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POSITION PAPER
Detection of Combined Events (Stacks)
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Scope

Combined event plant biotechnology products (sometimes called stacks) are those containing more than one biotechnology-derived event. Combined event products can be produced in different ways, but this position paper addresses only detection of combined event products produced through conventional breeding (crossing of plants carrying individual events), or by crossing of two plants containing different events during cultivation (for example in the farmers' fields). Events containing more than one gene in the same insertion site, which reside in the same locus in the plant genome (often referred to as vector or molecular stacks), are not covered by this document.

What is an event?

In plant biotechnology, this term refers to the specific genetic line characterised by the insertion of a particular foreign gene (or genes) sequence into the genetic material of a plant (expressed as a combination of what is inserted and where it appears in the plant genetic material). Events are typically created in the laboratory and then analysed in the greenhouse, growth chamber and eventually in the field. However, only a few of the many events produced will be identified as of commercial interest and only one carrying the desired trait will be commercialised.

What is a stacked event?

A stacked event contains two or more individually-created events residing in the same plant (or seed). An example is the combination of an event expressing herbicide resistance and an event expressing insect resistance. Such a combination may be produced by conventional breeding or by the pollination of one plant by another in the field.

Seed stacks, field stacks and seed mixtures

A seed stack is a stack in which the events have been deliberately combined during the breeding process to all be present in every seed in the lot. A field stack is an adventitious combination of events arising from open pollination in the field. Seed mixtures refer to the event combinations that may occur in the commodity chain when grain is mixed which may or may not contain the same events, different events, seed stacks, or field stacks. Thus commodity grain potentially contains seed mixtures of a variety of commercial events.

Detection and identification of individual events

An individual event can be detected by a number of means. However, if the intention is to detect the event specifically (to the exclusion of other possible related events) the standard method today is DNA analysis by locus- or event-specific Polymerase Chain Reaction (PCR).

Event-specific PCR usually relies on the anchoring of one PCR primer in the DNA inserted into the plant during the transformation process, and one PCR primer anchored in the DNA adjacent to the insertion site (known as flanking sequence). In real-time PCR, a probe will also be defined, which may be located either in the introduced DNA or the original plant DNA. PCR, when properly validated, will only amplify DNA from the specific event to the exclusion of non-transformed plant lines and all other events.

Detection and identification of stacks

In most, if not all cases, the inserted DNA of different events is located on different chromosomes or at different sites on the same chromosome, often many millions of base-pairs apart. Event-specific PCR cannot bridge gaps greater than a few hundred base-pairs, and thus cannot be designed to specifically identify stacks unless a single plant or seed is tested. By analyzing an individual plant or seed with multiple event-specific PCR methods, it can be established if the individual contains stacked events. However, the results cannot differentiate between intentional seed stacks and adventitious field stacks, because on a molecular level these two types of stacks appear identical.

If a number of seeds (or more commonly grains from a bulk shipment) are combined and subjected to PCR as a bulk sample results may be obtained that show that more than one event is present in a sample. However, the source of the events can be one of several options: a mix of seeds containing non-biotech and individual events; a mix containing non-biotech and stacked events; or a mix containing non-biotech, individual events and stacked events. This is especially difficult to analyse if the results indicate a low level of events in the sample. This can be illustrated by an example of samples each containing 1000 seeds/grains:

Table 1: Expected results of qualitative event-specific PCR tests on samples containing single and stacked events.

Sample #	Number of each type of seed in the sample					
	Event 1	Event 2	Event1 + Event2 stack	Non-transgenic	Theoretical Result PCR1	Theoretical Result PCR2
1	1	0	0	999	Positive	Negative
2	0	1	0	999	Negative	Positive
3	0	0	1	999	Positive	Positive
4	1	1	0	998	Positive	Positive
5	0	1	1	998	Positive	Positive
6	1	0	1	998	Positive	Positive
7	1	1	1	997	Positive	Positive

Samples 3 through 7 are indistinguishable as they all yield positive signals for both events, even though sample 4 contains no stacked seeds, samples 5-7 contain a mixture of stacked and individual events, and sample 3 contains only the stack. In this case, the only conclusions possible are the presence of event 1 in sample 1, of event 2 in sample 2 and of both events in samples 3-7. It is not possible to determine the source of the two events in samples 3-7 – that is, whether the signal is from single or stacked events.

The question then arises as to whether stacks can be detected using real-time quantitative PCR. Table 2 shows an example of the quantitative results that might be expected from the same types of samples as were examined in Table 1.

Table 2: Expected results of quantitative event-specific PCR tests on samples containing single and stacked events.

Sample #	Number of each type of seed in the sample					
	Event 1	Event 2	Event1 + Event2 stack	Non-transgenic	Theoretical Result PCR1	Theoretical Result PCR2
1	2	0	0	998	Positive (~0.2%)	Negative
2	0	2	0	998	Negative	Positive (~0.2%)
3	0	0	2	998	Positive (~0.2%)	Positive (~0.2%)
4	2	2	0	996	Positive (~0.2%)	Positive (~0.2%)
5	1	2	1	996	Positive (~0.1%)	Positive (~0.3%)
6	2	1	1	996	Positive (~0.3%)	Positive (~0.1%)
7	1	1	2	996	Positive (~0.2%)	Positive (~0.2%)
8	2	3	0	995	Positive (~0.2%)	Positive (~0.3%)
9	3	2	0	995	Positive (~0.3%)	Positive (~0.2%)
10	2	2	1	995	Positive (~0.3%)	Positive (~0.3%)

The first observation is that samples 3 and 4 give exactly the same results. In samples 5-7, there appears to be small differences that might be interpreted as a combination of seed mixtures and stacked events. However, there are other mixtures (8-9) which can give the same results without stacks being present or the same result with a different proportion of stacks present in the sample. Moreover, because of sampling and measurement uncertainty, quantitative PCR is not accurate enough to distinguish between, for example, 0.2% and 0.3% of an event in a sample.

Other approaches

To date there is no PCR or other molecular approach to testing that can distinguish between the presence of a low (or high) percentage of stacked events in a bulk sample, and the presence of a mixture of the two or more individual events that comprise the stack.

The International Seed Testing Association (ISTA) has proposed a statistical approach (to be implemented in Seedcalc9 www.seedtest.org) to quantify stacked and non-stacked seeds in a conventional seed lot. The approach is a pooled testing approach and involves the examination of as many as 10-20 pools. However, if the percentage of positive seeds in the sample is higher than a few percent of the seeds, the model may not give clear results.

Conclusions

Due to the nature of combined events (stacks) where two or more events are combined using plant breeding, it is not possible to use PCR or any other molecular approach to determine the presence of a stacked seed or grain as opposed to the presence of the two component events, unless single seeds/grains are examined. Even when a stack of two or more events is present, it cannot be determined in grain if this stack is an intentional combination, or one due to adventitious pollination in the field.

Reference materials (RM):

Reference materials for all commercialized events are available for the single events. As combined events (stacked) seeds and plants consist of combinations of these individual events, the single event reference materials are suitable for calibration or validation of methods used for the stacked events.