DAS-59122-7
Insect-Protected Maize (Corn)

Technical Bulletin

Dow AgroSciences™

History and Product Overview

Bacillus thuringiensis (*Bt*) is a common soil bacterium that produces proteins which kill specific insect larvae. Insecticide spray formulations with *Bt* organisms, or purified *Bt* proteins, have been available as a crop protective agent for at least 40 years, including use in recent years for organic farming operations. Beginning in the 1980's, the genes responsible for the expression of *Bt* proteins were isolated and successfully transferred into different transgenic crops, including potatoes, cotton and corn. Transgenic maize (corn) seed with *Bt* was first approved for commercial use in the mid-1990s. Compared to conventional *Bt* spray formulations, transgenic plants expressing *Bt* proteins provide greatly enhanced protection from insect damage throughout the growing season.

Event DAS-59122-7, utilizing the insertion of the *cry*34Ab1 and *cry*35Ab1genes, represents a new generation of transgenic insect protection traits for maize. This event was developed via collaboration between Dow AgroSciences LLC and Pioneer Hi-Bred International, Inc. The *cry*34Ab1 and *cry*35Ab1genes co-express a unique binary insecticidal crystal protein (called Cry34Ab1 and Cry35Ab1) derived from *Bt* strain PS149B1. DAS-59122-7 offers high levels of protection against larval stages of major corn rootworm pests, including western corn rootworm (*Diabrotica virgifera virgifera*), northern corn rootworm (*Diabrotica barberi*), and Mexican corn rootworm (*Diabrotica virgifera zeae*).

Corn rootworm larvae can destroy significant percentages of corn if left untreated. In the United States, current estimates show that 30 million acres of corn (out of 80 million grown) are infested with corn rootworms, and the number of infested acres is expected to grow over the next 20 years. Corn rootworms can cause significant reductions in yield and revenue for growers. The United States Department of Agriculture estimates that corn rootworms cause $1 billion in lost revenue each year, which includes $800 million in yield loss and $200 million in cost of treatment for corn growers. Recently, corn rootworms have been introduced into Europe and a new variant that is unaffected by crop rotations has developed underscoring the need for additional control tools.

DAS-59122-7 provides season-long root protection from larval corn rootworm feeding damage, which allows corn plants to remain healthier and less susceptible to environmental stresses throughout the growing season. Full-season protection against corn rootworms and other stress factors allows maize hybrids to reach their full genetic yield potential. DAS-59122-7 can also increase farmer's productivity by reducing various inputs (e.g., labor, fuel, equipment, pesticides) typically required for conventional corn rootworm control programs.

In addition to insect protection, maize hybrids containing event DAS-59122-7 provide tolerance to glufosinate-ammonium herbicides by the expression of the enzyme phosphinothricin acetyltransferase (generally referred to as PAT). Corn plants possessing this tolerance can be directly sprayed after emergence with glufosinate-ammonium herbicides, allowing for broad spectrum weed control without herbicide damage to the maize plant. Benefits to the farmer include convenient and effective weed control that ultimately enhances yield potential for the maize.

As of July 2007, event DAS-59122-7 is currently approved for cultivation in the United States and Canada, and goes by the trade name Herculex® RW *Rootworm Protection*. DAS-59122-7 is also approved for import of grain in Japan, Korea, Mexico, Philippines, Australia/New Zealand and Taiwan. DAS-59122-7 is the corn rootworm event in the combined trait product known as Herculex® XTRA *Insect Protection* (which also contains Bt event TC1507 (Herculex® I *Insect Protection*) for protection against above-ground, lepidopterous insects). Herculex XTRA is approved for cultivation in the US and Canada and for import of grain in Japan, Australia/New Zealand, Mexico and Korea.

Identity of DAS-59122-7

Table 1 summarizes the genetic elements and characteristics of DAS-59122-7

Table 1. Identity of DAS-59122-7	
Brand Names	Herculex® RW *Insect Protection,* Herculex® XTRA *Insect Protection*
Synonyms	DAS-59122-7, 59122-7, Cry34Ab1 and Cry35Ab1 maize (corn)
Trait Origin	*Bacillus thuringiensis* strain PS149B1
Common Protein Name	Cry34Ab1, Cry35Ab1, PAT (phosphinothricin acetyltransferase)
Gene Identification	*cry*34Ab1*, cry*35Ab1*, pat*
Crop	Maize (*Zea mays*)
Construct Number	PHP17662
Construct Description	See figure 1 in the next section.
Event	DAS-59122-7
Protein Weight/Size	Cry34Ab1 (123 amino acids, 13.6 kDa) Cry35Ab1 (383 amino acids, 43.8 kDa)
Marker Gene Protein:	PAT (phosphinothricin acetyltransferase)

Plant Transformation & Modification Characteristics

Gene Construct. The first step in creating DAS-59122-7 maize was the isolation and replication of DNA segments (genes) from *Bacillus thuringiensis* strain PS149B1 that were responsible for the expression of the desired insecticidal crystal proteins (Cry34Ab1 and Cry35Ab1). The isolated genes were then chemically re-synthesized to create the final transgenes, including plant-preferred DNA that optimizes expression of the insecticidal crystal protein in the plant.

As shown in Figure 1, the gene construct for DAS-59122-7 is comprised of a protein coding region (*cry*34Ab1 and *cry*35Ab1 genes), a preceding promoter element and a trailing regulatory element. The promoter element determines the strength at which a trait will be expressed in the plant. The promoter also determines in which plant tissues the trait will be expressed. The trailing regulatory element defines the length of the DNA to be expressed. In DAS-59122-7, the ubiquitin promoter from maize (UB1ZM) is used for the *cry*34 gene and the promoter from the *Triticum aestivum* (wheat) peroxidase gene (TA peroxidase pro) while its native leader sequence is used for the for *cry*35 gene. Trailing regulator element for both *cry* genes is from the *Solanum tuberosum* (potato) proteinase inhibitor II gene (PINII). The promoter and trailing regulatory elements have no effect on the structure of the Cry34Ab1 and Cry35Ab1 proteins, only on the expression of the gene.

A “selectable marker gene” is normally associated with a gene of interest during plant transformation. Selectable marker genes can be used in both the laboratory and the field to quickly determine if plants contain the desired genes and are expressing the desired proteins. For DAS-59122-7, the *pat* gene is used as the selectable marker. The *pat* gene, which is located next to the *cry*35Ab1 gene, is controlled by a different promoter and trailing regulatory element (both of which are from cauliflower mosaic virus - CaMV35S). The *pat* gene expresses the PAT protein and provides plant tolerance to glufosinate-ammonium herbicides. PAT facilitates the selection of plants containing Cry34Ab1 and Cry35Ab1 in the laboratory and field. It is critical that both Bt proteins and PAT protein expressions are maintained at their optimal level in order for the trait to exhibit its characteristics in corn plants.

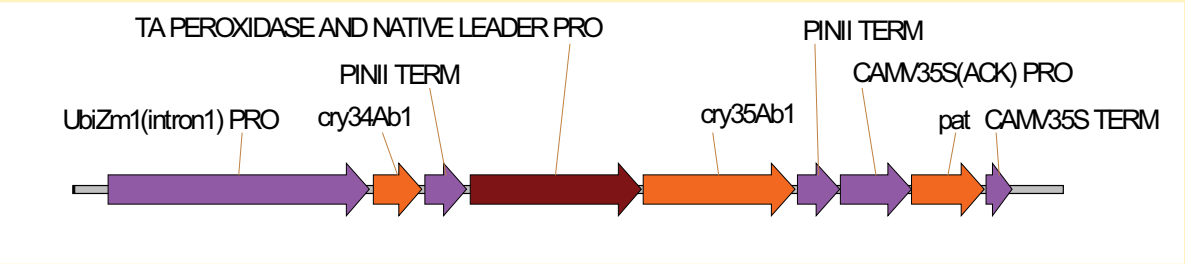

Fig. 1: DNA construct used to produce maize with DAS-59122-7

Characteristics & Genetic Stability of the Introduced DNA. Transgenic maize containing event DAS-59122-7 was produced by *Agrobacterium*-mediated transformation of the public maize line Hi-II with the gene construct shown in figure 1. Southern blot analysis of the genomic DNA of DAS-59122-7 demonstrated that the T-DNA from plasmid PHP17662 was inserted as a single, intact copy into the corn genome. All three novel genes, *cry*34Ab1, *cry*35Ab1, and *pat*, along with their respective promoter and trailing regulator elements, were completely integrated. The results from several rounds of backcrossing and self-crossing demonstrate that the *cry*34, *cry*35 and pat genes are stable. Observations of the phenotype indicated that the transgenes are inherited as a single dominant element according to Mendelian segregation patterns.

Expressed Material. The expression levels of Cry34Ab1, Cry35Ab1 and PAT proteins have been measured from different plant stages of field-grown DAS-59122-7 maize hybrids using ELISA (Enzyme Linked Immunosorbent Assay) specifically developed for each protein. Non-transgenic maize with comparable background genetics as DAS-59122-7 hybrids was also tested from the same field sites. Western immunoblot analysis was used to confirm the integrity of the newly expressed proteins. As shown in Table 2, Cry34Ab1 and Cry35Ab1 proteins are detectable in all plant tissues, and provide stable expression in root tissue across different growth stages.

Expression of the PAT protein was found in leaf tissue samples at levels are sufficient to confer tolerance to glufosinate-ammonium herbicide at the level of the whole plant. As expected, expression of the Cry34Ab1/Cry35Ab1 and PAT proteins was not detected in any samples from non-transgenic control plants with the same genetic background as the DAS-59122-7 hybrids.

Table 2. Expression of DAS-59122-7 in Plant Tissues from a Field-Grown Maize Hybrid

Stage and Tissue	ng Cry34Ab1/mg tissue dry weight	ng Cry35Ab1/mg tissue dry weight
V6 Roots	46.3 ± 14.0	13.5 ± 4.4
V9 Roots	51.8 ± 19.2	13.6 ± 5.2
R1 Roots	54.9 ± 20.6	10.4 ± 4.7
V9 Leaf	67.4 ± 20.7	43.3 ± 12.9
R1 Pollen	68.1 ± 5.3	0.14 ± 0.14
R1 Stalk	57.1 ± 6.1	17.5 ± 2.3
Maturity, Grain	45.7 ± 9.5	1.61 ± 0.07
Senescent whole plant	124.6 ± 38.2	33.4 ± 6.5

Mode of Action

Cry34Ab1 and Cry35Ab1. Cry34Ab1 and Cry35Ab1 proteins are both required for high activity against corn rootworm larvae. The mode of action for these proteins in DAS-59122-7 is generally similar to other *Bt* insecticidal crystal proteins. Like other *Bt* Cry proteins, Cry34Ab1 and Cry35Ab1 are delta endotoxins that must be ingested to kill the insect, and act by binding to and disrupting the integrity of the midgut membranes. The combination of Cry34Ab1 and Cry35Ab1 is lethal only when eaten by the larvae of certain coleopteran insects (i.e., corn rootworms).

In addition to direct toxicity, maize hybrids with DAS-59122-7 also show evidence of altering the feeding behavior of larval corn rootworms, resulting in less tunneling damage than typically seen in roots of the same non-*Bt* isoline.

PAT. Phosphinothricin-N-acetyltransferase (PAT) is an enzyme isolated from the common soil bacterium *Streptomyces viridochromogenes*, and is used as the selectable marker gene in event DAS-59122-7. PAT also imparts plants containing event DAS-59122-7 with tolerance to the herbicide glufosinate-ammonium. Glufosinate-ammonium (developed from the same bacteria as PAT) affects plant tissues by reducing glutamine levels and causing a corresponding increase in concentrations of ammonia. Excess ammonia disrupts cell membranes, stops photosynthesis and eventually results in plant death. PAT converts L-PPT (L-phosphinotricin), the active ingredient in glufosinate-ammonium herbicide, to its inactive form N-acetyl-L-PPT. Maize containing event DAS-59122-7 can be sprayed with this herbicide to control targeted weeds with no crop loss.

Biological Activity

Bt proteins are highly selective to certain categories and species of insects. Cry34Ab1 and Cry35Ab1 proteins in event DAS-59122-7 are specific to larvae of damaging corn rootworm species listed in Table 3. Multi-year field trials have shown maize hybrids with event DAS-59122-7 to provide outstanding and highly consistent root protection, even under adverse environmental conditions and high rootworm feeding pressure.

Cry34Ab1 and Cry35Ab1 proteins are expressed at effective concentrations during all growth stages of the maize plant. Thus, DAS-59122-7 maize provides season-long protection from corn rootworms, and allows maize hybrids to reach their full genetic yield potential. Root protection afforded by DAS-59122-7 maize can also result in better plant vigor and less stress under dry conditions when compared to non-*Bt* maize.

Table 3. List of Corn Rootworm Species Controlled by DAS-59122-7	
Scientific name	**English Common Name**
Diabrotica virgifera virgifera	Western corn rootworm
Diabrotica barberi	Northern corn rootworm
Diabrotica virgifera zeae	Mexican corn rootworm

Corn Rootworm Larvae in Soil

Corn Rootworm Larvae Burrowing in Root Tip

Adult Western Corn Rootworm

Toxicity & Allergenicity

The United States Environmental Protection Agency (EPA) found that DAS-59122-7 and other *Bt* lepidopteran-resistant maize products do not pose unreasonable risks to human health. Cry34Ab1, Cry35Ab1 and PAT are present in soil bacteria and the bacteria are not considered as pathogens for humans or animals.

Exposure to Cry34Ab1 and Cry35Ab1 via skin or inhalation is not likely since the protein is contained within plant cells, which essentially eliminates these exposure routes or reduces these exposure routes to negligible. Oral exposure, at very low levels, may occur from ingestion of processed maize products. However, rapid digestibility and a lack of mammalian toxicity for Cry34Ab1 and Cry35Ab1 has been demonstrated. Healthy mice demonstrated lack of acute toxicity after ingesting a dose of Cry34Ab1, Cry35Ab1 or PAT protein many thousand times the estimated dietary intake of humans (Table 4). In addition, a thirteen-week (90-day) oral toxicity feeding study in rats was carried out with grain from maize containing event DAS-59122-7 and non-transgenic maize grain. No significant diet-related differences were observed in the development or well being of the rats.

Neither Cry34/Cry35Ab1 nor PAT has the biochemical characteristics or homology (relevant similarities) with known food allergens or toxins, indicating that maize with event DAS-59122-7 is highly unlikely to pose any risk of allergic reaction. Additional information on toxicology and allergenicity testing on the proteins expressed in DAS-59122-7 can be found in the EPA documents (BRAD, Fact Sheets) listed in the references of this bulletin.

Table 4. Mammalian Toxicity of DAS-59122-7		
Test	**Species**	**Toxicity Parameters**
Acute Oral (PAT)	Mouse	LD_{50} > 5000 mg/kg
Acute Oral (Cry34Ab1)	Mouse	LD_{50} > 2700 mg/kg protein (highest dose tested)
Acute Oral (Cry34Ab1)	Mouse	LD_{50} > 1850 mg/kg protein (highest dose tested)
Acute Oral (Cry34Ab1 & Cry35Ab1)	Mouse	LD_{50} > 2000 mg/kg protein (highest dose tested)
90 Day Feeding (DAS-59122-7 grain as 35% of diet)	Rat	No adverse effects NOEL > 35% of diet

Compositional Analysis and Nutrition

A key component in the regulatory approval process is the demonstration that a crop derived through biotechnology is “substantially equivalent” to a non-biotech crop. Forage and grain from DAS-59122-7 maize have been analyzed for nutritional composition and compared to the nutritional composition of non-transgenic versions of the same maize hybrids. Nutrients like fat, protein, fiber, minerals and vitamins were measured and all values for nutrients in DAS-59122-7 remained within the normal range of variation reported for maize. The levels of potential anti-nutrient compounds in maize (including phytic acid and trypsin inhibitor), were analyzed in grain from maize with event DAS-59122-7 and non-transgenic line. There were no differences in the levels of antinutrients between the transgenic and non-transgenic lines. The results of these compositional analyses led to the conclusion that DAS-59122-7 forage and grain is not different in nutritional and anti-nutritional composition compared to maize hybrids currently marketed, grown and consumed.

Broiler chickens are sensitive to changes in nutrient quality in diets, and serve as a useful model species to evaluate the wholesomeness of food and feed. A chicken broiler study has been conducted on grain harvested from DAS-59122-7 hybrids. No differences in nutritional quality or growth of the broilers were observed between those animals that consumed the transgenic maize and those that were fed conventional maize. These results support the findings of the compositional analyses and indicate that maize with event DAS-59122-7 was equivalent to non-transgenic maize in the ability to provide adequate nutrition to rapidly growing broiler chickens.

Photo courtesy ofUSDA-ARS

In addition to poultry, cattle fed grain from DAS-59122-7showed no differences in field performance and carcass charateristics when compared to a non-transgenic near isoline hybrid.

Environmental Information

Following U.S. federal agency guidelines, maize with DAS-59122-7 was extensively tested to evaluate its impact on the environment.

Soil Metabolism. Maize with DAS-59122-7 produces minute quantities of the Cry34Ab1 and Cry35Ab1 proteins, contained in the plant and plant parts such as roots, leaves, and pollen. These proteins degrade rapidly in the soil, minimizing the potential for run-off or exposure to soil-dwelling non-target organisms. Based on a bioassay with the southern corn rootworm (*Diabrotica undecimpunctata*), purified Cry34Ab1/Cry35Ab1 proteins incorporated into test soils biodegraded with a half-life of approximately 3.2 days. This half-life is very comparable with the 4-7 days in published reports for other Cry proteins. Submitted registration data indicates that minimal short term accumulation of Cry34/35Ab1 protein in agricultural soil is expected.

Non-Target Organisms. A number of non-target invertebrates were tested as part of the registration process for event DAS-59122-7 and no adverse effects were observed at concentrations that greatly exceed levels of Cry 34Ab1/Cry35Ab1 proteins expected to be found in the environment. Some of the invertebrates tested in laboratory feeding studies included honeybees, earthworms, lady beetles, monarch butterfly larvae, springtails and green lacewings (Table 5).

Field studies have been conducted annually since 2001 to determine the impact of DAS-59122-7 maize on natural non-target arthropod populations. The results were compared with non-transgenic maize as well as conventional insecticides used for rootworm control. To date, no adverse effects have been observed when comparing the abundance of non-target organisms in DAS-59122-7 field plots compared to non-transgenic maize field plots.

Honeybee, *Apis melifera*

Lady Beetle, family Coccinellidae

Based on studies with bobwhite quail and broiler chickens, no adverse effects are expected on avian wildlife from incidental field exposure to DAS-59122-7 maize. Moreover, maize with DAS-59122-7 is also unlikely to present hazardous effects to fish and aquatic invertebrates, due to low toxicity (as measured on Rainbow Trout and Daphnia) and low exposure potential from maize.

Endangered species. Because of the selectivity of Cry34/Cry35Ab1 proteins, endangered species concerns primarily focus on the insect order Coleoptera. The distribution of most endangered coleopteran species does not overlap with maize production areas. The exception is the American burying beetle, which lives in areas where maize is grown. However, based on the preferred habitats and feeding behavior of the American burying beetle, the EPA concluded that there would be no exposure to significant levels of Cry34Ab1/Cry35Ab1 proteins (both larvae and adult insects feed on carrion with some limited adult predation on corn).

Outcrossing. Another important environmental consideration with a transgenic product is how cross-pollination will affect the environment. Gene exchange between maize with DAS-59122-7 and other cultivated maize varieties can occur where such lines are cultivated in close proximity. The exchange will be similar to that which occurs naturally between cultivated corn varieties at the present time. In the U.S. and Canada where DAS-59122-7 maize is approved for cultivation, there is no plant species closely related to maize in the wild; therefore, the risk of gene flow to other species is unlikely. Maize is not a weed and persists in the environment only with human intervention. There is no selective advantage for maize hybrids with DAS-59122-7 in the natural environment.

Table 5. Non-Target Test Results on DAS-59122-7

Study	Species	Value
Aquatic		
48-hr Acute toxicity	Water flea (*Daphnia magna*)	EC_{50} > 150 mg/L
Avian		
5-d Chronic dietary	Bobwhite quail	No adverse effects: NOEC > 57% grain in diet
42-d Oral dietary	Broiler chickens	No adverse effects: NOEC > 63% grain in diet
Earthworm		
14-d Acute toxicity	Earthworm (*Eisenia fetida*)	NOEC > 25.4 mg / kg dry soil
Bees and Beneficial Insects		
Dietary tox, development, 3-d-old larva to adult	Honeybee (*Apis mellifera*)	LD_{50} > 5.6 µg protein/larvae
Dietary, limit dose	Green lacewing larvae *(Chrysoperla carnea)*	LC_{50} > 280 µg protein/g diet
Dietary limit dose	Lady beetle adult *(Hippodamia convergens)*	LC_{50} > 280 µg protein/g diet
Dietary limit dose	Parasitic hymenopteran adult *(Nasinia vitripennis)*	LC_{50} > 280 µg protein/g diet
Other Non-Target Organisms		
9-d dietary	Monarch butterfly larvae (*Danaus plexippus*)	NOEC >3200 grains of pollen per larvae consumed at 96 h.
Chronic survival and reproduction	Collembola (springtail) (*Folsomia candida*)	NOEC > 12.7 mg/kg soil

Stewardship

Insect Resistance Management (IRM). There is a potential long-term risk of corn rootworm adaptation to the Cry34/Cry35Ab1 proteins leading to the possibility of reduced efficacy. In order to prolong the effectiveness of plant-expressed *Bt* insecticidal proteins, and the microbial spray formulations of these same proteins, regulatory authorities in Canada and the United States have required developers to implement specific Insect Resistance Management (IRM) Programs.

These programs are mandatory for all transgenic *Bt*-expressing plants, including DAS-59122-7 maize, and require that growers plant a certain percentage of their acreage to non-*Bt* varieties in order to reduce the potential for selecting Bt-resistant insect populations. For DAS-59122-7 maize, currently growers in Canada and the United States must agree to plant at least 20% of their maize acres in maize refuge (without corn rootworm specific *Bt*). In addition, the refuge must be planted within or adjacent to the field planted with DAS-59122-7 maize. The refuge can be managed with non-*Bt* insecticides including seed treatments, granular soil insecticides and foliar insecticides, depending on the pest target and population pressure.

Additional IRM options are available for hybrids with combined *Bt* gene products (e.g., Herculex XTRA) that target both above-ground insects and corn rootworms to ensure effective resistance management for the broader range of pests. Details on the specific design and requirements of individual IRM programs are defined by the relevant regulatory authority and subject to annual updates. IRM requirements for growers can also be found in registrant product use guides and web sites and should be checked at least annually.

Quality Assurance. A quality assurance (QA) program for DAS-59122-7 maize is in place with all licensees to ensure that maize seed sold with this event meets minimum requirements for product performance (e.g., gene expression, efficacy and agronomics). The QA program also ensures that parent maize seed with DAS-59122-7 contains the correct event and does not contain unintended events. Seed materials containing DAS-59122-7 are also expected to meet or exceed genetic purity standards established in the Federal Seed Act (95%, Regulations Part 201.7 and 201.11a), OECD Seed Schemes, and/or the Association of Official Seed Certifying Agencies (AOSCA).

Diagnostic Tests. Validated methods to detect the *cry*34Ab1 and *cry*35Ab1 genes, as well as the Cry34Ab1 and Cry35Ab1 proteins in transgenic plant material have been developed by independent companies for use in quality assurance programs and field investigations. For example, qualitative ELISA (Enzyme-linked immuno-sorbent assays) is used to detect the presence or absence of the Cry34Ab1 in the plant. Qualitative ELISA tests can be conducted in microtiter plates or with convenient strips, and can be performed in the laboratory or field. Quantitative ELISA tests estimate the amount of protein in the plant. They require an approved laboratory setting (microtiter plates) and significant calibration effort.

A PCR (polymerase chain reaction) method has also been developed for event-specific detection of DAS-59122-7 at the DNA level. This test ensures that seed contains the correct DAS-59122-7 event. PCR tests are also used to ensure that seed does not contain unintended events. Dow AgroSciences maintains a list of vendors with validated test kits.

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Additional references and information for DAS-59122-7 and other *Bt* traits can be found at AGBIOS web site: http://www.agbios.com.

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