions will appear top-heavy with most of the DNA concentrated at the higher molecular weights with little or no DNA in the lower nucleotide range. Samples which are digested with an excess amount of restriction enzyme have a good chance of complete digestion. However, some samples will not effectively digest completely due to impurities, etc., and must be repeated with another restriction digestion.

J. Certain sections of the gel will not be important in the overall analysis. Cutting these sections out of the gel allows for ease in transferring the gel

and less reagent usage. Determine the desired area of the gel for analysis and cut away the top and bottom parts of the desired gel area horizontally with a scalpel.

K. Photograph the gel under UV illumination.

L. Carefully slide the gel off the UV camera/illuminator onto the gel transfer device. Gel is now ready for the Southern transfer process.

SOUTHERN TRANSFER BY CAPILLARY BLOTTING

I. PURPOSE

DNA fragments that have been separated according to size by electrophoresis through an agarose gel are depurinated, denatured and transferred through an alkaline buffer onto a solid nylon membrane support by a technique described by Southern (1975). The relative positions of the DNA fragments in the agarose gel are preserved during their transfer to the nylon membrane. Once the DNA fragments are transferred onto the nylon membrane, they are immobilized by UV crosslinking in a Stratalinker.

II. MATERIALS AND EQUIPMENT

1) Depurination solution (O.25 N HCl)
2) Denaturation solution (0.5 N NaOH/0.6 M NaCl)
3) Neutralization buffer (10.05 M Tris pH 6.8/1M NaCl)
4) Distilled water
5) Filter paper
6) Paper towels
7) ONCOR nylon membrane
8) Trays
9) Glass plates
10) Stratalinker
11) Tweezers
12) Orbital shaker
13) Gel transfer device

III. PROCEDURE

A. Slide gel off of gel transfer device into a tray containing 400 ml. of **Depurination solution.** Place tray on orbital shaker set at '2'. Let rotate for 10 minutes. As an internal control the bromphenol blue should be completely yellow.

B. After depurination, drain solution and add 500 ml of distilled water to the tray. Briefly rinse gel and then drain water away.

C. Add 400 ml. of **Denaturation solution** into the tray, rotate for 15 minutes, drain.

D. Add 400 ml. of denaturation solution into the tray, rotate for 30 minutes, drain.

E. Repeat step D.

F. While the gel is denaturing, cut 1 sheet of S&S GB002 filter paper to match the size of the gel. Wearing gloves, also cut a piece of ONCOR membrane to match the size of the gel.

G. Using another gel transfer device, place the gel onto plastic wrap.

H. Wet the membrane in denaturation solution. Carefully place the membrane in contact with the gel. Watch for bubbles. Roll a pipette across the membrane gently to insure even contact between the gel and membrane and remove trapped air.

I. Place precut piece of dry filter paper on top of the gel-membrane complex.

J. Cover the filter paper with a stack of paper towels 1.5 inches thick. Cover the paper towels with a glass plate. Place a weight approximately 70 g on the glass plate.

K. Allow the transfer to proceed for at least three hours.

L. After the transfer is complete, crosslink the DNA to the membrane by UV irradiation in the Stratalinker as described:

1. Place one or two sheets of absorbent paper lightly dampened with transfer buffer on the floor of the Stratalinker UV crosslinker. Place the membrane (to obtain the best results the membrane should still be damp from the transfer) on top of the absorbent paper with the side with the attached nucleic acids facing upwards, enabling direct irradiation of the nucleic acids by the ultraviolet bulbs.
2. Close the door.
3. Press the Autocrosslink button. When the UV light bulbs turn on, the display will immediately begin to count down from the entered value (120,000 μjoules/cm^2, or 1200 on the LED display). If the door is opened at any point during the exposure, the irradiation will stop and the remaining exposure value will be displayed. (Close the door to continue irradiation.)
4. When the irradiation is complete, the beeper will sound for approximately 3-4 seconds. The autocrosslink setting should take approximately 25-50 seconds.
5. Remove the membrane and close the door. The irradiation is now complete. Clean the floor of the unit after each use to remove any residual salt.
6. Wash blot in 300 ml **Neutralization buffer** for 15 minutes at room temperature.
7. Put the membrane in a plastic bag and seal. Store at room temperature until ready to hybridize with DNA probes. (Storing in a sealed bag is not necessary if hybridization will occur the same day as the transfer.)

M. Place the flattened gel in a tray with distilled water and a few drops of Ethidium bromide for 15 minutes. (Precise measurement of Ethidium bromide is not necessary, but concentrations above 0.5 μg/ml are not required for quick staining and are more dangerous to dispose of.) After staining, photograph gel under UV illumination. Absence of visible DNA in the gel post-transfer represents a successful transfer of DNA from the agarose gel to the nylon membrane. Significant amounts of residual DNA remaining in the lanes on the post-transfer gel indicates a poor transfer and may result in absent or faint bands appearing on the final autoradiograph.

ISOTOPIC DNA PROBE LABELING AND SOUTHERN BLOT HYBRIDIZATION

I. PURPOSE

DNA probe(s) are labeled with a radioactive marker. The nylon membrane containing the DNA fragments is incubated with the probe in a special hybridization solution overnight. This solution will allow maximum sensitivity in specific binding of probe to complementary DNA sequences incorporated in the membrane. The method for labeling the DNA probe with radioactive markers is called "random-primed" DNA labeling, utilizing a kit supplied by Boehringer Mannheim Biochemica.

II. MATERIALS AND EQUIPMENT

1) Boehringer Mannheim random primed DNA labeling kit
2) [P^{32}]-dCTP (NEN-Dupont)
3) Ice bath
4) 37°C water bath
5) Micropipettor and tips
6) Ultramicropipettor and tips
7) Heating block
8) 1.5 ml screw-cap microcentrifuge tubes
9) Table top centrifuge
10) 5'-3' D50 select purification columns (TE version)
11) Hybridization oven and supplies
12) Hybrisol I (ONCOR)
13) Trays
14) TE (10 mM Tris-HCl, pH 8.0/1 mM EDTA)
15) PLUS appropriate materials for safe radioisotope handling:
16) Isotope monitoring supplies
17) Designated work area
18) Beta counter
19) Beta shield
20) Appropriate disposal containers
21) Gloves
22) Eye protection
23) Radiation dosimeter
24) Laboratory coat

III. PROCEDURE

Notes: DNA to be labeled must be linear, isotope should only be on first half-life.

A. Labeling
1. Add 25 ng of DNA plasmid and distilled water to a volume of 9 ul and place in an eppendorf tube with a tiny hole punched in the cap. Cap the tube and denature the DNA/water mixture by heating for 5 minutes in a boiling water bath and subsequent cooling in an ice bath.
2. After denaturation, give tube a quick spin in a centrifuge to remove condensation in tube. Add 3 µl of dNTP's (prepared by making a 1+1+1 mixture of dATP, dGTP and dTTP-from kit) to the tube. Add 2 µl of reaction mixture(from kit) to the tube.
3. Behind the beta shield, add 5 µl of isotope to the tube.
4. Obtain Klenow enzyme from kit in the freezer, place in ice bath and transport to the beta shield. Add 1 µl of enzyme to the tube.
5. Incubate tube for 30 minutes at 37°C.
6. Place sample on ice, add 30 µl of TE to stop the reaction.

B. Purification
1. Let a 5'-3' D50 select column warm to room temperature, invert the column several times to resuspend the gel inside the column. Remove the top cap first, then the bottom cap from the column, allow excess buffer to drain from column.
2. Place the column in the column collection tube with cap entirely removed. Spin the tube and column together for two minutes at 1000-1500 g. Discard tube and excess buffer. Keep column in upright position.
3. Place the column in a fresh collection tube and carefully add the 50 µl probe mixture to the top of the gel in the column. DO NOT apply the mixture to the sides of the column. Spin the column and tube for 4 minutes at 1000-1500 g.
4. The fluid contained in the tube is the purified probe. Measure the amount of counts in both the tube and the column to access level of radioactive incorporation into the probe. Discard column into radioactive waste container.

C. Hybridization
1. Prewarm Hybrisol I and hybridization tube to 45°C.
2. Prehybridize the nylon membrane (blot) by placing in a prewarmed hybridization tube, DNA side in with 5 mls of prewarmed Hybrisol I. Always use gloves when handling the membrane to avoid fingerprints on final autoradiograph.
3. Prepare probe for hybridization: Boil the probe for 5 minutes and quickly cool on ice. Place the denatured probe mixture in a fresh prewarmed 5 mls of Hybrisol I, mix and place in tube with the blot.
4. Place the entire assembly in a hybridization oven overnight at 45°C.

SOUTHERN BLOT WASHING & AUTORADIOGRAPHY

I. PURPOSE

Autoradiography is used to visualize and quantitate on X-ray film radioactive molecules hybridized to nylon membranes. The isotope P^{32} emits beta particles which activate silver bromide crystals on a film emulsion to the silver metal form. This form is visualized during the photographic development process.

II. MATERIALS AND EQUIPMENT

1) Wash Solution (0.1X SSC 0.1%SDS)
2) Orbital Shaker
3) Orbital Shaking Waterbath
4) Tweezers
5) Darkroom
6) Darkroom chemicals and supplies
7) Film cassette holder
8) Clamps
9) Saran wrap
10) -80°C. freezer
11) Gel transfer device
12) 0.4 N NaOH
13) 2X SSC/0.1% SDS.

III. PROCEDURE

A. Stringency washes: After overnight hybridization, the membrane is given a series of washes at high stringency to remove nonspecific binding of probe to membrane but allows specific probe/DNA sequence interactions to remain intact.

B. Take hybridization tube out of hybridization oven, pour off excess hybridization fluid in designated radioactive sink. Add approx. 250 mls of **Wash Solution** to the tube. The membrane can be moved to an alternative container at this point. Place container on an orbital shaker set at '2'. Rotate for 15 minutes. Pour off old **Wash Solution** and replace with new. Wash in this manner for a total of 3 times.

C. Add 250 ml **Wash Solution** to the container containing the membrane.

D. Place the container in a 52°C water bath to wash for 1 hour.

E. Prepare a piece of saran wrap twice the size of the membrane. Lay the saran wrap out on a clean flat surface. Remove wrinkles.

F. When the washes are completed, remove the membrane from the bag and place it on the saran wrap. Fold the remaining saran wrap over to cover both sides of the membrane. Place in a film cassette holder and go to the darkroom.

G. In the darkroom, in the dark, place a sheet of unexposed X-ray film and an intensifying screen in the film cassette holder with the membrane in this order: intensifying screen, film, top of blot facing film. Close film cassette holder and sandwich between two gel transfer devices. Seal with clamps and place in the -80°C. freezer overnight.

H. The next day, thaw out film holder and develop the X-ray film according to darkroom chemical specifications. Dry film and analyze.

I. When all data has been analyzed, the membrane can be stripped of the radioactive probe for storage or for reprobing with another probe.

J. Stripping: Place blot in a tray with approx. 400 mls of **0.4 N NaOH** and rotate in the orbital shaking waterbath at 42°C for 30 minutes.

K. Pour solution out and add approximately 400 mls of **0.1X SSC 0.1% SDS** to the tray, rotate at 65°C for 30 minutes. Repeat two more times.

L. Remove last wash solution, reseal in plastic bag and expose to film to ensure removal of hybridized counts. If there are still counts repeat the stripping process again and do a final Beta/Geiger counter check after that.

M. Store the cleaned membranes in a sealed bag with 20 ml **2X SSC/0.1% SDS.**

PROTEIN CONCENTRATION DETERMINATION OF PLANT LYSATES

I. PURPOSE

To prepare plant protein lysates and determine their soluble protein concentrations.

II. MATERIALS AND EQUIPMENT REQUIRED

1) Mortar and pestle
2) Liquid nitrogen
3) 1X Sample buffer: 0.05 M Tris HCl pH 6.8
   1% SDS
   5% beta mercaptoethanol(BME)
   10% glycerol
4) Microcentrifuge tubes - 1.5 ml and 2.0 ml
5) Microcentrifuge
6) Heating Block
7) 5X Sample buffer: 0.25M Tris HCl pH6.8
   5% SDS
   25% BME
   50 % glycerol
8) Pierce's Albumin Standard: 2 mg/ml bovine serum albumin (BSA) in 0.9% NaCl and 0.05% NaN3, Product #23209
9) Deionized distilled water (ddH20)
10) 0.15%(w/v) sodium deoxycholate solution
11) 72% trichloroacetic acid (TCA) solution
12) Solubilizing solution : 5% SDS(w/v) in 0.1 N NaOH
13) Pierce's BCA Protein Assay Reagent , Product # 23225
14) 12 x 75 mm test tubes
15) Disposable polystyrene cuvettes
16) Beckman DU-64 Spectrophotometer or equivalent

III. PROCEDURE

A. Sample Lysate Preparation
   1. Weigh out approximately 0.5 grams of each tissue to be tested.
   2. Using a mortar and pestle and liquid nitrogen, grind the tissue to a fine powder.
   3. Place ground tissue in a 2.0 ml microfuge tube.
   4. Add 400ul of sample buffer and vortex.

The volume of sample buffer added to the ground tissue sample was calculated using the following formula:

Micrograms of tissue weighed X 0.8 = uls of sample buffer added
This ratio of plant tissue to sample buffer produces protein concentrations ranging from approximately 1000 -1500ug/ml.

5. Heat for 5 mins. at 95-100°C.
6. Centrifuge for 10-15 minutes at 13K rpm, 4°C.
7. Remove supernant from debris pellet and place in new centrifuge tube. Discard pellet. Divide supernants into three or four aliquots and store at -20°C.

B. BSA Protein Standards Preparation

1. Prepare BSA protein standards at 200-1600 ug/ml as outlined in the table below:

BSA Std. Conc.	2 mg/ml BSA	5X Sample Buffer	dd H20
200 ug/ml	50 ul	100 ul	350 ul
400 ug/ml	100 ul	100 ul	300 ul
600 ug/ml	150 ul	100 ul	250 ul
800 ug/ml	200 ul	100 ul	200 ul
1000 ug/ml	250 ul	100 ul	150 ul
1200 ug/ml	300 ul	100 ul	100 ul
1400 ug/ml	350 ul	100 ul	50 ul
1600 ug/ml	400 ul	100 ul	0 ul

C. TCA Precipitation of Protein

1. Pipet 100 uls of each BSA protein standard or sample lysate into a 1.5 ml microfuge tube. For blank use 100 uls of 1X sample buffer. Set up three tubes of each BSA standard and blank and two tubes of each sample lysate.
2. TCA precipitate the proteins by adding the following reagents to each tube, in the given order:
   a. 400 uls ddH20
   b. 100 uls 0.15 % deoxycholate soln.
   c. 100 uls 72% TCA soln.
3. Allow the tubes to stand at room temperature for 15 minutes.
4. Vortex , then centrifuge reactions for 10 minutes at 13K rpm
5. Pour off the supernatant from each protein pellet and add 100 uls of ddH20. Break up pellet by vortexing or pipeting up and down with a pipetman.
6. Add 100 uls 0.15% deoxycholate soln. then 100uls 72% TCA soln. to each tube.
7. Repeat steps 3 and 4.
8. Pour off the supernatant. Add 100 uls solubilizing soln. to each protein pellets and vortex to resuspend.

D. BCA Protein Assay

1. Pipet 50 uls of each protein ppt. soln. into a test tube.
2. Prepare BCA working reagent by mixing 50 parts of Reagent A with 1 part of Reagent B.
3. Add 1.0 ml of the BCA working reagent to each test tube and mix well.
4. Incubate all tubes at 37C for 30 minutes.
5. After incubation, cool tubes to room temperature.
6. Measure the absorbance at 562 nm of each tube versus water reference.

6. Subtract the absorbance of the blank from the value found for the standards or unknowns.
7. Prepare a standard curve by plotting the net (blank corrected) absorbance at 562 nm versus BSA std.protein concentration. Using this standard curve, determine the protein concentration for each unknown lysate sample.

RIBONUCLEASE PROTECTION ASSAY

I. PURPOSE

This assay is used to detect the existence of SAMase RNA in plants.

II. MATERIALS AND EQUIPMENT REQUIRED

1) RPA II™ Kit [Ambion #1410].
2) Anti-sense SAMase RNA probe.
3) Ubiquitin RNA probe.
4) Plant RNA.
5) Total RNA sample.
6) 30% Acrylamide [Boehringer 100670]
7) 2% Bis-acrylamide [Boehringer 100675]
8) 10% Ammonium persulfate.
9) TEMED [Bio-Red #161-0800].
10) Urea [Stratagene #300191].
11) 100% EtOH.
12) 10xTBE buffer: 890 mM Tris base, 890 mM Boric Acid, 20 mM EDTA.
13) Heating blocks.
14) Microcentrifuge [Brinkmann 5415].
15) Electrophoresis apparatus [Bio-Red Mini Protean II].
16) Micropipets capable of accurately delivering 1-20 µL, 10-100 µL, 100-1000 µL.
17) KODAK film.
18) Scintillation counter [Beckman LS 6000SC.
19) Scintillation fluid [Beckman #158735].

III. PROCEDURE

A. Hybridization of Probe and Sample RNA:

1. For each 1.7 ml tube, mix $2x10^4$ cpm probe with sample RNA (25 ug-50 ug).
2. Set up 2 yeast RNA control tubes for the probe to be used by mixing 2 ul yeast RNA with the same amount of probe used above.
3. Set up a standard control tube by mixing 100 pg SAMase RNA with probe.
4. Adjust the concentration of NH_4OAc to 0.5 M and add 2.5 volumes of EtOH to precipitate samples in -20°C freezer for 30 minutes.
5. Spin tubes in microcentrifuge for 15 minutes at 4°C. Remove

supernatant from each tube.

6. Resuspend each pellet in 20 ul Hybridization Solution A. Heat all tubes at 90°C for 3-4 minutes.
7. Incubate tubes in 42°-45°C heating block overnight.

B. RNase Digestion of Hybridized RNAs:
1. Prepare RNase buffer by 1:100 dilution of solution R with solution Bx.
2. Add 200 µl of RNase buffer to all tubes, except one yeast RNA control tube is added with 200 µl of solution Bx.
3. Incubate at 37°C for 30 minutes.
4. Add 300 µl solution Dx to each tube, mix well. Precipitate RNA in -20°C freezer for 1 hour.
5. Spin all tubes for 15 minutes at 4°C. Remove all supernatant from each tube. Dissolve pellet in 8 µl loading buffer and heat tubes for 3 minutes at 90°±5°C.

C. Preparation of 5% Polyacrylamide/8 M Urea Gel:
1. Add followings into a 50 ml tube:
3.6 g of Urea
0.75 ml of 10x TBE
1.23 ml of 30% Acrylamide
0.25 ml of 2% Bis-acrylamide
1.67 ml of water.
Heat in 37°C water bath until all dissolved
2. Add 60 µl of 10% APS, 8 µl of TEMED, mix well. Pour a 0.75 mm gel in a Bio-Rad vertical electrophresis apparatus. Allow it sit at room temperature for 30 minutes to polymerized.

D. Separation and Detection of Protected Fragments:
1. Load reactions into gel, run gel with 1x TBE buffer at 200 volts until all the dyes just come off the bottom of the gel.
2. Transfer gel to a blot paper, cover by Saran Wrap, dry it in Bio-Rad gel drier for 2 hours.
3. Change the Saran Wrap, then expose gel to KODAK film for 2 hours and 16 hours.
4. Develop film.

E. Calculations:
1. Locate SAMase band in the gel, cut out the band and transfer to a vial.
2. Add 5 ml scintilation fluid, count in Beckman scintillation counter.

ETHYLENE ANALYSIS

I. PURPOSE

To measure plant ethylene production level by gas chromatograph.

II. MATERIALS AND EQUIPMENT REQUIRED

1) Gas chromatograph FID [HP 5890 series II].
2) Mason jars with lids.
3) Rubber stoppers.
4) Air tight 2 ml syringe: Pressure-lok® [Dynatech #050033].
5) Side-port needles [Alltech #943052].
6) 10.8 ppm ethylene in nitrogen [Aldrich #32,128-1].
7) 125 ml gas Sampling bulbs [Alltech #6940].
8) 390 ml vacuum bottles [Alltech #8090].
9) 20 ml Vacutainer [Becton Dickinson #6433]

III. PROCEDURE

A. Turn on the computer in the equipment room near scintillation counter, open Windows 3.1 and doublle-click the GC-FID icon to run FID software per FID manual instructions.

B. Turn on control valves of air and hydrogen gas tank. Press the ignition button to start the FID.

C. Prepare settings on chromatograph (see manufacturer's instructions).

D. Prepare ethylene standards :

1. Flush sampling bulbs and vacuum bottles with air. Close all of openings.
2. Prepare standards as follows:
   -Dilute 2 ml of 10.8 ppm ethylene into a 390 ml bottle to reach final concentration of 0.0553 ppm.
   -Dilute 4 ml of 10.8 ppm ethylene into a 390ml bottle to reach final concentration of 0.1108 ppm.
   -Dilute 4 ml of 10.8 ppm ethylene into a 125 ml sampling bulb to reach final concentration of 0.3456 ppm.
   -Dilute 2 ml of 10.8 ppm ethylene into a 20 ml Vacutainer tube to reach final concentration of 1.08 ppm.

-Dilute 4 ml of 10.8 ppm ethylene into a 20 ml Vacutainer tube to reach final concentration of 2.16 ppm.
-Flush a 125 ml sampling bulb with 10.8 ppm ethylene.

E. Program the analysis methods as follows:
   1. Injector information :
      Manual injection, dual injectors.
   2. Zone temperature:
      Set both injector temperatures at 200°C, both detectors at 250 °C.
   3. Oven parameters:
      Set oven equilibration time for 0.5 minutes, oven maximum temperature at 190 °C; oven initial and final temperature at 60°C, and initial time for 2.2 minutes.
   4. Signal information:
      Set detector A and B as the sources for signal 1 and 2, respectively. Set both peak width for 0.020 minute, data rate as 20 Hz. Start saving data at 0 minute and stop at 2.2 minutes.
   5. Valves/relays information
      Set initial setpoints for valve 1 and 2 off, valve 3 and 4 on.
   6. Detector information:
      Set both FIDs on.
   7. Signal plot information:
      Set attenuation at -1, offset 10%; set time as 1 minute.
   8. Integration events:
      Set initial area reject value as 1, initial peak width as 0.04, shoulder detection off, and initial threshold as -3. Set integrator off at 2.2 minutes.
   9. Report specification:
      Reports are based on area of peaks. Use external standard for calculations. Send report to screen.
   10. Calibration settings:
      Set both reference and non-reference window for 0.5 minute. Units of amount is nl/ml and multiplier is 1.
   11. Multilevel information:
      Choose linear curve fit and ignore the origin in the calculation.

F. Set up standard curves for both detectors:
   Inject 2 ml of a standard into each GC-FID injector and start analysis program. Set retention time at 1.9 minutes which is the time ethylene peak starts. Enter the amount of the ethylene standard at the end of each run. Repeat twice with same standard to ensure accuracy. Plot the curve and check the r^2 value. The r^2 value should be .98 or better.

G. Ethylene analysis:
   1. Place sample in jar, close lid and tighten the rubber stopper. Let sit at room temperature for 30-60 minutes or until ethylene amount falls within a detectable range. Record the close time.
   2. Use air tight syringe to withdraw a 2 ml gas sample from Mason jar

through the rubber stopper.

3. Inject sample into GC-FID and start the analysis program.
4. Record the sampling time and the amount of ethylene from the computer screen.
5. Repeat steps 1 through 4 for all samples.
6. Clean columns at the end of each day by setting the oven temperature just below the maximum temperature, approximately 175°C. Finish the cleaning when the signal is parallel with baseline and no peak is present.

H. Data analysis:

Record the sample name and weight in an Excel spread sheet. Enter the close time and the measure time, as well as the amount of ethylene into Excel. Also record the volume of the mason jar. Calculate the nanoliters of ethylene produced per hour per gram of tissue, minus the weight of the tomato.

PREPARATION OF SINGLE STRANDED RNA PROBE

I. PURPOSE

To prepare high specific activity single-stranded RNA probes used in ribonuclease protection assay.

II. BACKGROUND

In vitro transcription reaction and radioactive nucleotides are used to generate RNA probe.

III. MATERIALS AND EQUIPMENT REQUIRED

1) pGEM® Express System II [Promega P2490].
2) Heating block.
3) CsCl banded DNA template pGEM·7Zf(+)SAM·K. pAG #924.
4) Temed [Bio-Rad #161-0800].
5) RQ1 RNase-free DNase [Promega #M6101].
6) a-^{32}P UTP [NEN #NEG-007H].
7) Micropipets capable of accurately delivering 1-20 µl, 10-100 µl, 100-1000 µl.
8) Beckman Scintillation counter.
9) Scintillation fluid [Beckman #158735].
10) RNAse-free G-50 spin column (STE SELECT D(RF) G-50 spin column, 5 Prime 3 Prime)

IV. PROCEDURES

A. Preparation of DNA template:
   1. Digest pGEM·7Zf(+)SAM·K with NcoI, then extract DNA with phenol/chloroform once, chloroform/isomyl alcohol once.
   2. Adjust NaOAc concentration to 0.3 M, add 2.5 volumns of EtOH to precipitate DNA at -20°C.
   3. Spin for 10 minutes. Wash with 75% ethanol, dry the pellet and dissolve in TE to a final concentration at 1 µg/µl.

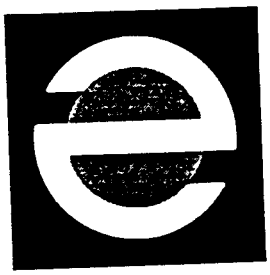
B. In vitro transcription reaction: [Promega kit P-2490 or equivalent]
   1. Add following components into 1.7 ml tube:
      4 µl of 5x transcription buffer

2 µl of 100 mM DTT
0.5 µl of RNasin ribonuclease inhibitor (40u/µl)
4 µl of ATP, GTP, and CTP (2.5 mM each)
2.4 µl of 100 µM UTP
1 µl of linearized template DNA (1 µg/µl)
5 µl of [a-^{32}P]UTP
1 µl of T7 RNA polymerase (15 u/µl)

2. Incubate for 60 minutes at 37-40¤C.

C. Removal of DNA template:
Add RQ1 RNase-free DNase to a concentration of 1u/µg of template DNA. Incubate at 37°C for 15 minutes.

D. Purification of RNA probe:
1. Invert G-50 spin column several times to fully resuspend the gel. Remove the top closure first, then the bottom closure and allow the buffer to drain from the column.
2. Place the column in one of the collection tubes provided with the column and centrifuge at 1100*g for two minutes with a swinging bucket or horizontal rotor.
3. Discard the collection tube and collected buffer.
4. Place the column in a second collection tube, apply reaction directly to the center of the gel bed, and centrifuge at 1100*g for four minutes.
5. The RNA probe will be recovered in the collection tube. Remove one µl of probe and place in a scintillation vial. Add five ml of scintillation cocktail and determine the radioactivity by scintillation counter, per mfgr. instructions.
6. Store the probe at -20¤C.



AGRITOPE, INC.
8505 SW CREEKSIDE PL.
BEAVERTON, OREGON 97008

(503) 641-6115
(503) 643-2781 FAX

General:

USDA Notification Number:	**94-143-09-N**
Cooperator Name:	**George Clough Ph.D.**
Field Trial Location (State, County):	**Oregon, Umatilla**
Type of Trial :	**Efficacy/Seed production**
Agritope Field Representative:	**Matthew Kramer**

Seed Storage Information:

Date seed received:	April 28,1994
Seed received by (give name):	G. Clough
Seed lot number:	see entry list
Amount received:	150 seed
Amount remaining after planting:	0
Disposition of seed remaining:	n/a

Give location of the secure locked seed storage facility:

Horticultural lab, Hermiston Agricultural Research & extension Center, Oregon State University, Hermiston OR

Planting Information:

Date planted:

Greenhouse: April 28, 1994

Field: June 2, 1994

Number of transgenic seeds planted:	150
Number of non-transgenic seeds planted:	25
Date of seedling emergence:	n/a (greenhouse)
Estimated percent of germination:	85%
Width in feet of tomato-free buffer:	5,280 feet

Containment Information:

Did any seeds or other plant material inadvertently escape from the trial site?

NO

If yes, explain:

_____

_____

Equipment Cleaning:

1. **During flowering--Give date(s) that field equipment was examined before leaving the trial site with all flowers removed from the machinery:**

   No equipment entered field

2. **During harvest (if applicable)--Give date(s) that field equipment was examined before leaving the trial site with all plant material, fruit and seeds removed from the machinery.**

   No equipment entered field

Plant Observations:

1. **Date of first observed open flower:**

   June 2, 1994

2. **Presence of crown gall disease symptoms on any of the transgenic or non-transgenic tomato plants? If yes, explain:**

   NONE

3. **Any of the transgenic tomato plants have abnormal appearance? If yes, explain:**

   NONE

Herbicide Treatments:

1. **What herbicides were applied as your standard regime?**

a. **Pre-plant incorporated, please describe:**

   Vapam @ 60 gal./acre, roto tilled April 5, 1994

b. **Pre-emergence, please describe:**

None

c. **Post-directed, please describe:**

None

2. **Any additional treatments, please list:**

None

Trial Completion:

1. **Date harvested (if applicable):** October 17, 1994

2. **Date trial destroyed. Describe how trial was destroyed:**

October 22, 1994, flail-chopped plants, disced field 2X

Site Monitoring:

1. **What crop was planted after the field trial was destroyed?**

Wheat, Barley

2. **Were there any volunteer tomato plants after trial was destroyed? If yes, explain and state when and how they were destroyed:**

NO

Chronological Log:

Date	Plant Stage	Activities/Comments
4/5/94	pre-plant	fumigated w/ Vapam @ 60gal/acre
4/9/94	pre-plant	broadcast fertilizer
4/17/94	pre-plant	sub-soil roto-tilled
4/28/94	Seed	ship to HAREC, plant to greenhouse
5/15/94	pre-plant	roto-tilled, lay drip tape, apply mulch
6/2/94	Seedling	transplant to field
8/15/94	Fruit set	estimate maturity for seed harvest, evaluate ripening phenotype.
10/17/94	Mature fruit	harvest each genotype for seed production.
10/22/94	Post harvest	destroy trial
10/23/95	Seed	final seed shipped to Agritope, Inc.
4/12/95	Monitoring visit	walk field to detect presence of volunteer tomato plants-none detected
5/15/95	Monitoring visit	walk field to detect presence of volunteer tomato plants-none detected
6/5/95	Monitoring visit	walk field to detect presence of volunteer tomato plants-none detected
8/9/95	Monitoring visit	walk field to detect presence of volunteer tomato plants-none detected

Other Observations:

None

Conclusions:

This trial was completed with no observed symptoms of *Agrobacterium* infection or loss of containment. The objectives of the trial were met in that the various ripening phenotypes of the different transgenic lines were evaluated and seed was produced from the selected materials. Monitoring of the site was completed during the spring and summer of the following growing season with no volunteer plants detected.

APPENDIX 9

Chromatographs of Tomatine Measurements in Large Red Cherry Control and Transgenic pAG 5420 Transformants

FIELD TRIAL ENTRY LIST

HERMISTON AGRICULTURAL RESEARCH AND EXPERIMENT CENTER
SUMMER, 1994

GENOTYPE:

TORC 5420 35-1
TORC 5420 22-1
TORC 5420 40A-1
LARGE RED CHERRY CONTROL

PREPARATION OF SAMase RNA

I. PURPOSE

To produce SAMase RNA used as standard in Ribonuclease Protection Assay.

II. BACKGROUND

pGEM® Express System II [Promega P2490] is used to synthesize SAMase RNA by In vitro transcription reaction.

III. MATERIALS AND EQUIPMENT REQUIRED

1) pGEM® Express System II [Promega P2490].
2) Heating block.
3) CsCl banded DNA template pGEM·5Zf(+)SAM·K (pAG 923).
4) RQ1 RNase-free DNase [Promega #M6101].
5) Micropipets capable of accurately delivering 1-20 µL, 10-100 µL, 100-1000 µL.
6) Phenol:chloroform [Amresco #0883]
7) 7.5 M NH_4OAc.
8) 3 M NaOAc.
9) 100% EtOH.
10) Chloroform:Isoamyl alcohol [Amresco #X205].
11) Beckman spectophotometer.

IV. PROCEDURES

A. Preparation of DNA template:
   1. Digest pGEM·5Zf(+)SAM·K (pAG 923) with Asp1718, then extract DNA with phenol/chloroform once, chloroform/isomyl alcohol once.
   2. Adjust NaOAc concentration to 0.3 M, add 2.5 volumns of EtOH to precipitate DNA at -20°C.
   3. Spin for 10 minutes, dry the pellet, dissolve in TE to a final concentration at 1 µg/µl.

B. In vitro transcription reaction:
   1. Add following components into 1.7 ml tube:
      40 µl of 5x transcription buffer
      20 µl of 100 mM DTT
      5 µl of RNasin ribonuclease inhibitor (40µ/µl)

40 µl of ATP, GTP, UTP and CTP (2.5 mM each)
4 µl of linearized template DNA (1 µg/µl)
4 µl of T7 RNA polymerase (15 µ/µl)
87 µl of H_2O

2. Incubate for 2 hours at 37-40 degree.

C. Removal of DNA template:
Add RQ1 RNase-free DNase to a concentration of 1u/µg of template DNA. Incubate at 37°C for 15 minutes.

D. Purification of RNA:
1. Extract RNA with phenol:chloroform once, then with chloroform/ isoamyl alcohol once.
3. Add 0.5 volumn of 7.5 M NH_4OAc and 2.5 volumn of 100% EtOH to precipitate RNA at -20°C.
4. Spin for 15 minutes, dry pellet, dissolve in H_2O.

E. Determine RNA concentration:
Make 1:100 dilution of RNA, and determine the concentration on the UV spectophotometer.

TOTAL RNA PREPARATION (TOMATO)

I. PURPOSE

A modified RNA miniprep is used to extract total RNA from tomato plant tissues. this procedure may be applicable to other plant species.

II. MATERIALS AND EQUIPMENT REQUIRED

1) Plant leaf tissue, or fruit tissue.
2) Saturated Phenol, Biotechnology grade.[Amresco #0945].
3) Extraction Buffer: 100 mM LiCl, 100 mM Tris pH 8.0, 10 mM EDTA pH 8.0, 1% SDS.
4) Liquid Nitrogen.
5) Chloroform [Aldrich #31,998-8].
6) 4 M LiCl.
7) 30 M NaOAc (pH 5.2).
8) 100% absolute ethanol.
9) Phase Lock Gel™ I Heavy [5'–>3' #1183182].
10) Mortar and pestle.
11) Heating block set at 80°C.
12) Microcentrifuge Tomy MC-150 or Eppendorf 5510.
13) Micropipets capable of accurately delivering 1-20 µL, 10-100 µL, 100-1000 µL.
14) Beckman Spectrophotometer.

III. PROCEDURE

A. Mix 1:1 phenol and extraction buffer, heat in the 80°C heating block.
B. Pour liquid nitrogen into mortar, grind about 1 g plant tissue with liquid nitrogen until it becomes a powder. Transfer the powder into a 1.7 ml microfuge tube.
C. Vortex 1:1 mixture of phenol and extraction buffer and add 500 µL to the microfuge tube. Vortex 1 minute until all tissue is thawed.
D. Add 250 µL chloroform, vortex 30 seconds.
E. Transfer contents into a phase lock gel tube, spin 5 minutes in a micro-centrifuge at 4°C.
F. Transfer the top aqueous phase into a 1.7 ml tube. Add an equal volume of 4M LiCl and vortex. Precipitate at least 2 hours at -20°C.
G. Spin 10 minutes in a microcentrifuge, remove all of the supernatant, air dry the pellet, dissolve in 50 µL H_2O.

H. Determine concentration of RNA sample by Beckman Spectrophotometer, according to SOP ARD-0002.

I. RNA preparations are stored precipitated at -20°C. Add 1/10 volume 3.0M NaOAc then 2.5 volumes 100% ethanol. Vortex and place in -20°C freezer for storage.

DOUBLE STRANDED DNA SEQUENCING

I. PURPOSE

To elucidate DNA sequence information.

II. MATERIALS AND EQUIPMENT REQUIRED

1) Double stranded plasmid DNA as template.
2) Single stranded oligomer (10-30 bp), 0.5 pm/µl as a primer.
3) USB Sequenase® Version 2.0 DNA Sequencing Kit consisting of:
   - 5X Sequenase Reaction Buffer
   - Control DNA M13mp18
   - Primer (-40), 17-mer
   - 0.1 M, DTT
   - 5X labeling Mix (dGTP)
   - 5X labeling Mix (dITP)
   - ddGTP Termination mix (dGTP)
   - ddATP Termination mix (dGTP)
   - ddTTP Termination mix (dGTP)
   - ddCTP Termination mix (dGTP)
   - Sequence Extending Mix (dGTP)
   - ddGTP Termination mix (dITP)
   - ddATP Termination mix (dITP)
   - ddTTP Termination mix (dITP)
   - ddCTP Termination mix (dITP)
   - Sequence Extending Mix (dITP)
   - Sequenase® Version 2.0 T7 DNA polymerase, 13 U/µl
   - Pyrophosphatase, 4 U/µl
   - Enzyme dilution buffer
   - Mn Buffer (dGTP)
   - Glycerol dilution buffer
   - Stop Solution
   - Protocol booklet
4) [35S]dATP, 1000-1500 Ci/mmol.
5) 75% Ethanol.
6) 100% Ethanol.
7) 70% Ethanol.
8) Distilled, deionized H_2O.
9) 7.5 M Ammonium Acetate (AmOAc).
10) 3 M Sodium Hydroxide (NaOH).
11) 10X TBE.
12) Long Ranger® (AT Biochem).
13) 10% Ammonium Persulfate (APS), made on the day of use.
14) TEMED.
15) Neutral pH detergent (Beckman).
16) Sodium bicarbonate ($NaHCO_3$).

17) Urea
18) Siliconizing agent (Gel Slick, AT Biochem, or equiv.)
19) Constant temperature bath or heating block for 37°C, 65°C and 75°C.
20) 3000 volt power supply.
21) Microfuge.
22) BRL model SA sequencing apparatus or equivalent and accessories:
    - Pair of glass plates
    - 0.4 mm spacers
    - 0.4 shark's tooth comb, 24 or 48 well
    - Porous bottom spacer (AT Biochem)
    - 1" binder clamps
    - Gel temperature sensing strip
23) Large format autoradiography film and film holder.
24) Filter paper (Schleicher & Schuell GB002 or equiv.)
25) BioRad® model 583 gel dryer or equivalent.

III. PROCEDURE

A. Denaturing double stranded DNA

1. These volumes can be proportionately increased for multiple reactions:

DNA	x (x contains 3-5 µg double stranded DNA/reaction)
H2O	9-x
3 M NaOH	1
	10

2. Incubate for 5 minutes at room temperature.
3. Add 2.7 µl 7.5 M AmmOAc + 50.8 µl EtOH.
4. Incubate on ice for 10 minutes.
5. Microfuge 15 minutes, decant.
6. Wash with 75% EtOH, dry.
7. Resuspend template DNA in 7 µl di H20.

B. Annealing of primer to template DNA

1. Combine:

Primer	1 µl
Seq. buffer	2 µl
Template DNA	7 µl
	10 µl Total

2. Heat to 65°C for 2 min.
3. Allow to cool to 35°C slowly (20-30 minutes).

C. Labeling of template DNA

1. Choose a dilution to use based on desired results:

Normal	will be able to read ~ 50 bp from primer
Intermediate	will be able to read ~ 25 bp from primer
Close	will be able to read ~ 5 bp from primer

2. Dilute as described below for normal, intermediate or close:
   a. Dilute labeling mix (dGTP) 1:5 (1 µl + 4 µl H2O) - normal.
   b. Dilute labeling mix (dGTP) 1:7 (1 µl + 6 µl H2O) - intermediate.
   c. Dilute labeling mix (dGTP) 1:10 ((1 µl + 10 µl H2O) - close.

3. Dilute Sequenase® Version 2.0 1 µl + 7 µl cold enzyme dilution buffer. Use within 60 minutes of diluting the enzyme.
4. Combine:

Template-Primer	10 µl
Diluted labeling mix	2 µl
0.1 M DTT	1 µl
35S-dATP	0.5 µl
Diluted Sequenase® Version 2.0	2 µl
	15.5 µl Total

5. Mix thoroughly, **spin briefly** in a microfuge to force all solution to the bottom of the tube. Total incubation time 3-4 minutes at room temperature. (Labeling temperature may be modified according to the USB protocol booklet.)

D. Termination of sequence reaction
1. Put 2.5 µl of each ddNTP (ddGTP, ddATP, ddCTP, ddTTP termination mixes for dGTP) into a separate tube; one set for each sequencing reaction.
2. Pre-warm to 37°C. (Termination temperature may be modified according to the USB protocol booklet.)
3. When labeling reaction is complete transfer 3.5 µl of the labeling reaction to each ddNTP tube. Mix thoroughly by pipeting up and down 3-5 times.
4. Return to 37°C, incubate for ≥ 5 minutes. (Termination temperature may be modified according to the USB protocol booklet.)
5. Add 4 µl of stop solution to each reaction.
6. Mix and place on ice to cool; store at -20°C.

E. Sequencing gel
1. Prepare gel
   a. Clean plates prior to pouring the gel. Detergent, dust, fingerprints, grease, etc. if present on the plates can interfere with pouring a sequencing gel. Be sure one of the plates is water-repellent with a siliconizing agent. If not, follow the directions from a siliconizing agent to make one and only one of the plates water repellent.
   b. Use a neutral pH detergent to clean. If an abrasive is needed try sodium bicarbonate.
   c. Rinse many times with deionized water.
   d. Use 70% ethanol to rinse the plates. Dry thoroughly with a paper towel.
   e. Assemble the glass plates with side and bottom spacers in place. Clamp with 1" binder clamps.
   f. Make a gel solution.
      1. Combine the following with di H_2O to < 60 ml:

Urea	25.2 g
10X TBE	7.2 ml
Long Ranger	6 ml

2. Mix until urea is dissolved. Placing in 37°C bath for a brief period speeds up the dissolution of urea.
3. Add distilled H_2O to 60 ml.

g. Use a disposable 0.2 or 0.45 µ filter unit, attach to vacuum. Transfer the gel solution to the filter unit. Filter and de-gas 5-10 minutes.

h. Remove the plunger from disposable 60 cc syringe. Wrap small piece of parafilm around tip of syringe. Pour gel solution into the barrel of the syringe.

i. Add 300 µl 10% APS & 20 µl TEMED to the gel solution. Replace plunger at barrel opening. Mix gently to avoid introducing air bubbles.

j. Remove parafilm from tip of syringe. Using slow, even pressure on plunger, expel gel solution through tip of syringe into the assembled gel apparatus. *Watch for bubbles!* Pounding on the glass plate with a pipetman to prevent bubble formation is an accepted practice. **Note:** If bubbles develop in the gel that would interfere with running the gel, the gel must be discarded and a new gel made.

k. Place shark's tooth comb between the two glass plates with the straight edge facing the bottom of the gel. Clamp the comb in place with 1" binder clamps.

l. Allow the gel to set for up to 24 hours.

F. Run gel

1. Remove binder clamps from gel apparatus, then place the gel apparatus in the sequencing apparatus.
2. Pre-run gel with 0.8X TBE at 80 W until gel temperature reaches 50°C.
3. Heat the samples at 75-80°C for 2 minutes, then place on ice. Load 3 µl of each reaction per lane, making note of the order in which they were loaded.
4. Run the gel at 80 W for 3-4 hours (for a long read). Replace the buffer in the upper chamber with fresh 0.8X TBE.
5. Another 3 µl per lane of each reaction are loaded in new lanes, making note of the order loaded.
6. Run the gel at 80 W for another 2 hours (for a short read). (The long read total run is 5-6 hours.)
7. Turn off the power supply, drain the upper chamber. Remove the gel apparatus from the sequencing apparatus. Separate the glass plates. Gel should remain on one plate. Lay a sheet of filter paper on top of the gel and lift the gel off the glass. The top 16 cm should be discarded (it contains no useful sequence data and will not fit in our gel dryer).
8. Lay the gel, filter side down, on the base support of the gel dryer. Cover the gel with saran wrap. Lay the plastic gel dryer sheet on top of the gel. Fold the rubber sheet over the whole assembly. Attach vacuum and turn on the dryer to 80°C for 2 hours.
9. Expose the gel to film.

G. Develop the autorad
   1. Follow the directions for the film used.
   2. Exposures vary from overnight to several days depending upon the amount and ratio of template and primer DNA and upon the age of the isotope used.

WESTERN BLOTTING PROCEDURE

I. PURPOSE

The transfer of proteins from SDS-PAGE gels to a solid support for immunological detection of immobilized proteins

II. MATERIALS AND EQUIPMENT

1) 5X Protein Loading Buffer:
   325mM Tris HCl, pH 6.8
   50% glycerol
   5% SDS
   .005% bromophenol blue
   Add 50 µl B-mercaptoethanol to 950 µl of sample buffer prior to use.
2) BioRad Mini-PROTEAN II Cell or equivalent gel electrophoresis apparatus and accessories: Mini-gel glass plates
   1.5 mm spacers
   1.5 mm teflon combs
   gel clamp assemblies
   casting stand
   inner cooling core
   electrophoresis tank and lid
3) 125 ml vacuum flask
4) 30% liquid acrylamide (Boehringer Mannheim Biochemicals , cat.# 100670)
5) 2% liquid bis-acrylamide (Boehringer Mannheim Biochemicals, cat. #100675)
6) 4X Tris Cl/SDS, pH 6.8 Stacking buffer: 0.5 M Tris. Cl, 0.4 % SDS, pH 6.8
7) 4X Tris Cl/SDS, pH 8.8 Separating buffer: 1.5M Tris. Cl, 0.4% SDS, pH 8.8
8) Deionized distilled water (ddH_2O)
9) 10% (w/v)Ammonium persulfate (APS)
10) TEMED
11) Isobutanol, H_2O-saturated
11) Purified SAMase protein from pMalcRI.SAM fusion protein
12) Amersham Rainbow Protein Molecular Weight Markers or equivalent
13) Electrophoresis buffer: 25 mM Tris, 192 mM glycine, 0.1% SDS
14) BioRad Mini Trans-Blot Electrophoretic Transfer Cell or equivalent transblot apparatus and accessories: gel holder cassettes
   fiber pads
   Bio-Ice cooling unit
   electrode module
   transblot tank and lid
15) Blotting paper (Schleicher and Schuell GB003 GelBlot paper or equivalent)
16) Millipore Immobilon-P membrane or equivalent PVDF membrane
17) 100% Methanol
18) Transblot buffer: 25 mM Tris, 192mM glycine, 15% methanol pH ~ 8.3

19) 10X Phosphate Buffered Saline (10X PBS): $NaH_2PO_4 . H_2O$ 2.03 g
Na_2HPO_4 11.49 g
NaCl 85 g
ddH_2O to 1 liter

The pH of the 10X solution is 6.7 to 6.9. The pH of the 1X working solution should be 7.3 to 7.5 (if not, adjust the 1X)

20) PBS: 1:10 dilution of 10X PBS in ddH_2O
21) PBS.Tween 20 solution: PBS with 0.05% (v/v)Tween 20
22) Blocking Solution: 5%(w/v)Non-Fat Powder Milk in PBS.Tween20
23) Wash Solution: 3.5% (w/v) Non-Fat Powder Milk in PBS.Tween 20
24) SAMase mouse monoclonal antibody - Epitope SAM10-9A3.1.3 Mab
25) Kirkegaard and Perry Laboratory peroxidase labeled goat antimouse IgG+IgM(H+L) antibody, 1 mg/ml
26) DuPont NEN Renaissance Western Blot Chemiluminescence reagent: enhanced luminol reagent and oxidizing reagent
27) Kodak XAR X-ray film

III. PROCEDURE

A. Preparation of Two 16% SDS-PAGE Minigels, 1.5 mm thick
1. Assemble the minigel plates according to the manufacturer's instructions, using 1.5 mm spacers.
2. Prepare separating gel solution by combining the four reagents below in a vacuum flask:
10.38 ml 30% acrylamide
4.32 ml 2% bis-acrylamide
5.0 ml 4X Tris/SDS(pH8.8)
0.3 ml ddH_2O
3. Degas separating gel solution for 10-15 minutes.
4. Add 40 µl 10% APS and 20 µl TEMED, swirl to mix.
5. Using a Pasteur pipet, pour separating gel solution into the gap between the glass plates until the height of the gel solution is approximately 6.0 cm.
6. Carefully overlay gel solution with H_2O saturated isobutanol.
7. Let gels polymerize at room temperature for 45-60 minutes.
8. Pour off the isobutanol overlay and rinse the tops of the gels with ddH_2O. Drain off as much of the H_2O as possible. Before pouring stacker, remove any remaining H_2O with the edge of a piece of blotting paper.
9. Prepare 4.5% stacking gel solution by combining the four reagents below in a vacuum flask:
1.1 ml 30% acrylamide
.45 ml 25 bis-acrylamide
1.88 ml 4X Tris/SDS (pH 6.8)
4.07 ml ddH_2O
10. Degas stacking gel solution for 10-15 minutes.
11. Add 75 µl 10% APS and 9 µl TEMED, swirl to mix.

12. Pour stacking gel solution onto separating gel.
13. Insert 1.5 mm comb into stacking gel solution. If necessary, add more stacking gel solution to fill in spaces in the comb. Be careful not to trap any air bubbles in between the teeth of the comb.
14. Let gel polymerize at room temperature for 30-45 minutes.

B. Gel Electrophoresis of Protein Samples
1. Remove the combs from the gels. Using a squirt bottle, wash the wells out with ddH_2O to remove any unpolymerized acrylamide.
2. Assembly gels in electrophoresis apparatus according to manufacturer's instructions.
3. Fill upper and lower buffer reservoirs with cold electrophoresis buffer.
4. Prepare molecular weight markers and SAMase standards for loading on the gel. Combine markers or standards with a 1/4 volume of 5X protein sample buffer. Use 7 µl markers + 1.75 µl sample buffer for 15 well gels and 10 µl markers + 2.5 µl sample buffer for 10 well gels. Heat for 1 minute at 95-100 C°. For SAMase, prepare with the 1/4 volume sample buffer but heat for 2-3 minutes at 95-100 C°.
5. If frozen , thaw plant lysates to room temperature.
6. Load lysate samples, standards, and markers on gel.
7. If necessary, add more cold electrophoresis buffer to the electrophoresis tank until it is at least 1/2 full.
8. Electrophorese gels at 150 volts for approximately two hours or until the magenta 14.3 kilodalton molecular marker is one cm. from the bottom of the gel.

C. Transblotting Proteins to a Membrane
1. Remove gels from glass plates and soak in transblot buffer for 15 minutes.
2. Cut Immobilon-P membranes and blotting paper to the same size of the gels (approximately 5.5 X 8.5 cm). Note: always use forceps or wear gloves when handling membranes.
3. Prepare membranes by soaking in 100% methanol for one minute, washing with ddH_2O for 2-3 minutes, and finally soaking in transblot buffer for 5 minutes.
4. Prewet fiber pads and blotting paper in transblot buffer.
5. Assemble gels in transblot "sandwich" as follows:
   a. Open up gel cassette holder and lay flat on the benchtop. On the black half of the holder (cathode (-) side), begin stacking the following components on top of each other in the following order: (bottom to top)
      1. cathode(-) side of gel cassette (black)
      2. fiber pad
      3. blotting paper
      4. gel
      5. membrane
      6. blotting paper
      7. fiber pad
      8. anode (+) side of gel cassette(clear)
   b. When placing gel onto the blotting paper, wet gel with more buffer and smooth out gel removing any air bubbles between paper and gel.

c. Place membrane directly onto the gel. Using a pipet as a rolling pin , remove any air bubbles between gel and membrane.

6. Place gel cassette between the electrode module of transblot apparatus with the black side of the cassette facing the cathode(black) and the clear side of the holder facing the anode (red).
7. Add a one inch stir bar and cooling unit to transblot tank and fill to the top with cold transblot buffer.
8. Place the transblot apparatus in the walk-in cold room (4 C°). Electrophorese at 100 volts for 2 hours, stirring the buffer during the run .
9. Take apart transblot sandwich and remove membrane. Rinse with PBS to remove any gel stuck to the membrane.
10. Let membrane air dry for 30-60 minutes. Store in a cool dry place.

D. Immunological Detection of Target Protein

Note: All incubations and washes are performed at room temperature on an orbital shaker.

1. Rewet membranes for 1 minute in 100% methanol then wash with ddH_2O for 2-3 minutes and finally soak in transblot buffer for 5 minutes.
2. Block open sites by incubating in blocking solution for one hour .
3. Rinse blots with washing solution twice.
4. Dilute the SAMase Mab (SAM10-9A3.1.3) to 1 µg/ml in washing solution
5. Incubate blots with SAMase Mab for 2 hours.
6. Wash blots five times: 1X 10 minutes, 4X 5 minutes in washing solution
7. Dilute the peroxidase labelled conjugate (Goat anti-mouse IgG+IgM(H+L) Ab) 1 to 10,000 in washing solution.
8. Incubate blots with conjugate for 2 hours .
9. Wash blot five times: 1X 10 minutes, 3X 5 minutes in washing solution
   1X 5 minutes in PBS
10. Prepare chemiluminescence working reagent just before using,by mixing equal volumes of enhanced luminol reagent (brown bottle) and oxidizing reagent (white bottle). Use at least 0.125 ml per cm^2 membrane - approximately 6 ml/blot.
11. Incubate blots in chemiluminescence working reagent for 1 minutes.
12. Remove excess chemiluminescence reagent from the blots. Place blots between the cover of a polypropylene sheet protector. Gently smooth out any air pockets.
13. Expose blots to film. For detection of SAMase, expose blots for one hour and overnight.

NEW ENGLAND BioLabs

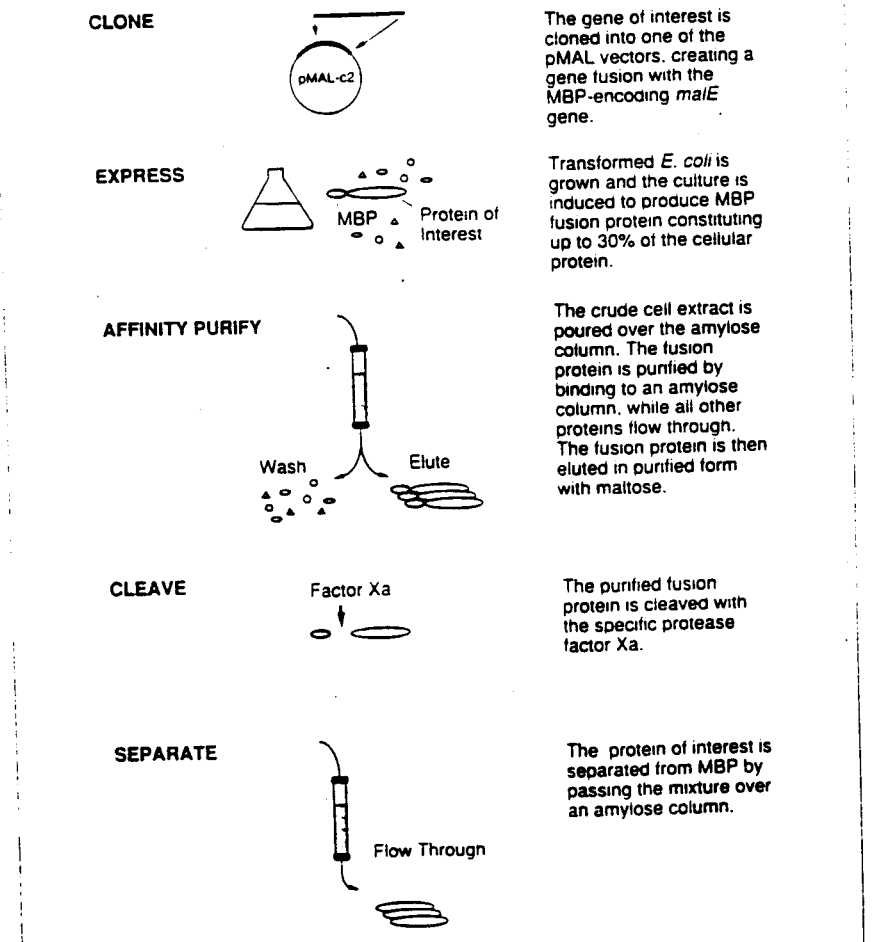
1995 CATALOG

Protein Fusion and Purification System

#800 ..... **$400**

The System Includes:

- *pMAL-c2: 5 µg*
- *pMAL-p2: 5 µg*
- *Amylose Resin: 15 ml (binding capacity ~40 mg)*
- *Factor Xa: 50 µg*
- *anti-MBP antiserum: 25 µl (for Western blot analysis)*
- *MBP2*: 10 µg (marker for SDS-polyacrylamide gels)*
- *MBP2-paramyosin-ΔSal: 100 µg (control for factor Xa cleavage)*
- *E. coli Host*
- *A Comprehensive Instruction Manual*



SDS-polyacrylamide gel electrophoresis of fractions from the purification of MBP-paramyosin-ΔSal. A. Lane 1: uninduced cells. Lane 2: induced cells. B. Lane 1: purified protein eluted from amylose column with maltose. Lane 2: purified protein after factor Xa cleavage. Lane 3: paramyosin fragment eluted from second amylose column.

In the Protein Fusion and Purification System, the cloned gene is inserted into a pMAL vector downstream from the *malE* gene, which encodes maltose-binding protein (MBP). This results in the expression of an MBP-fusion protein (1,2,3). The technique uses the strong P_{tac} promoter and the translation initiation signals of MBP to express large amounts of the fusion protein. The fusion protein is then purified by a one-step affinity purification for MBP (4).

The system uses the pMAL-2 vectors which are designed so that insertion interrupts a *lacZ*α gene allowing a blue-to-white screen for inserts on X-gal (5). pMAL-c2 has an exact deletion of the *malE* signal sequence, resulting in cytoplasmic expression of the fusion protein. pMAL-p2 contains the normal *malE* signal sequence, which directs the fusion protein through the cytoplasmic membrane. pMAL-p2 fusion proteins capable of being exported can be purified from the periplasm. Between the *malE* sequence and the polylinker there is a spacer sequence coding for 10 asparagine residues. This spacer insulates MBP from the protein of interest, increasing the chances that a particular fusion will bind tightly to the amylose resin. The vectors also include a sequence coding for the recognition site of the protease factor Xa (cleaves after the amino acids Ile-Glu-Gly-Arg). This allows the protein of interest to be cleaved from MBP after purification, without adding any vector-derived residues to the protein (6). For this purpose, the polylinker includes an *Xmn* I site superimposed on the sequence coding for the factor Xa site. This is where the gene of interest is inserted. An *Eco*R I site in the same reading frame as that of λgt11 and a number of other useful sites are present directly after the factor Xa sequence. The vectors also include the M13 origin of DNA replication which allows the production of single-stranded DNA for sequencing and mutagenesis.

Expression from the pMAL vectors yields up to 100 mg fusion protein from a liter of culture (gel photo). While no expression system works with every cloned gene, the Protein Fusion and Purification System gives substantial yields of protein in about 75% of the cases tested so far. A chapter in *Current Protocols in Molecular Biology* (3) provides an in-depth analysis of the use of the pMAL vectors. The System's instruction manual is available separately upon request.

References:

(1) Guan, C. et al. (1987) *Gene* 67, 21–30.
(2) Maina, C.V. et al. (1988) *Gene* 74, 365–373.
(3) Riggs, P., in Ausebel, F.M. et al. (eds), *Current Protocols in Molecular Biology* (1990) Greene Associates/Wiley Interscience, New York.
(4) Kellerman and Ferenci (1982) *Methods in Enzymol.* 90, 459-463.
(5) Yanisch-Perron, C. et al. (1985) *Gene* 33, 103–119.
(6) Nagai, K and Thøgersen, H. C. (1987) *Methods Enzymol.* 153, 461–481.

APPENDIX 5

Field Trial Reports

AGRITOPE

Agritope
8505 SW Creekside Place
Beaverton, Oregon 97005
503 641 6115
Fax 503 643 2781

October 19, 1993

Dr. Arnold S. Foudin
Deputy Director, Biological Permits
BBEP-APHIS, USDA
Federal Building, Room 844
6505 Belcrest Road
Hyattsville, MD 20782

Dear Dr. Foudin:

This final report is being submitted for Biotechnology Permit Number 92-085-01 in compliance with Supplemental Permit #3.

Our field test was initiated on June 12, 1992—the approval date of the permit application. The field test was terminated on September 30, 1992 with all non-collected plant material being disced into the soil. The test site has been kept fallow for the past year and observed to be free of any volunteer plants. There was no observed horizontal movement, altered survival characteristics or unusual phenotypes in any of the field plants.

Fruit was harvested from the field-grown plants at various stages of ripening. Molecular, biochemical and physiological measurements were made to assess the extent of gene expression, the effect on ethylene biosynthesis, and postharvest properties. Leaf area, plant dry weight, fruit number, fruit size, pH, soluble solids and titratable acidity were also measured. The results of these analyses are described in the attachment.

All transgenic plants had normal appearance. All material has been handled in accordance to the specifications contained in the permit and supplemental conditions attached.

I hope that this report provides all necessary information to complete the permit conditions. Thank you for cooperation throughout the permit application procedure and conduct of these field trials.

Sincerely,

Richard K. Bestwick, Ph.D.
Sr. Vice President, Research & Development

RKB/jrd

Visual Observations

Weekly visual observations were made from transplanting until first harvest. ESKN 37 plants appeared small, with lighter colored foliage than the other lines. CGN 30 plants, while larger than ESKN 37 and of normal color, also were small; the remaining lines appeared normal in size and color. These differences were apparent throughout the observational period.

Mid-season plant growth

Eight weeks after transplanting, 2 plants/plot were cut at ground level. Leaves and fruit were removed. Plant leaf areas were measured with a LI-3100 LAM. Fruit were counted and weighed. Leaves and stems were recombined, dried in a forced air oven at 65 C, and plant dry weight was taken. Data were analyzed using the SAS general linear models procedure; ESKN 18 was compared to the check (35A) by single degree of freedom orthogonal contrast. Mean separation between constructs was by Duncan's multiple range test.

Leaf area, plant dry weight, and fruit number were similar for all 6 lines evaluated (Table 1). However, total and average fruit weights differed between constructs. Total fruit weight of ESKN 18 did not differ significantly from the other lines, but average fruit weight was greater for ESKN 18 than any other.

Table 1. Tomato plant characteristics, Hermiston, Oregon, 1992.

	Plant		Fruit		
Line	Leaf area (cm^2)	Dry wt. (g)	No.	Wt. (g)	Ave. wt. (g)
35A	31571	323	312	1774 b[z]	5.4 c
CGN 8	25140	344	163	1260 b	7.1 c
CGN 30	49020	530	431	4247 a	10.2 b
ESKN 1D	42535	530	225	1458 b	7.9 bc
ESKN 18	53137	583	201	2681 ab	13.0 a
ESKN 37	26228	321	255	1826 b	6.6 c

[z]Means followed by different letters significantly different at P=0.05 (Duncan's multiple range test).

Fruit quality evaluation

Procedures:

12-15 red ripe fruit/plot were combined and homogenized in a Waring blender. Homogenate was strained to remove solids and vacuum-filtered to produce a clear solution. Soluble solids were determined with an Atago hand-held refractometer, and pH was measured with an Accumet 825MP specific ion/pH meter. Titratable

acidity was determined by titration of 5 ml of solution to pH 8.2 with 0.1N NaOH. Data were analyzed using the SAS general linear models procedure; ESKN 18 was compared to the check (35A) by single degree of freedom orthogonal contrast. Mean separation between constructs was by Duncan's multiple range test.

pH, soluble solids and titratable acidity differed significantly between lines (Table 2). When ESKN 18 was compared to 35A, pH was significantly higher; soluble solids and titratable acidity were similar.

Table 2. Tomato fruit characteristics, Hermiston, Oregon, 1992.

Line	pH	Soluble solids	Titratable acidity (meq)
35A	4.11 c[z]	5.78 ab	124.0 ab
CGN 8	3.98 c	5.15 c	154.0 a
CGN 30	4.05 c	5.23 c	139.5 ab
ESKN 1D	4.26 b	6.10 a	119.5 b
ESKN 18	4.45 a	5.48 bc	107.0 b
ESKN 37	4.32 ab	5.40 bc	115.5 b

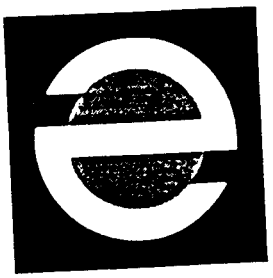
[z]Means followed by different letters significantly different at P=0.05 (Duncan's multiple range test).

Table 3. Tomato fruit ripening, Hermiston, Oregon, 1992.

Line	Heat units[z] to		
	Breaker	Pink	Red
35A	1044 c[y]	1095 d	1143 d
CGN 8	1124 b	1233 b	1288 b
CGN 30	1060 c	1122 d	1177 d
ESKN 1D	1136 b	1207 bc	1253 bc
ESKN 18	1227 a	1367 a	1433 a
ESKN 37	1113 b	1151 cd	1192 cd

[z] Heat units from anthesis with 45°F. base, 100°F. maximum.

[y] Means followed by different letters significantly different at P=0.05 (Duncan's multiple range test).



AGRITOPE, INC.
8505 SW CREEKSIDE PL.
BEAVERTON, OREGON 97008

(503) 641-6115
(503) 643-2781 FAX

General:

USDA Notification Number: 93-050-01

Cooperator Name: George Clough Ph.D.

Field Trial Location (State, County): Oregon, Umatilla

Type of Trial : Evaluate Phenotype

Agritope Field Representative: Richard K. Bestwick, Ph.D.

Seed Storage Information:

Date seed received: April 27, 1993

Seed received by (give name): G. Clough

Seed lot number: see entry list

Amount received: 1500 seed

Amount remaining after planting: 0

Disposition of seed remaining: n/a

Give location of the secure locked seed storage facility:

Horticultural lab, Hermiston Agricultural Research & Extension Center, Oregon State University, Hermiston OR

Planting Information:

Date planted:

Greenhouse: April 28, 1993

Field: June 2, 1993

Number of transgenic seeds planted: 1008

Number of non-transgenic seeds planted: 128 vector transformed control
128 untransformed control

Date of seedling emergence: n/a (greenhouse)

Estimated percent of germination: 88.6%

Width in feet of tomato-free buffer: 5,280 feet

Containment Information:

Did any seeds or other plant material inadvertently escape from the trial site?

NO

If yes, explain:

_____

_____

Equipment Cleaning:

1. **During flowering--Give date(s) that field equipment was examined before leaving the trial site with all flowers removed from the machinery:**

   No equipment entered field

2. **During harvest (if applicable)--Give date(s) that field equipment was examined before leaving the trial site with all plant material, fruit and seeds removed from the machinery.**

   No equipment entered field

Plant Observations:

1. **Date of first observed open flower:**

   June 30, 1993

2. **Presence of crown gall disease symptoms on any of the transgenic or non-transgenic tomato plants? If yes, explain:**

   NONE

3. **Any of the transgenic tomato plants have abnormal appearance? If yes, explain:**

   NONE

Herbicide Treatments:

1. **What herbicides were applied as your standard regime?**

a. **Pre-plant incorporated, please describe:**

   Vapam @ 60 gal./acre, roto tilled April 2, 1993

b. **Pre-emergence, please describe:**

None

c. **Post-directed, please describe:**

None

2. **Any additional treatments, please list:**

None

Trial Completion:

1. **Date harvested (if applicable):** August 26-September 10, 1993

2. **Date trial destroyed. Describe how trial was destroyed:**

October 21, 1993, flail-chopped plants, disced field 2X

Site Monitoring:

1. **What crop was planted after the field trial was destroyed?**

Wheat, Barley

2. **Were there any volunteer tomato plants after trial was destroyed? If yes, explain and state when and how they were destroyed:**

NO

Chronological Log:

Date	Plant Stage	Activities/Comments
4/2/93	pre-plant	fumigate with Vapam @ 60 gal/acre
4/9/93	pre-plant	broadcast fertilizer
4/15/93	pre-plant	sub-soil roto-tilled
5/18/95	pre-plant	roto-tilled, lay drip tape
6/2/93	seedling	transplant
6/16/93	seedling	stake field, first tie
6/17/93	seedling	first evaluation for vigor and growth
6/22/93	fruit set	tied plants
6/23/93	fruit set	fertigation
6/30/93	fruit set	second evaluation for vigor, growth and fruit set
7/1/93	fruit set	Asana application for Colorado potato beetle
7/12/93	fruit set	tied plants
7/12/93	fruit set	measure plant height
7/14/93	fruit set	fertigation
7/23/93	fruit set	tied plants
7/27/93	ripening	sample leaf area
8/26-27/93	ripe fruit	harvest
9/2/93	ripe fruit	harvest
9/9-10-/93	ripe fruit	harvest
10/21/93	n/a	trial destroyed, plants chopped
10/29/93	n/a	field disced
5/9/94	monitoring visit	no voluteer tomato detected
6/2/94	monitoring visit	no voluteer tomato detected

Other Observations:

None

Conclusions:

This trial was completed with no observed symptoms of *Agrobacterium* infection or loss of containment. The objectives of the trial were met in that the various ripening phenotypes of the different transgenic lines were evaluated and seed was produced from the selected materials. Monitoring of the site was completed during the spring and summer of the following growing season with no volunteer plants detected.

FIELD TRIAL ENTRY LIST

HERMISTON AGRICULTURAL RESEARCH AND EXPERIMENT CENTER
SUMMER, 1993

GENOTYPE:

TORC 5420 35-1
TORC 5420 22-1
TORC 5420 40A-1
LARGE RED CHERRY CONTROL
H 100 CONTROL
TOHC5420
RIO GRANDE CONTROL
TORG5420
UC82B CONTROL
TOUC5420

AGRITOPE, INC.
8505 SW CREEKSIDE PL.
BEAVERTON, OREGON 97008

(503) 641-6115
(503) 643-2781 FAX

General:

USDA Notification Number: 94-143-09-N

Cooperator Name: George Clough Ph.D.

Field Trial Location (State, County): Oregon, Umatilla

Type of Trial : Efficacy/Seed production

Agritope Field Representative: Matthew Kramer

Seed Storage Information:

Date seed received: April 28,1994
Seed received by (give name): G. Clough
Seed lot number: see entry list
Amount received: 150 seed
Amount remaining after planting: 0
Disposition of seed remaining: n/a
Give location of the secure locked seed storage facility:
Horticultural lab, Hermiston Agricultural Research & extension Center, Oregon State University, Hermiston OR

Planting Information:

Date planted:
Greenhouse: April 28, 1994
Field: June 2, 1994
Number of transgenic seeds planted: 150
Number of non-transgenic seeds planted: 25
Date of seedling emergence: n/a (greenhouse)
Estimated percent of germination: 85%
Width in feet of tomato-free buffer: 5,280 feet

Containment Information:

Did any seeds or other plant material inadvertently escape from the trial site?

NO

If yes, explain:

_____

_____

Equipment Cleaning:

1. **During flowering--Give date(s) that field equipment was examined before leaving the trial site with all flowers removed from the machinery:**

   No equipment entered field

2. **During harvest (if applicable)--Give date(s) that field equipment was examined before leaving the trial site with all plant material, fruit and seeds removed from the machinery.**

   No equipment entered field

Plant Observations:

1. **Date of first observed open flower:**

   June 2, 1994

2. **Presence of crown gall disease symptoms on any of the transgenic or non-transgenic tomato plants? If yes, explain:**

   NONE

3. **Any of the transgenic tomato plants have abnormal appearance? If yes, explain:**

   NONE

Herbicide Treatments:

1. **What herbicides were applied as your standard regime?**

a. **Pre-plant incorporated, please describe:**

   Vapam @ 60 gal./acre, roto tilled April 5, 1994

b. **Pre-emergence, please describe:**

None

c. **Post-directed, please describe:**

None

2. **Any additional treatments, please list:**

None

Trial Completion:

1. **Date harvested (if applicable):** October 17, 1994

2. **Date trial destroyed. Describe how trial was destroyed:**

October 22, 1994. flail-chopped plants, disced field 2X

Site Monitoring:

1. **What crop was planted after the field trial was destroyed?**

Wheat, Barley

2. **Were there any volunteer tomato plants after trial was destroyed? If yes, explain and state when and how they were destroyed:**

NO

Chronological Log:

Date	Plant Stage	Activities/Comments
4/5/94	pre-plant	fumigated w/ Vapam @ 60gal/acre
4/9/94	pre-plant	broadcast fertilizer
4/17/94	pre-plant	sub-soil roto-tilled
4/28/94	Seed	ship to HAREC, plant to greenhouse
5/15/94	pre-plant	roto-tilled, lay drip tape, apply mulch
6/2/94	Seedling	transplant to field
8/15/94	Fruit set	estimate maturity for seed harvest, evaluate ripening phenotype.
10/17/94	Mature fruit	harvest each genotype for seed production.
10/22/94	Post harvest	destroy trial
10/23/95	Seed	final seed shipped to Agritope, Inc.
4/12/95	Monitoring visit	walk field to detect presence of volunteer tomato plants-none detected
5/15/95	Monitoring visit	walk field to detect presence of volunteer tomato plants-none detected
6/5/95	Monitoring visit	walk field to detect presence of volunteer tomato plants-none detected
8/9/95	Monitoring visit	walk field to detect presence of volunteer tomato plants-none detected

Other Observations:

None

Conclusions:

This trial was completed with no observed symptoms of *Agrobacterium* infection or loss of containment. The objectives of the trial were met in that the various ripening phenotypes of the different transgenic lines were evaluated and seed was produced from the selected materials. Monitoring of the site was completed during the spring and summer of the following growing season with no volunteer plants detected.

APPENDIX 9

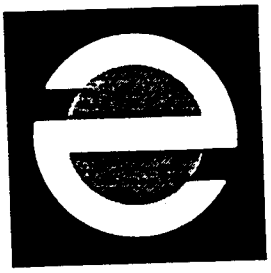
Chromatographs of Tomatine Measurements in Large Red Cherry Control and Transgenic pAG 5420 Transformants

FIELD TRIAL ENTRY LIST

HERMISTON AGRICULTURAL RESEARCH AND EXPERIMENT CENTER
SUMMER, 1994

GENOTYPE:

TORC 5420 35-1
TORC 5420 22-1
TORC 5420 40A-1
LARGE RED CHERRY CONTROL



AGRITOPE, INC.
8505 SW CREEKSIDE PL.
BEAVERTON, OREGON 97008

(503) 641-6115
(503) 643-2781 FAX

General:

USDA Notification Number: 93-361-01N

Cooperator Name: Dr. Steve Czaplewski

Field Trial Location (State, County): Florida, Hendry

Type of Trial : Phenotype evaaluation

Agritope Field Representative: Matthew Kramer

Seed Storage Information:

Date seed received: August 24, 1994
Seed received by (give name): Dr. Steve Czaplewski
Seed lot number: see entry list
Amount received: 2500
Amount remaining after planting: none
Disposition of seed remaining: n/a
Give location of the secure locked seed storage facility:
Rogers Seed Company Naples, Field Station

Planting Information:

Date planted:
Greenhouse: August 26, 1994
Field: October 4, 1994
Number of transgenic seeds planted: 2500
Number of non-transgenic seeds planted: 600
Date of seedling emergence: n/a (greenhouse)
Estimated percent of germination: 77%
Width in feet of tomato-free buffer: > 100 ft.

Containment Information:

Did any seeds or other plant material inadvertently escape from the trial site?

NO

If yes, explain:

_____

_____

Equipment Cleaning:

1. **During flowering--Give date(s) that field equipment was examined before leaving the trial site with all flowers removed from the machinery:**

   No equipment entered field during flowering, all cultivation and weeding and chemical applications done by hand.

2. **During harvest (if applicable)--Give date(s) that field equipment was examined before leaving the trial site with all plant material, fruit and seeds removed from the machinery.**

   No equipment entered field, all harvest was done by hand.

Plant Observations:

1. **Date of first observed open flower:**

   November 3, 1994

2. **Presence of crown gall disease symptoms on any of the transgenic or non-transgenic tomato plants? If yes, explain:**

   NONE

3. **Any of the transgenic tomato plants have abnormal appearance? If yes, explain:**

   NONE

Herbicide Treatments:

1. **What herbicides were applied as your standard regime?**
   a. **Pre-plant incorporated, please describe:**

      Methyl Bromide fumigation @ 200 lbs./acre

   b. **Pre-emergence, please describe:**

      None

c. **Post-directed, please describe:**

See chronological log

2. **Any additional treatments, please list:**

Various fungicide and bacteriacide applications were done by hand with a backpack sprayer.

Trial Completion:

1. **Date harvested (if applicable):** January 23 - February 28, 1995

2. **Date trial destroyed. Describe how trial was destroyed:** March 3, 1995

Plants were pulled and left in the field. Field was disced 2 X and site was fumigated with Methyl Bromide @ 200 lbs./acre.

Site Monitoring:

1. **What crop was planted after the field trial was destroyed?**

Melons

2. **Were there any volunteer tomato plants after trial was destroyed? If yes, explain and state when and how they were destroyed:**

NO

Chronological Log:

Date	Plant Stage	Activities/Comments
8/23/94	Seed	Ship to Rogers Seed CO. Naples. FL Research Station
8/26/94	Seed	Seed sown in greenhouse
10/4/94	Seedling stage	Transplanted to field
10/10/94	Seedling stage	Stake the trial
10/14/94	Seedling stage	Spray diquat. backpack sprayer
10/20/94	Seedling stage	Prune and sucker
11/4/94	Flowering	Apply admire for whitefly control
11/15/94	Flowering	Tie trial
11/28/94	Fruit set	Prune and weed by hand
12/6/94	Fruit set	Spray Kocide, Manex, Agrimek and Induce to control bacteria and leaf miner
12/8/94	Fruit set	Spray lannate and Javelin to control fruit worm
12/29/94	Fruit set	Spray lannate and Javelin to control fruit worm
1/5/95	Fruit set	Evaluate trial
1/13/95	Fruit set	Evaluate trial
1/23/95	Harvest	Begin harvest
2/6/95	Harvest	Evaluate trial
2/22/95	Harvest	Continue harvest
2/28/95	Harvest	Complete harvest
3/3/95	Post-harvest	Destroy field. fumigate

Other Observations:

None

Conclusions:

This trial was completed with no observed symptoms of *Agrobacterium* infection or loss of containment. No abnormal plant growth was observed and no enhanced weediness was observed on the site. The objectives of the trial were met in that the various ripening phenotypes of the different transgenic lines were evaluated and seed was produced from the selected materials. Fruit for tomatine and nutritional studies was also harvested from this trial. Post destruction fumigation with methyl bromide was used to insure the lack of any re-growth of transgenic tomatoes on the site.

FIELD TRIAL ENTRY LIST

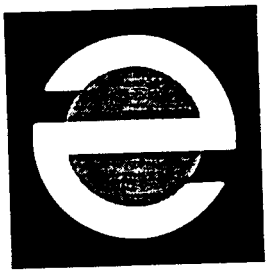
Rogers NK Seed Co.
Greenway Rd., Naples, FL

WINTER, 1994

GENOTYPE:

TORC 5420
TORC 5520
Large Red Cherry Control

TOK 1 5420
TOK 2 5420
TOK 3 5420
TOK 4 5420
TOK1 Control
TOK2 Control
TOK3 Control
TOK4 Control



AGRITOPE, INC.
8505 SW CREEKSIDE PL.
BEAVERTON, OREGON 97008

(503) 641-6115
(503) 643-2781 FAX

General:

USDA Notification Number: **94-353-01N**

Cooperator Name: **Mark Draper**

Field Trial Location (State, County): **California, Riverside County**

Type of Trial : **Efficacy/Seed production**

Agritope Field Representative: **Matthew Kramer**

Seed Storage Information:

Date seed received: December 10, 1994

Seed received by (give name): Speedling, Inc. Nipomo CA

Seed lot number: see entry list

Amount received: 4,000

Amount remaining after planting: n/a

Disposition of seed remaining: 400 excess plants disposed on field site

Give location of the secure locked seed storage facility:

Speedling, Inc. Nipomo California

Planting Information:

Date planted:

Greenhouse: December 26, 1994

Field: February 17, 1995

Number of transgenic seeds planted:

Number of non-transgenic seeds planted:

Date of seedling emergence: n/a (greenhouse)

Estimated percent of germination: 70%

Width in feet of tomato-free buffer: > 200 feet

Containment Information:

Did any seeds or other plant material inadvertently escape from the trial site?

NO

If yes, explain:

_____

_____

Equipment Cleaning:

1. **During flowering--Give date(s) that field equipment was examined before leaving the trial site with all flowers removed from the machinery:**

   No equipment entered field, all weeding done by hand.

2. **During harvest (if applicable)--Give date(s) that field equipment was examined before leaving the trial site with all plant material, fruit and seeds removed from the machinery.**

   No equipment entered field, harvest was completed by hand.

Plant Observations:

1. **Date of first observed open flower:**

   March 21, 1995

2. **Presence of crown gall disease symptoms on any of the transgenic or non-transgenic tomato plants? If yes, explain:**

   NONE

3. **Any of the transgenic tomato plants have abnormal appearance? If yes, explain:**

   NONE

Herbicide Treatments:

1. **What herbicides were applied as your standard regime?**

a. **Pre-plant incorporated, please describe:**

   NONE

b. **Pre-emergence, please describe:**

   NONE, weed control was accomplished by hand hoeing.

c. **Post-directed, please describe:**

NONE, weed control was accomplished by hand hoeing.

2. **Any additional treatments, please list:**

NONE, all weed control was accomplished by hand hoeing.

Trial Completion:

1. **Date harvested (if applicable):** June 7, 1994

2. **Date trial destroyed. Describe how trial was destroyed:**

June 22, 1995, flail-chopped plants, disced field 2X

Site Monitoring:

1. **What crop was planted after the field trial was destroyed?**

fallow

2. **Were there any volunteer tomato plants after trial was destroyed? If yes, explain and state when and how they were destroyed:**

TBD

Chronological Log:

Date	Plant Stage	Activities/Comments
12/10/94	Seed	shipped to Speedling, Inc, Nipomo CA for transplant production.
2/1/95	pre-plant	roto-till field, shape beds
2/7/95	pre-plant	lay drip irrigation tape
2/15/95	pre-plant	lay plastic and set up plots
2/17/95	seedling	transplant to field
3/23/95	seedling	stake field, first tie
3/25/95	seedling	first evaluation for vigor and growth
4/7/95	fruit set	tied plants
5/3/95	fruit set	second evaluation for vigor, growth and fruit set
5/29/95	fruit set	third evaluation for vigor, growth and phenotype
6/7/95	mature fruit	harvest
6/7/95	harvest	ship fruit to Agritope for seed production
6/22/95	post harvest	destroy field

Other Observations:

None

Conclusions:

This trial was completed with no observed symptoms of *Agrobacterium* infection or loss of containment. The objectives of the trial were met in that the various ripening phenotypes of the different transgenic lines were evaluated and seed was produced from the selected materials. Monitoring of the site was completed during the spring and summer of the following growing season with no volunteer plants detected.

FIELD TRIAL ENTRY LIST

Mark Draper Enterprises, Inc.
40555 Monroe, Indio, CA

SPRING 1995

GENOTYPE:

TORC 5420
TORC 5520
LARGE RED CHERRY CONTROL

APPENDIX 6

DNA Sequence of the Integrated Backbone DNA from pAG 5420

Bac ne sequence
Monday, November 13, 1995 12:42 PM

```
                10        20        30        40        50        60        70
                *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
CTCCATGGGC TTTTTCTCCT CGTGCTCGTA AACGGACCCG AACATCTCTG GAGCTTTCTT CAGGGCCGAC

                80        90        100       110       120       130       140
                *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
AATCGGATCT CGCGGAAATC CTGCACGTCG GCCGCTCCAA GCCGTCGAAT CTGAGCCTTA ATCACAATTG

                150       160       170       180       190       200       210
                *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
TCAATTTTAA TCCTCTGTTT ATCGGCAGTT CGTAGAGCGC GCCGTGCGCC CGAGCGATAC TGAGCGAAGC

                220       230       240       250       260       270       280
                *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
AAGTGCGTCG AGCAGTGCCC GCTTGTTCCT GAAATGCCAG TAAAGCGCTG GCTGCTGAAC CCCCAGCCGG

                290       300       310       320       330       340       350
                *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
AACTGACCCC ACAAGGCCCT AGCGTTTGCA ATGCACCAGG TCATCATTGA CCCAGGCGTG TTCCACCAGG

                360       370       380       390       400       410       420
                *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
CCGCTGCCTC GCAACTCTTC GCAGGCTTCG CCGACCTGCT CGCGCCACTT CTTCACGCGG GTGGAATCCG

                430       440       450       460       470       480       490
                *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
ATCCGCACAT GAGGCGGAAG GTTTCCAGCT TGAGCGGGTA CGGCTCCCGG TGCGAGCTGA AATAGTCGAA

                500       510       520       530       540       550       560
                *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
CATCCGTCGG GCCGTCGGCG ACAGCTTGCG GTACTTCTCC CATATGAATT TCGTGTAGTG GTCGCCAGCA

                570       580       590       600       610       620       630
                *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
AACAGCACGA CGATTTCCTC GTCGATCAGG ACCTGGCAAC GGGACGTTTT CTTGCCACGG TCCAGGACGC

                640       650       660       670       680       690       700
                *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
GGAAGCGGTG CAGCAGCGAC ACCGATTCCA GGTGCCCAAC GCGGTCGGAC GTGAAGCCCA TCGCCGTCGC

                710       720       730       740       750       760       770
                *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
CTGTAGGCGC GACAGGCATT CCTCGGCCTT CGTGTAATAC CGGCCATTGA TCGACCAGCC CAGGTCCTGG
```

Bac ne sequence
Monday, November 13, 1995 12:41 PM

```
      780        790        800        810        820        830        840
      *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
CAAAGCTCGT AGAACGTGAA GGTGATCGGC TCGCCGATAG GGGTGCGCTT CGCGTACTCC AACACCTGCT

      850        860        870        880        890        900        910
      *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
GCCACACCAG TTCGTCATCG TCGGCCCGCA GCTCGACGCC GGTGTAGGTG ATCTTCACGT CCTTGTTGAC

      920        930        940        950        960        970        980
      *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
GTGGAAAATG ACCTTGTTTT GCAGCGCCTC GCGCGGGATT TTCTTGTTGC GCGTGGTGAA CAGGGCAGAG

      990        1000       1010       1020       1030       1040       1050
      *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
CGGGCCGTGT CGTTTGGCAT CGCTCGCATC GTGTCCGGCC ACGGCGCAAT ATCGAACAAG GAAAGCTGCA

      1060       1070       1080       1090       1100       1110       1120
      *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
TTTCCTTGAT CTGCTGCTTC GTGTGTTTCA GCAACGCGGC CTGCTTGGCC TCGCTGACCT GTTTTGCCAG

      1130       1140       1150       1160       1170       1180       1190
      *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
GTCCTCGCCG GCGGTTTTTC GCTTCTTGGT CGTCATAGTT CCTCGCGTGT CGATGGTCAT CGACTTCGCC

      1200       1210       1220       1230       1240       1250       1260
      *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
AAACCTGCCG CCTCCTGTTC GAGACGACGC GAACGCTCCA CGGCGGCCGA TGGCGCGGGC AGGGCAGGGG

      1270       1280       1290       1300       1310       1320       1330
      *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
GAGCCAGTTG CACGCTGTCG CGCTCGATCT TGGCCGTAGC TTGCTGGACC ATCGAGCCGA CGGACTGGAA

      1340       1350       1360       1370       1380       1390       1400
      *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
GGTTTCGCGG GGCGCACGCA TGACGGTGCG GCTTGCGATG GTTTCGGCAT CCTCGGCGGA AAACCCCGCG

      1410       1420       1430       1440       1450       1460       1470
      *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
TCGATCAGTT CTTGCCTGTA TGCCTTCCGG TCAAACGTCC GATTCATTCA CCCTCCTTGC GGGATTGCCC

      1480       1490       1500       1510       1520       1530       1540
      *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
```

Bac one sequence
Monday, November 13, 1995 12:52 PM

CGACTCACGC CGGGGCAATG TGCCCTTATT CCTGATTTGA CCCGCCTGGT GCCTTGGTGT CCAGATAATC

1550 1560 1570 1580 1590 1600 1610

CACCTTATCG GCAATGAAGT CGGTCCCGTA GACCGTCTGG CCGTCCTTCT CGTACTTGGT ATTCCGAATC

1620 1630 1640 1650 1660 1670 1680

TTGCCCTGCA CGAATACCAG CGACCCCTTG CCCAAATACT TGCCGTGGGC CTCGGCCTGA GAGCCAAAAC

1690 1700 1710 1720 1730 1740 1750

ACTTGATGCG GAAGAAGTCG GTGCGCTCCT GCTTGTCGCC GGCATCGTTG CGCCACTCTT CATTAACCGC

1760 1770 1780 1790 1800 1810 1820

TATATCGAAA ATTGCTTGCG GCTTGTTAGA ATTGCCATGA CGTACCTCGG TGTCACGGGT AAGATTACCG

1830 1840 1850 1860 1870 1880 1890

ATAAACTGGA ACTGATTATG GCTCATATCG AAAGTCTCCT TGAGAAAGGA GACTCTAGTT TAGCTAAACA

1900 1910 1920 1930 1940 1950 1960

TTGGTTCCGC TGTCAAGAAC TTTAGCGGCT AAAATTTTGC GGGCCGCGAC CAAAGGTGCG AGGGGCGGCT

1970 1980 1990 2000 2010 2020 2030

TCCGCTGTGT ACAACCAGAT ATTTTTCACC AACATCCTTC GTCTGCTCGA TGAGCGGGGC ATGACGAAAC

2040 2050 2060 2070 2080 2090 2100

ATGAGCTGTC GGAGAGGGCA GGGGTTTCAA TTTCGTTTTT ATCAGACTTA ACCAACGGTA AGGCCAACCC

2110 2120 2130 2140 2150 2160 2170

CTCGTTGAAG GTGCTGACTC TTATACACAA GTAGCGTCCT GAACGGAACC TTTCCCGTTT TCCAGGATCT

2180 2190 2200 2210 2220 2230 2240

GACTTCCATG TGACCTCCTA ACATGGTAAC GTTCATGATA ACTTCTGCTC TTCATCGTGC GGCCGACTGG

2250 2260 2270 2280 2290 2300 2310

Bac ne sequence
Monday, November 13, 1995 12:52 PM

```
                                                                    *   *
GCTAAATCTG TGTTCTCTTC GGCGGCGCTG GGTGATCCTC GCCGTACTGC CCGCTTGGTT AACGTCGCCG

      2320       2330       2340       2350       2360       2370       2380
   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
CCCAATTGGC AAAATATTCT GGTAAATCAA TAACCATCTC ATCAGAGGGT AGTGAAGCCA TGCAGGAAGG

      2390       2400       2410       2420       2430       2440       2450
   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
CGCTTACCGA TTTTACCGCA ATCCCAACGT TTCTGCCGAG GCGATCAGAA AGGCTGGCGC CATGCAAACA

      2460       2470       2480       2490       2500       2510       2520
   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
GTCAAGTTGG CTCAGGAGTT TCCCGAACTG CTGGCCATTG AGGACACCAC CTCTTTGAGT TATCGCCACC

      2530       2540       2550       2560       2570       2580       2590
   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *   *
AGGTCGCCGA AGAGCTTGGC AAGCTGGGCT CTATTCAGGA TAAATCCCGC GGATGGTGGG TTCACTCCGT

      2600
   *   *
TCTCTTGCCC GTC
```

APPENDIX 7

Complete Protein Sequence of *S*-adenosylmethionine hydrolase

sam-k Nucleotide and Aligned Amino Acid Sequences

```
          10          20          30          40
     *    *    *      *    *      *    *      *    *
ATG GTT TTC ACT AAA GAG CCT GCG AAC GTC TTC TAT GTA CTG GTT TCC
Met Val Phe Thr Lys Glu Pro Ala Asn Val Phe Tyr Val Leu Val Ser

50          60          70          80          90
*    *      *    *      *    *      *    *      *    *
GCT TTC CGT TCT AAC CTC TGC GAT GAG GTG AAT ATG AGC AGA CAC CGC
Ala Phe Arg Ser Asn Leu Cys Asp Glu Val Asn Met Ser Arg His Arg

  100         110         120         130         140
   *    *      *    *      *    *      *    *      *
CAC ATG GTA AGC ACT TTA CGT GCC GCA CCG GGT CTT TAT GGC TCC GTT
His Met Val Ser Thr Leu Arg Ala Ala Pro Gly Leu Tyr Gly Ser Val

    150         160         170         180         190
*    *      *    *      *    *      *    *      *    *
GAG TCA ACC GAT TTG ACC GGG TGC TAT CGT GAG GCA ATC TCA AGC GCA
Glu Ser Thr Asp Leu Thr Gly Cys Tyr Arg Glu Ala Ile Ser Ser Ala

       200         210         220         230         240
  *    *      *    *      *    *      *    *      *    *
CCA ACT GAG GAA AAA ACT GTT CGT GTA CGC TAC AAG GAC AAA GCG CAG
Pro Thr Glu Glu Lys Thr Val Arg Val Arg Tyr Lys Asp Lys Ala Gln

          250         260         270         280
     *    *      *    *      *    *      *    *      *
GCA CTC AAT GTT GCA CGC CTA GCT TGT AAT GAG TGG GAG CAA GAT TGC
Ala Leu Asn Val Ala Arg Leu Ala Cys Asn Glu Trp Glu Gln Asp Cys

290         300         310         320         330
*    *      *    *      *    *      *    *      *    *
GTA CTG GTA TAC AAA TCA CAG ACT CAC ACG GCT GGT CTG GTG TAC GCT
Val Leu Val Tyr Lys Ser Gln Thr His Thr Ala Gly Leu Val Tyr Ala

  340         350         360         370         380
   *    *      *    *      *    *      *    *      *
AAA GGT ATC GAC GGG TAT AAG GCT GAA CGT CTG CCG GGT AGT TTC CAA
Lys Gly Ile Asp Gly Tyr Lys Ala Glu Arg Leu Pro Gly Ser Phe Gln

    390         400         410         420         430
*    *      *    *      *    *      *    *      *    *
GAG GTT CCT AAA GGC GCA CCG CTG CAA GGC TGC TTC ACT ATT GAT GAG
Glu Val Pro Lys Gly Ala Pro Leu Gln Gly Cys Phe Thr Ile Asp Glu

       440         450
  *    *      *    *      *
TTC GGT CGC CGC TGG CAA GTA CAA TAA
Phe Gly Arg Arg Trp Gln Val Gln ***
```

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O| |O IntelliGenetics
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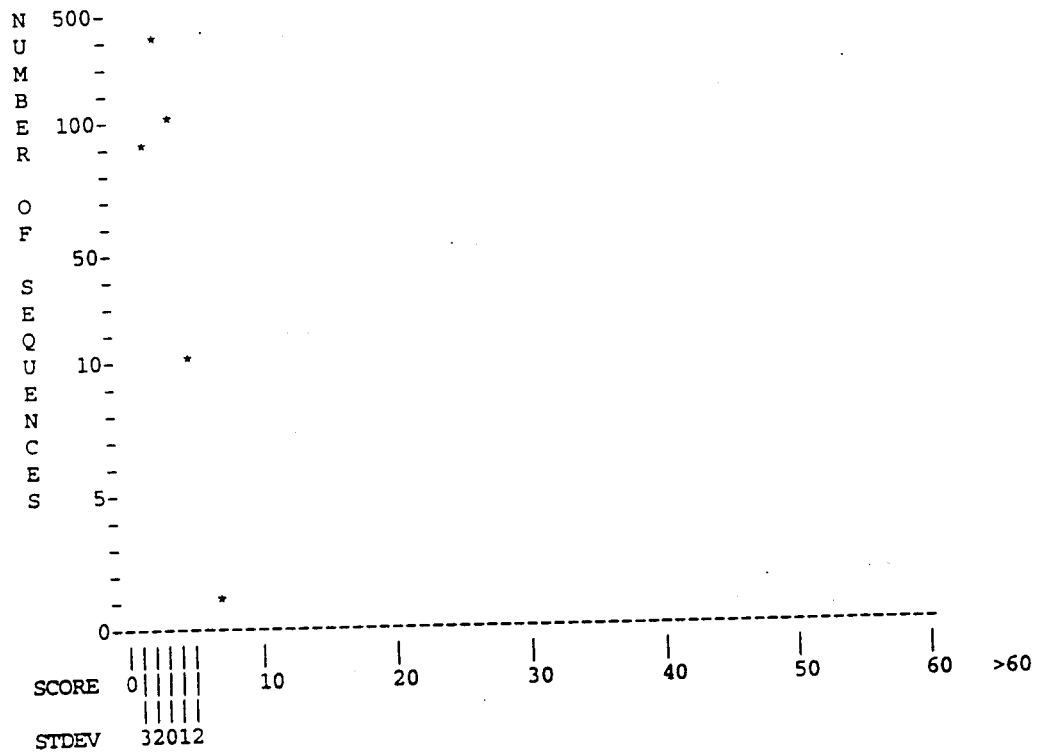
January 19, 1995

IFind - Sequence Data Bank Searching Program Version 5.4
(Based on the algorithm of Wilbur and Lipman.)

Begin recording for user rbestwic at 18-Jan-95 12:06pm Swiss-Protein databank -toxin as keyword toxin.pep compared to SAMSYN1.pep



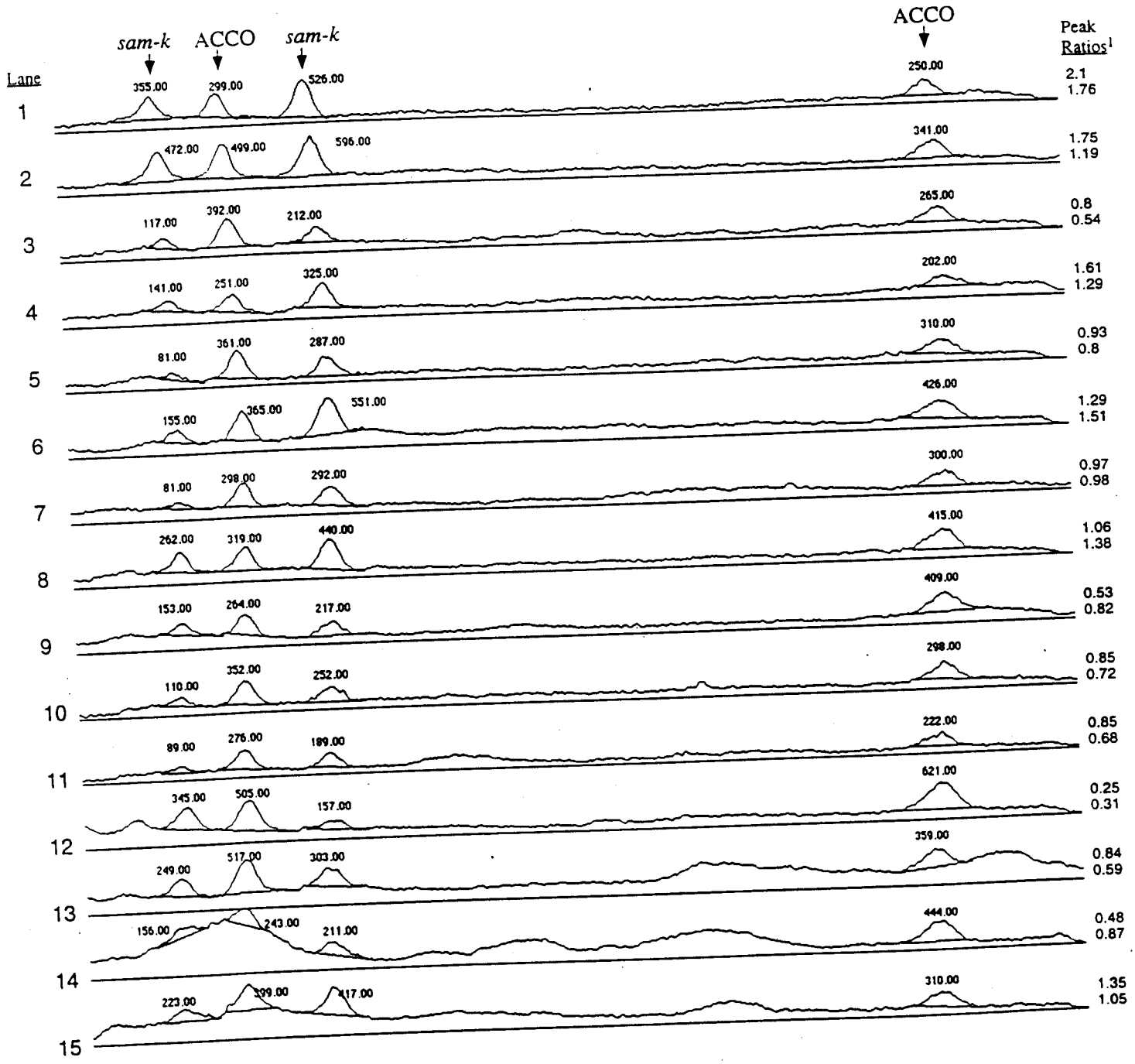
Mean score=3.14, Standard deviation=.68.
IFIND search completed. Number of sequences compared: 675

RANK	SEQUENCE	LENGTH	SCORE	STANDARD-DEVIATION-FROM-MEAN
1	HLY1_ECOLI	L=1023	7	5.65
2	CX3_NAJHA	L=60	5	2.72
3	CX4_NAJHA	L=60	5	2.72
4	CX8_NAJHA	L=60	5	2.72
5	THNB_VISAL	L=103	5	2.72
6	TTR_PSESY	L=177	5	2.72
7	RAC1_HUMAN	L=192	5	2.72
8	RAC1_MOUSE	L=192	5	2.72
9	SPEA_STRPY	L=251	5	2.72
10	RIPC_PHYAM	L=313	5	2.72
11	PHLC_CLOBI	L=398	5	2.72
12	CEAC_ECOLI	L=561	5	2.72
13	HLYA_ECOLI	L=1024	5	2.72
14	HSER_HUMAN	L=1073	5	2.72
15	HSER_RAT	L=1075	5	2.72
16	BXE_CLOBO	L=1250	5	2.72
17	BXE_CLOBU	L=1250	5	2.72
18	HTS1_COCCA	L=5217	5	2.72
19	CXOC_CONMA	L=29	4	1.26
20	TXAM_METSE	L=36	4	1.26

APPENDIX 8

Scanning Densitometry of *sam-k* and ACC Oxidase Band Intensities in R_1 Progeny of Line 35-1-N

Scanning Densitometry of 35-1-N R_1 Southern Analysis (Fig. 2)

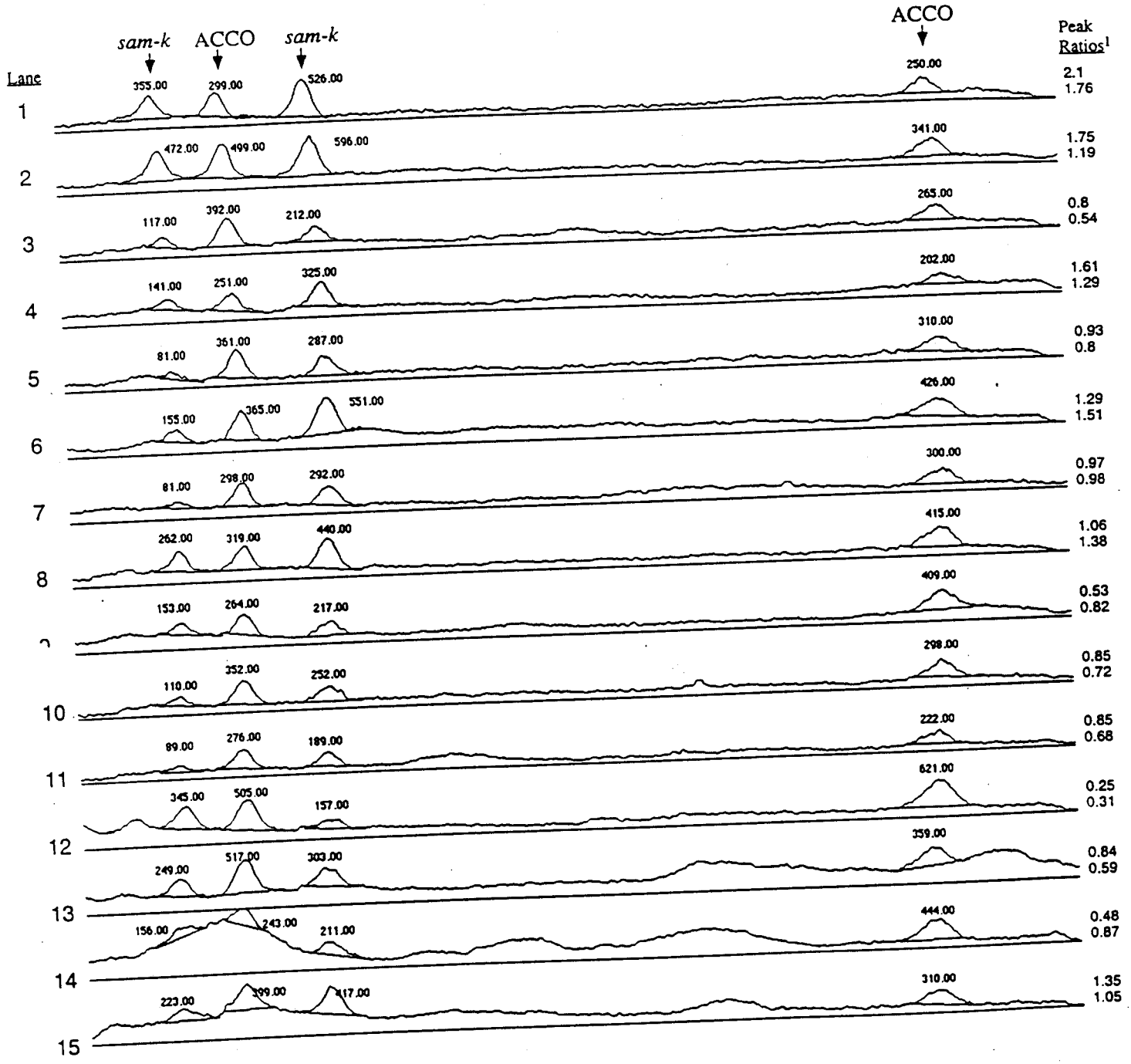


[1] Peak ratios represent the integrated band intensity ratios for the second *sam-k* peak to either the right most ACCO peak (top number) or the left most ACCO peak (bottom number).

APPENDIX 8

Scanning Densitometry of *sam-k* and ACC Oxidase Band Intensities in R_1 Progeny of Line 35-1-N

Scanning Densitometry of 35-1-N R_1 Southern Analysis (Fig. 2)

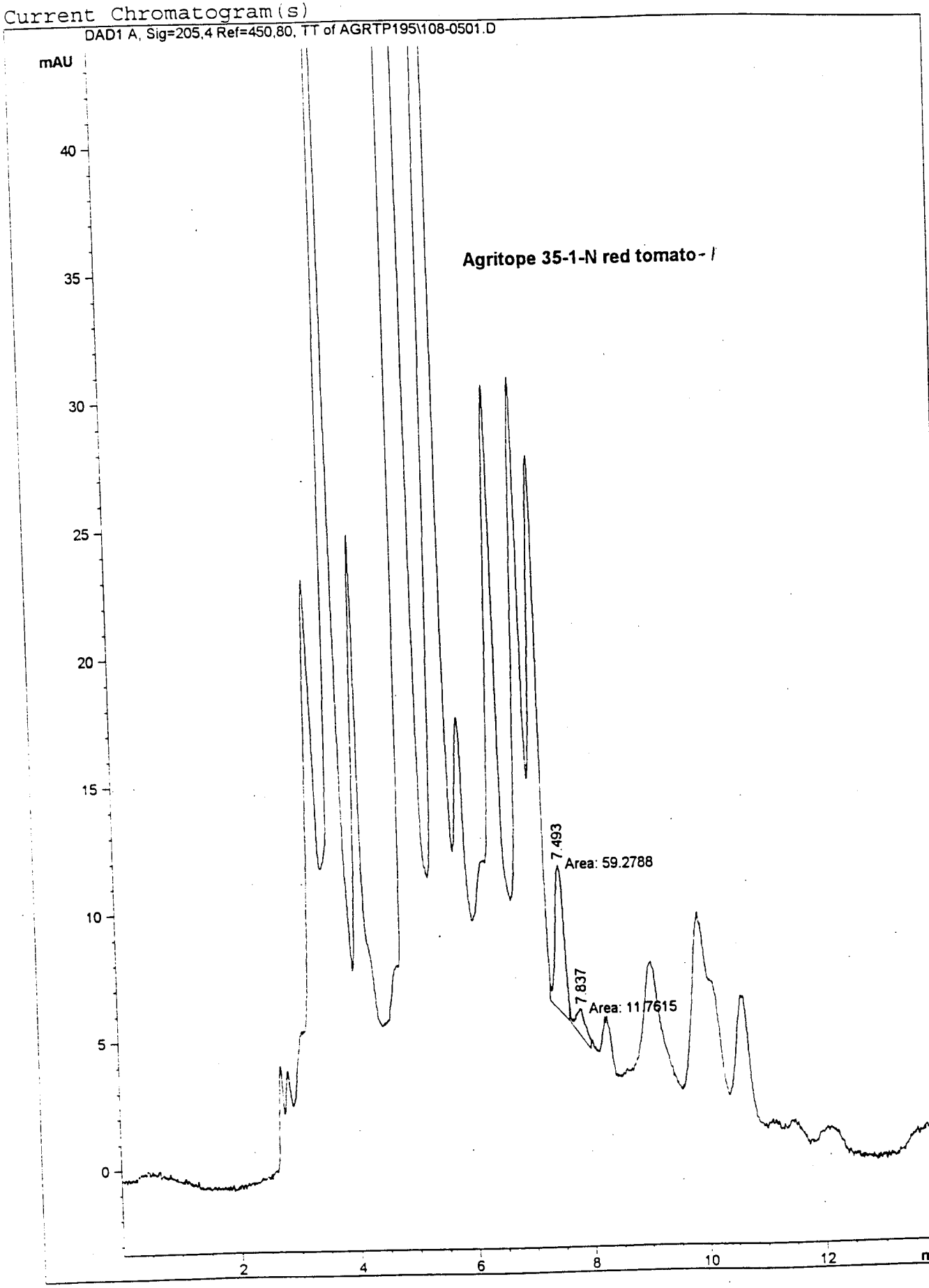


[1] Peak ratios represent the integrated band intensity ratios for the second *sam-k* peak to either the right most ACCO peak (top number) or the left most ACCO peak (bottom number).

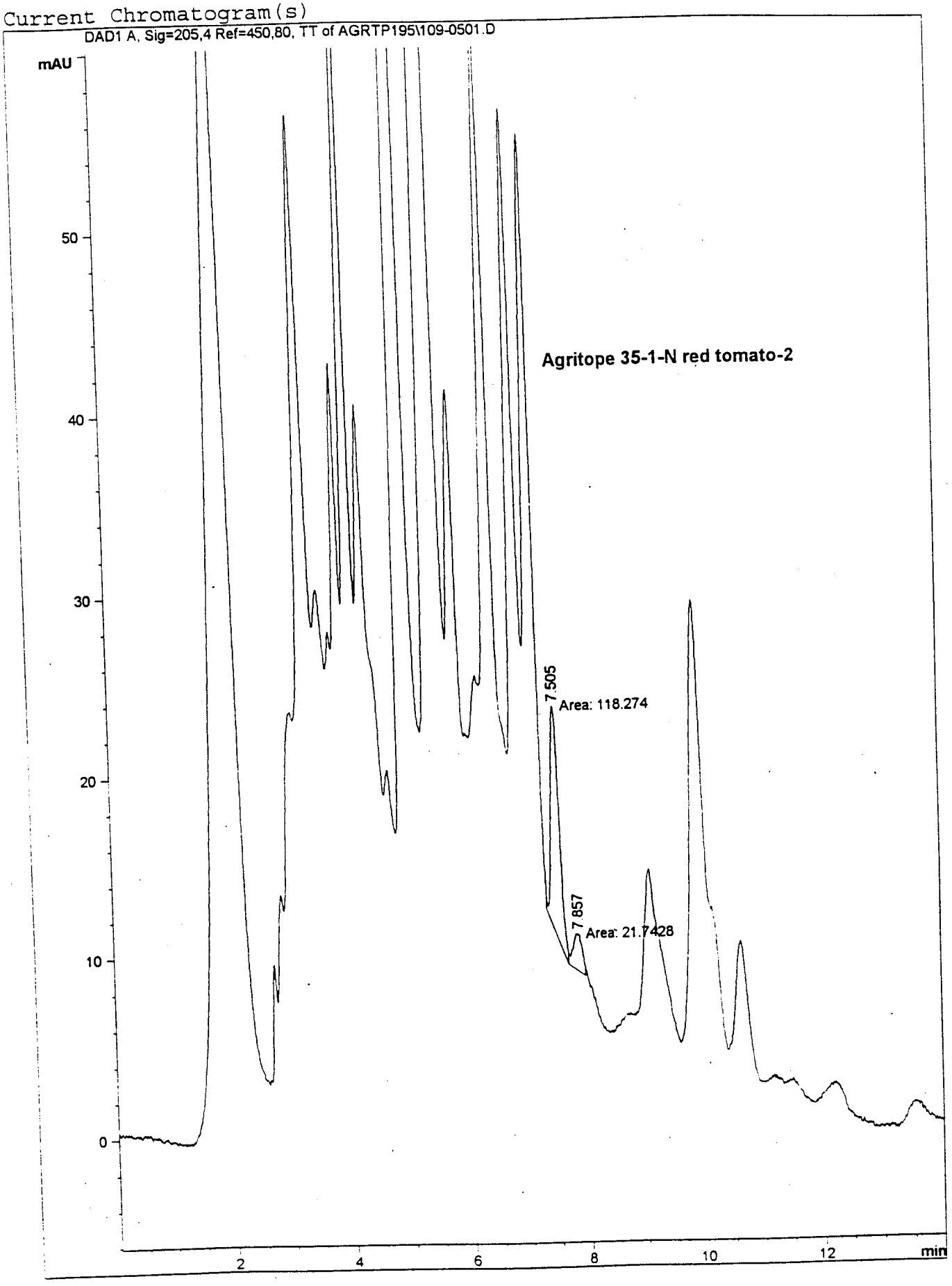
APPENDIX 9

Chromatographs of Tomatine Measurements in Large Red Cherry Control and Transgenic pAG 5420 Transformants

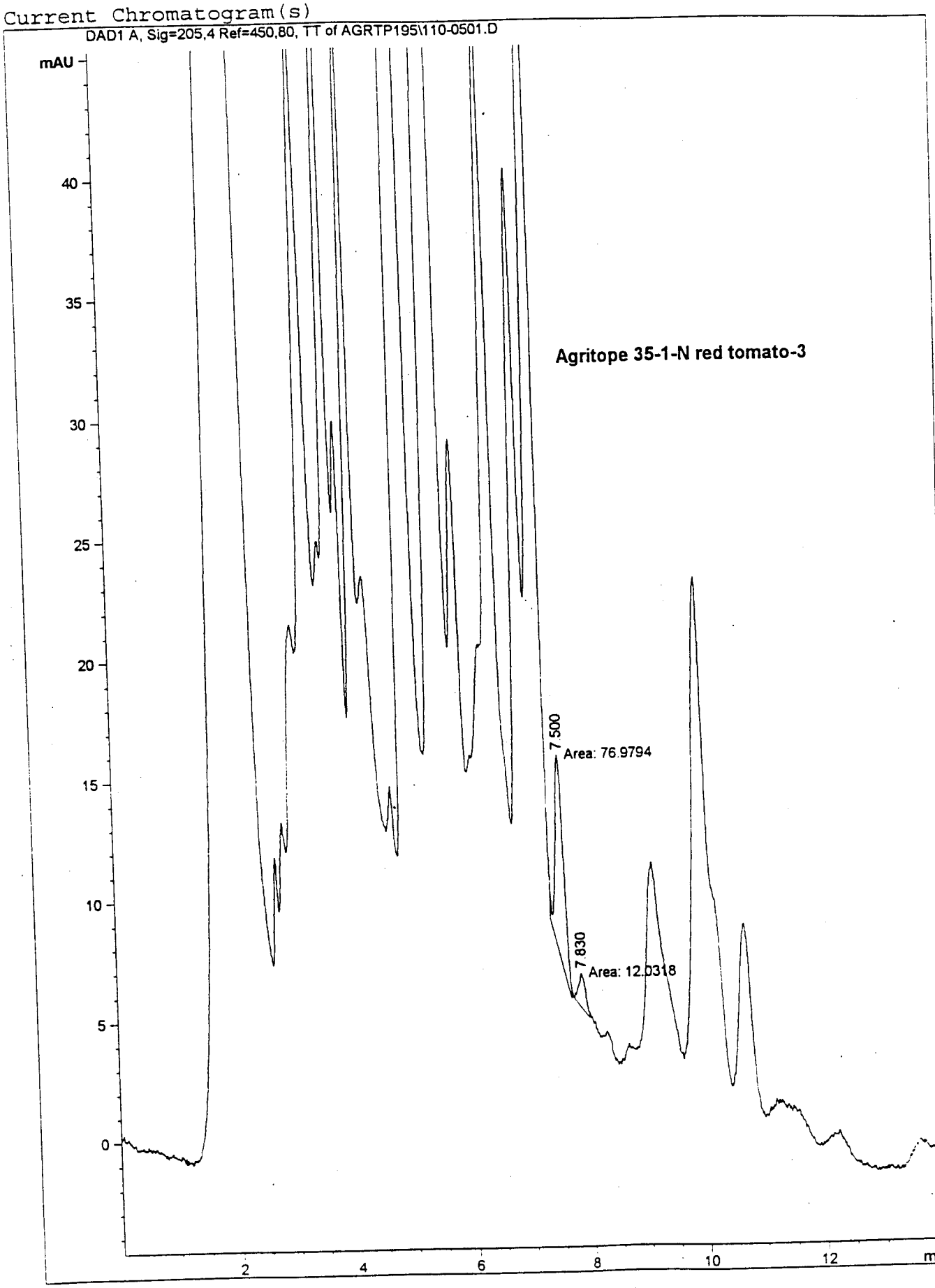
Current Chromatogram(s)



Current Chromatogram(s)



Current Chromatogram(s)



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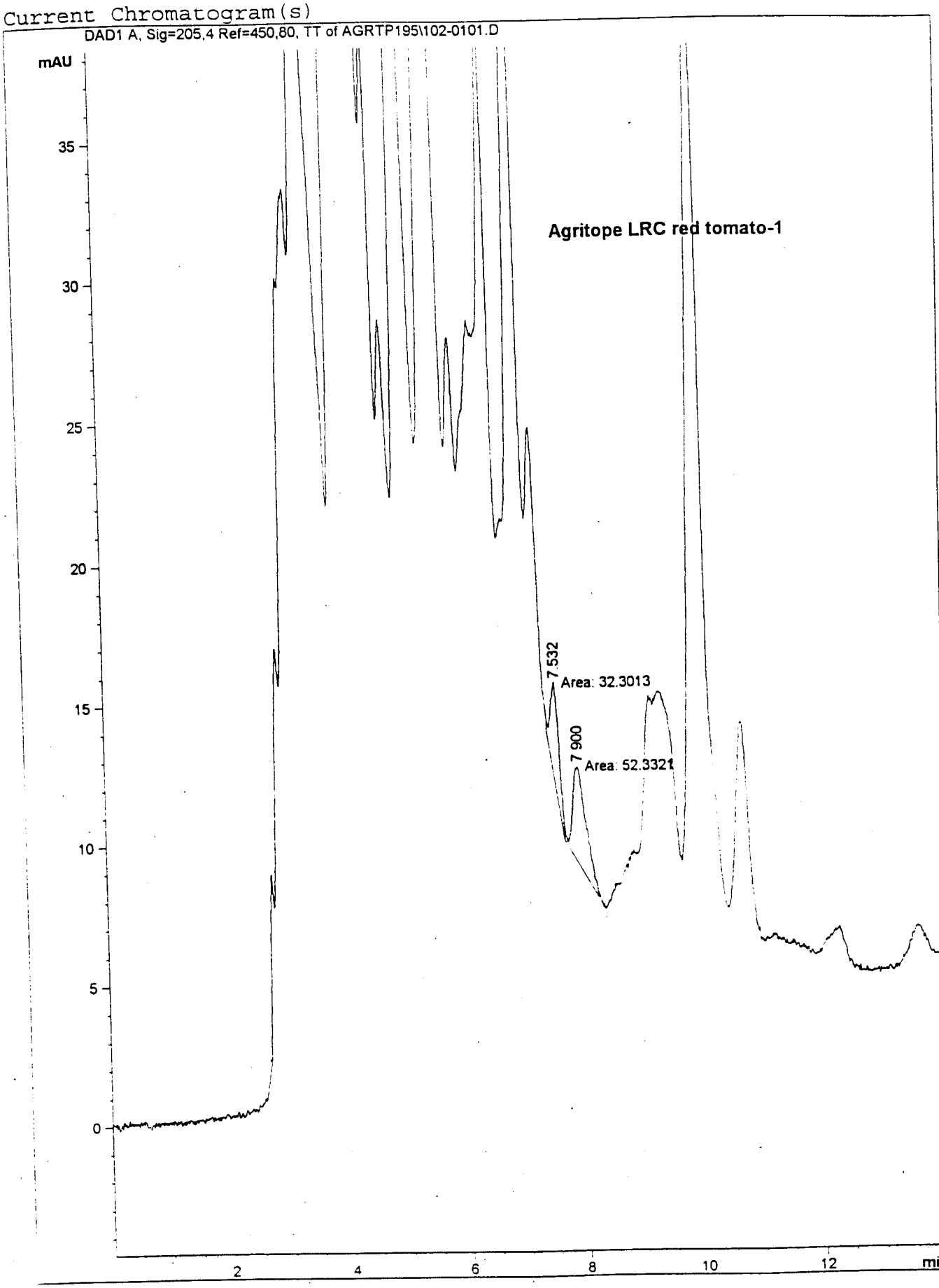
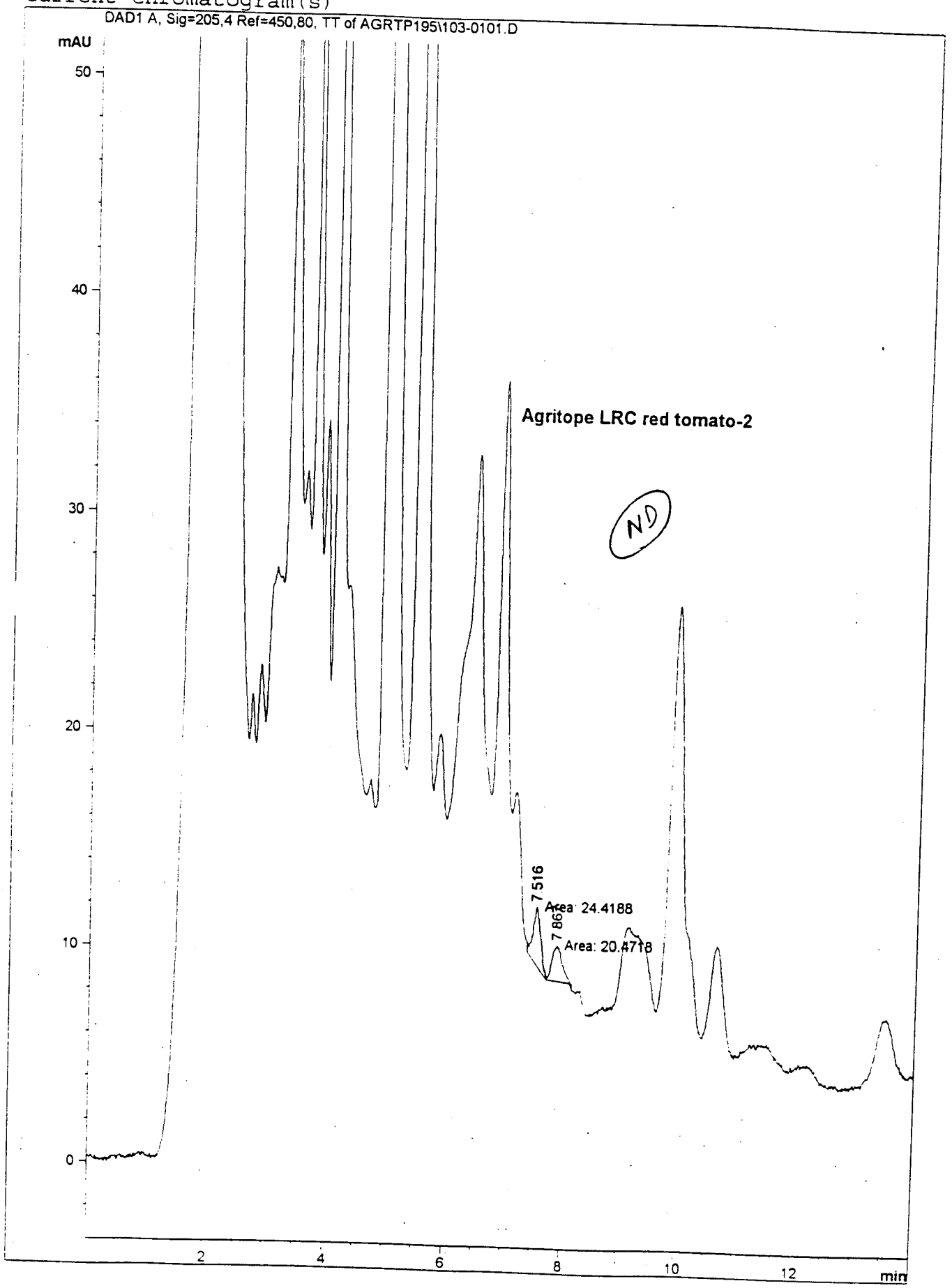


of window 38: Current Chromatogram(s)

Page 1 of 1

Current Chromatogram(s)



Current Chromatogram(s)