Bayerisches Landesamt für
Gesundheit und Lebensmittelsicherheit

LGL

Evaluation of methods for the unequivocal identification of single-mutations derived from NGT

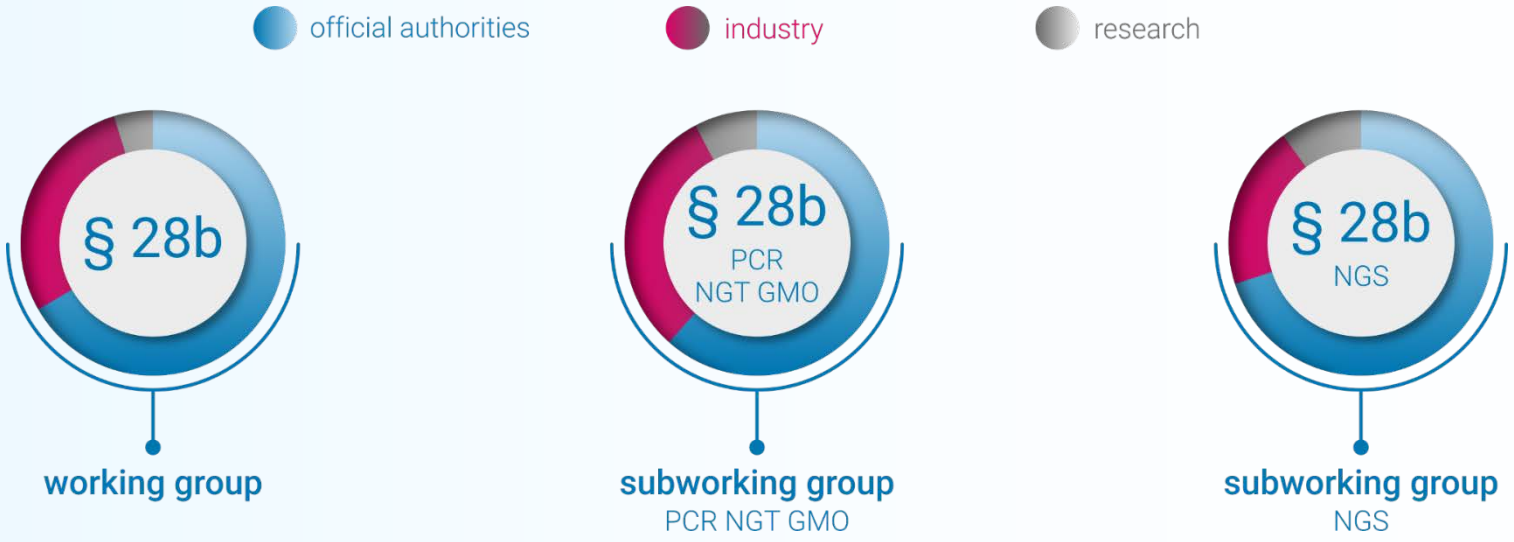
Dr. Patrick Gürtler / § 28b Genetic Engineering Act (GenTG) Working Group

§ 28b German Genetic Engineering Act (GenTG) Working Group

§ 28b Genetic Engineering Act (GenTG)

(1) The competent higher federal authority [BVL, Federal Office of Consumer Protection and Food Safety] shall, in consultation with the authorities responsible for food and feed legislation, publish an official collection of methods for sampling and examination of samples carried out or used in the context of the monitoring of genetic engineering activities, genetic engineering facilities, release of genetically modified organisms and the placing on the market.

(2) The procedures shall be established with the participation of experts from the fields of official control, research and the industry involved. The collection shall be kept up to date on an ongoing basis.



§ 28b Genetic Engineering Act (GenTG) Working Group

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Detection (and identification) of NGT organisms

Challenges for method development and NGT identification

01 – collecting information on NGT organisms

- technique used for modification
- modification introduced into NGT organism
- sequence information

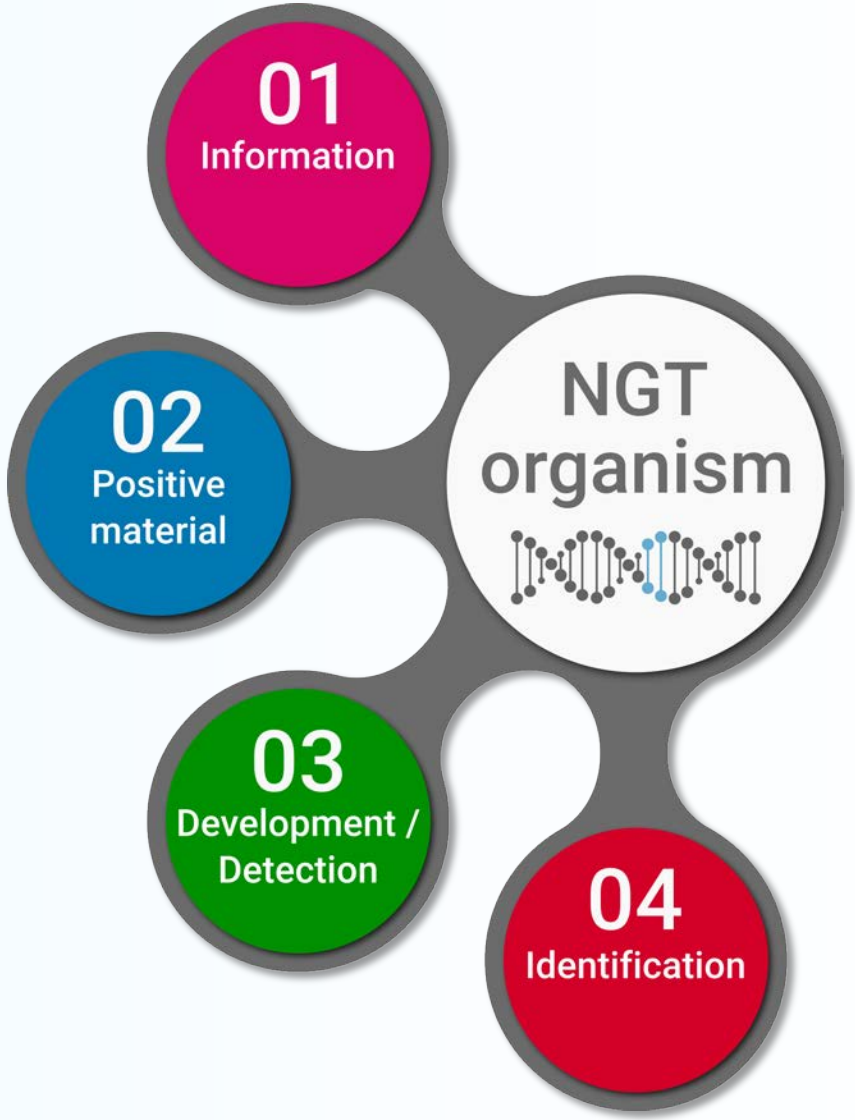
02 – availability of positive material

- material provided by companies or commercially available material
  ⇨ ideally certified reference material (CRM)
- synthetic plasmids

03 – development of modification-specific methods / detection

- screening methods
- specific methods ⇨ suitable for routine analysis
- innovative methods and new approaches

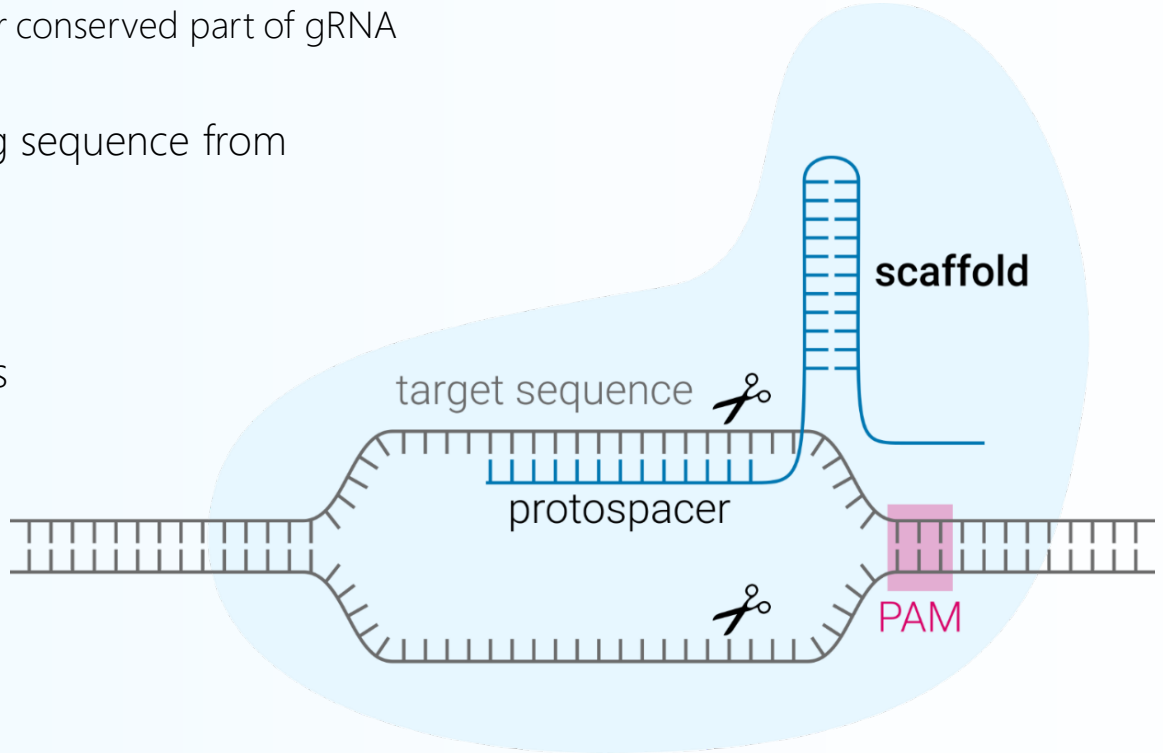
04 – identification



Detection (and identification) of NGT organisms

Screening for remaining residues of **CRISPR/Cas9 machinery**

- Cas9
- guide RNA (gRNA)
  - **protospacer sequence** ⇨ variable
  - scaffold structure ⇨ **gRNA scaffold coding sequence** ⇨ rather conserved part of gRNA
- method development for detecting the gRNA scaffold coding sequence from
  - *Streptococcus pyogenes*
  - *Staphylococcus aureus*
- ongoing interlaboratory trial with 14 participating laboratories (Germany, Austria, Switzerland, Italy)
- applicable in certain cases
  - monitoring of stably transformed NGT plants
  - monitoring activities at genetic engineering facilities
  - monitoring of ornamental plants



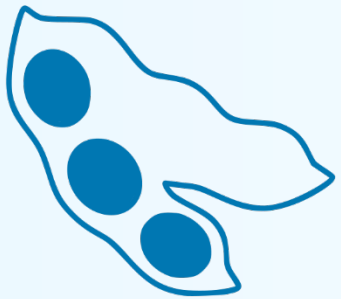
Detection (and identification) of NGT organisms

Several market-relevant NGT organisms have been identified for which information is available

Oilseed rape

Cibus

single base substitution
herbicide tolerance



Soybean

Calyxt

small deletions
altered fatty acid composition

Corn

Corteva Agriscience

large deletion
altered starch composition

Tomato

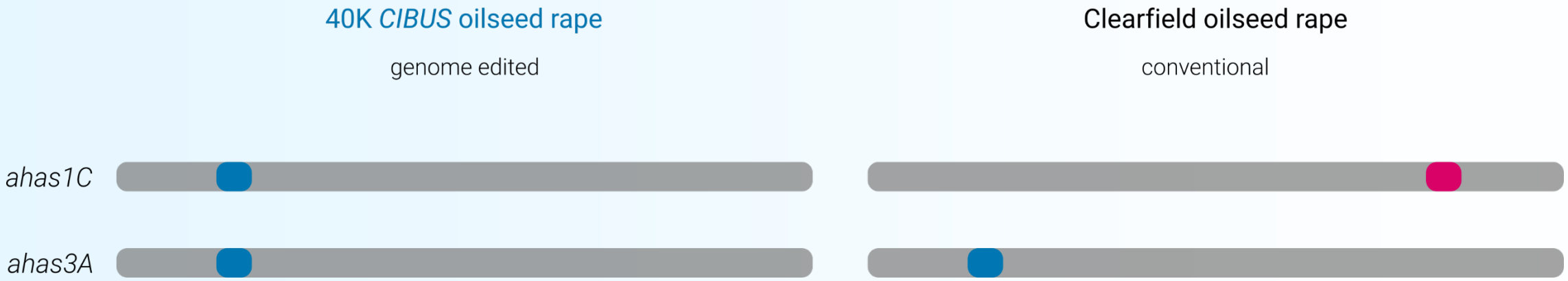
Sanatech Seed

single base insertion
high GABA content

Detection (and identification) of NGT organisms

Herbicide-tolerant (HT) oilseed rape (*Cibus*)

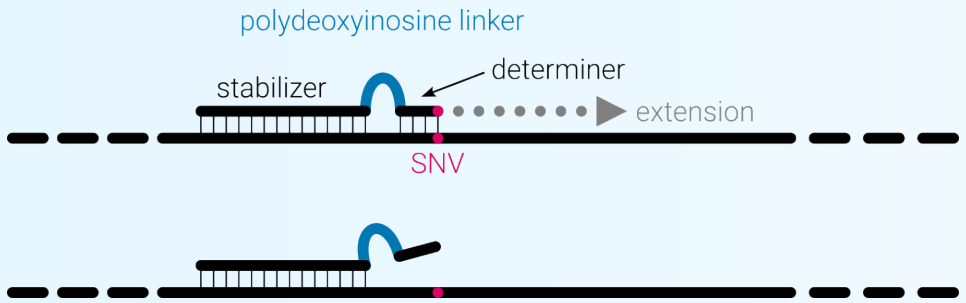
- **sequence** information
  - HT oilseed rape: SNV in *ahas1C* and *ahas3A* confers tolerance to sulfonylurea (SU) herbicides due to amino acid substitution
  - *Clearfield* oilseed rape (conventional): SNV in *ahas3A* and downstream *ahas1C*
- information on the incorporated **mutation** and the applied **modification technique** (as part of an ODM application)
- **positive material** provided by *Cibus* under a Material Transfer Agreement (MTA)
- development / optimization phase (qPCR/dPCR/NGS)
- additional mutations are taken into account for a combined detection approach

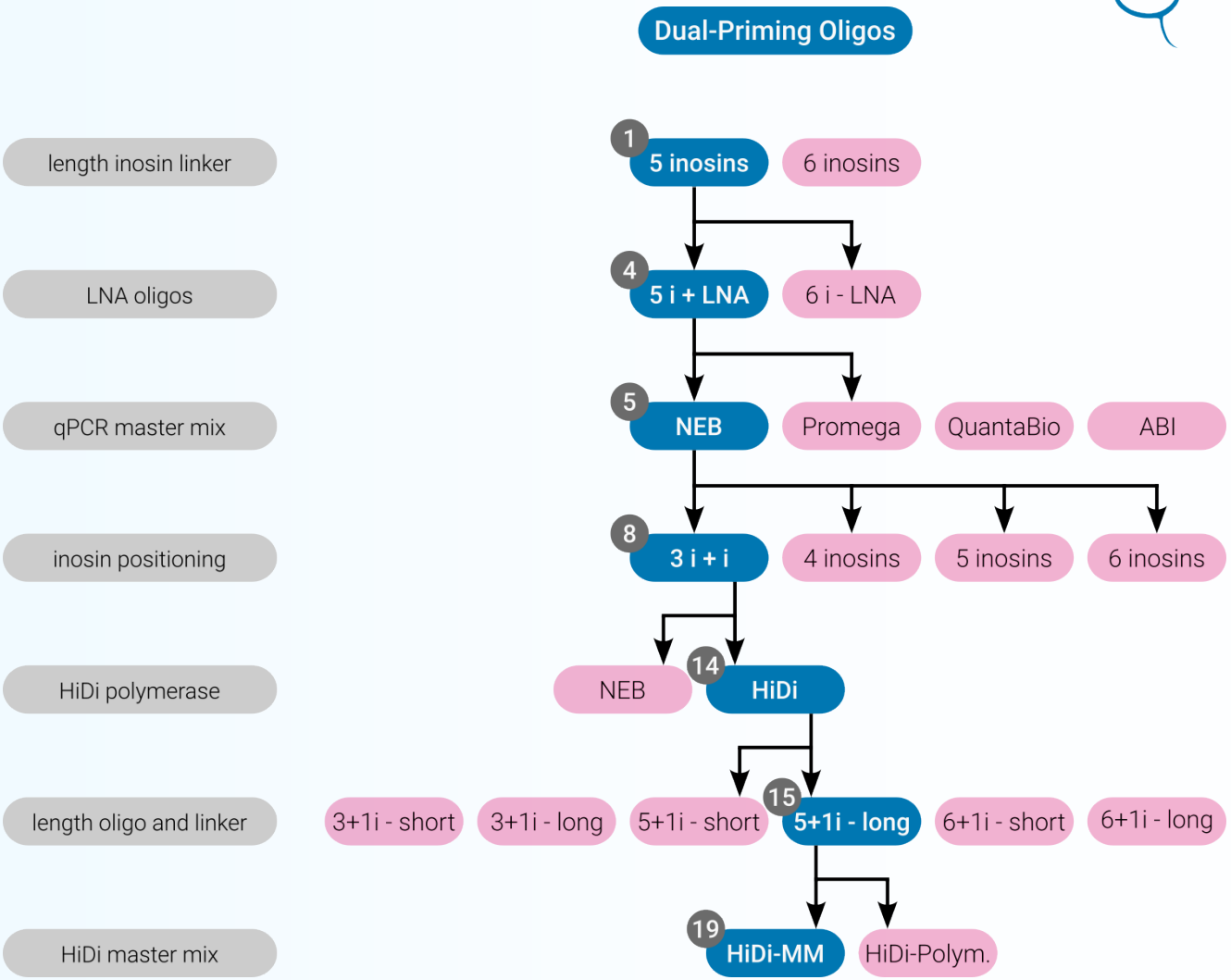


Detection (and identification) of NGT organisms

Herbicide-tolerant oilseed rape –
Development of modification-specific methods

- qPCR- / dPCR- and NGS-based methods
  - still ongoing development / optimization
  - unspecific amplification signals
- Project* at LGL (**Dr. Steffen Heinz**) explores new approaches for SNV detection
  - dual-priming oligos (DPO; Seegene)
  - high discrimination polymerase (HiDi; myPOLS)



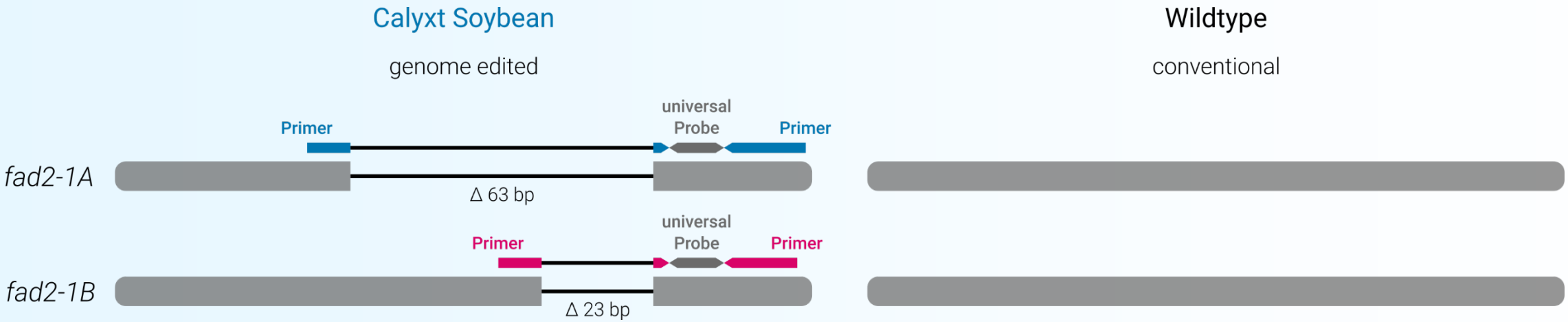


*funded by Bavarian State Ministry of the Environment and Consumer Protection (StMUV); figure based on work by **Dr. Steffen Heinz**

Detection (and identification) of NGT organisms

Calyno™ Soybean (*Calyxt*)

- **sequence** information published
  - 63 bp deletion in *fad2-1A* gene variant
  - 23 bp deletion in *fad2-1B* gene variant
- **altered fatty-acid composition** (▲oleic acid content, ▼linoleic acid content)
- information on the incorporated **modification** and the applied **modification technique** (TALEN)
- **no positive material** available ⇨ synthetic plasmids used for method development and in-house-validation



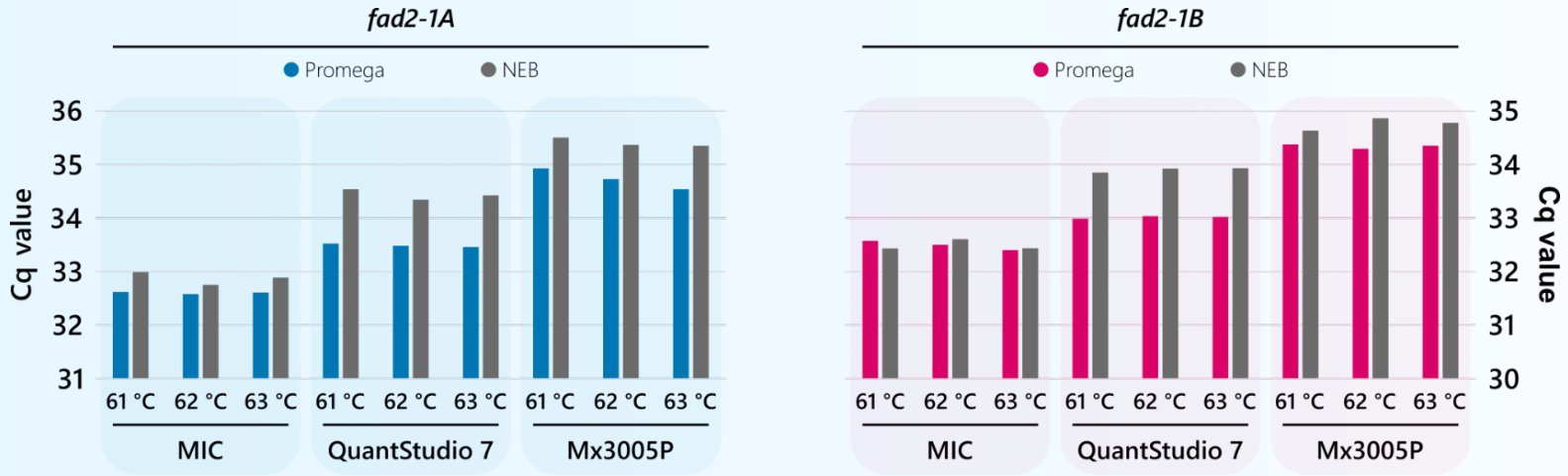
Haun, W., Coffman, A., Clasen, B. M., Demorest, Z. L., Lowy, A., Ray, E., ... & Zhang, F. (2014). Improved soybean oil quality by targeted mutagenesis of the fatty acid desaturase 2 gene family. *Plant biotechnology journal*, *12*(7), 934-940.

Detection (and identification) of NGT organisms

Calyno™ Soybean (*Calyxt*)

- in-house validation of developed detection methods for deletions in ***fad2-1A*** and ***fad2-1B***
  - $LOD_{95\%}$ of 3-5 cp/PCR and qPCR efficiency of >94 %
  - no unspecific amplification signals
  - Robustness

    Variation of oligos (-30%), temperature (± 1 °C), qPCR cycler and master mix product



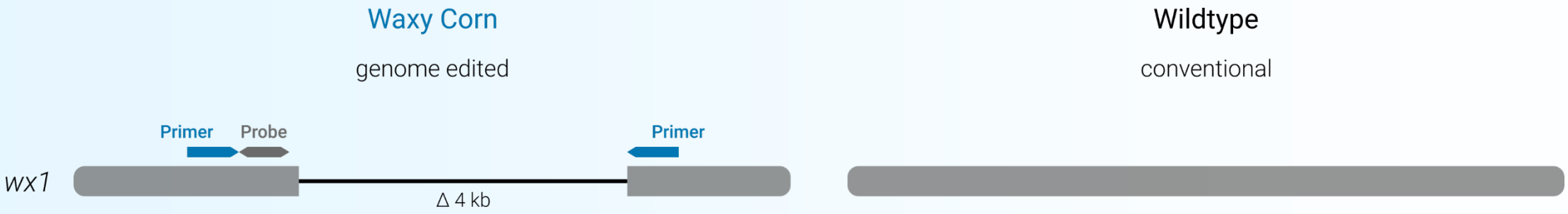
- Interlaboratory trial planned for 2023

		fad2-1A	fad2-1B
Plasmid	pFAD2-1A	✓	✗
	pFAD2-1B	✗	✓
Soybean	wild-type	✗	✗
	DP-305423	✗	✗
	MON87701	✗	✗
	MON87708	✗	✗
	MON87769	✗	✗
	MON89788	✗	✗
	DAS-44406	✗	✗
	DAS-81419	✗	✗
	GTS 40-3-2	✗	✗
Corn	MON810	✗	✗
	MON88017	✗	✗
	MON87427	✗	✗
Oilseed rape	MON88302	✗	✗
	DP-73496	✗	✗

Detection (and identification) of NGT organisms

Waxy Corn (*Corteva Agriscience*)

- **sequence** information
  - PH184C line: 4 kb deletion in *waxy gene (wx1)*
- ratio amylopectin and amylose increased from 70-75 % to 95-100 %
- information on the incorporated **mutation** and the applied **modification technique** (CRISPR/Cas9)
- **positive material** provided by *Corteva Agriscience* under a Material Transfer Agreement (MTA)
- verification of the published method (Gao *et al.*, 2020; PCR conditions – personal communication)

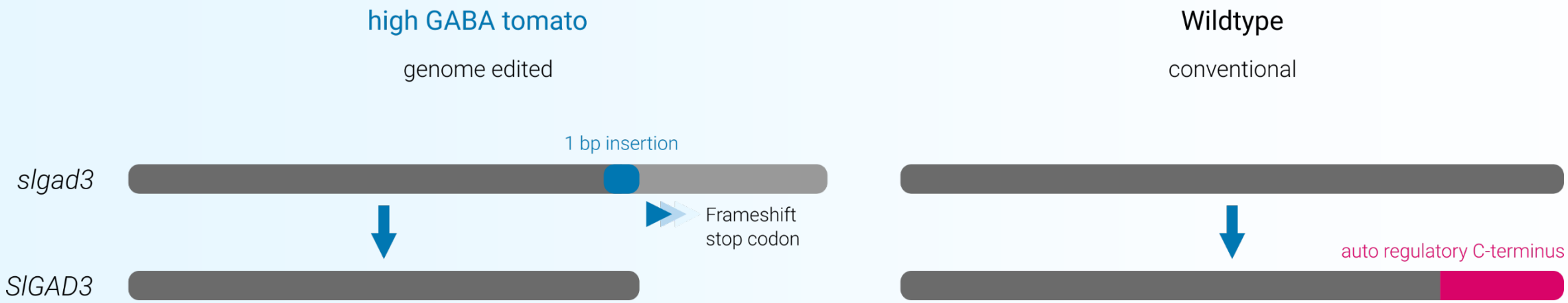


Gao, H., Gadlage, M. J., Lafitte, H. R., Lenderts, B., Yang, M., Schroder, M., ... & Meeley, R. B. (2020). Superior field performance of waxy corn engineered using CRISPR–Cas9. *Nature biotechnology*, *38*(5), 579-581.

Detection (and identification) of NGT organisms

High GABA tomato (*Sanatech Seed*)

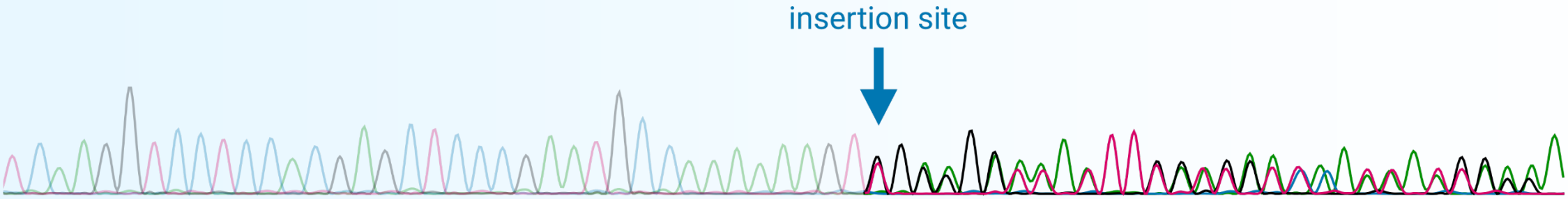
- **sequence** information on *slgad3* gene, but not exact position of the mutation
  - 1 bp insertion (predicted to be a T)
  - position predicted using a bioinformatical online tool (CRISPR-P)
- **frameshift** and generation of a new **stop codon** ⇨ truncated protein and loss of **auto regulatory domain**
- japanese patent information on the incorporated **mutation** and the applied **modification technique** (CRISPR/Cas9)
- **positive material** available (DNA and seeds)



Detection (and identification) of NGT organisms

High GABA tomato (*Sanatech Seed*)

- verification of the predicted insertion by DNA sequencing (Sanger and NGS)
  - **confirmation of insertion site** by Sanger Sequencing and Next Generation Sequencing (NGS)
  - seed material **heterozygeous** for this mutation



- development of a **NGS detection pipeline** (Planton GmbH; member of § 28b GenTG working group)
- considerations to develop qPCR/dPCR methods
- interlaboratory trial planned

Summary (1)

Oilseed rape (Cibus)
- ➔ methods with increased specificity

01 Information | 02 Positive material | 03 Development / Detection

Soybean (Calyxt)
- ➔ synthetic plasmids as positive material
- ➔ interlaboratory trial planned for 2023

01 Information | 02 Positive material | 03 Development / Detection | 04 Identification

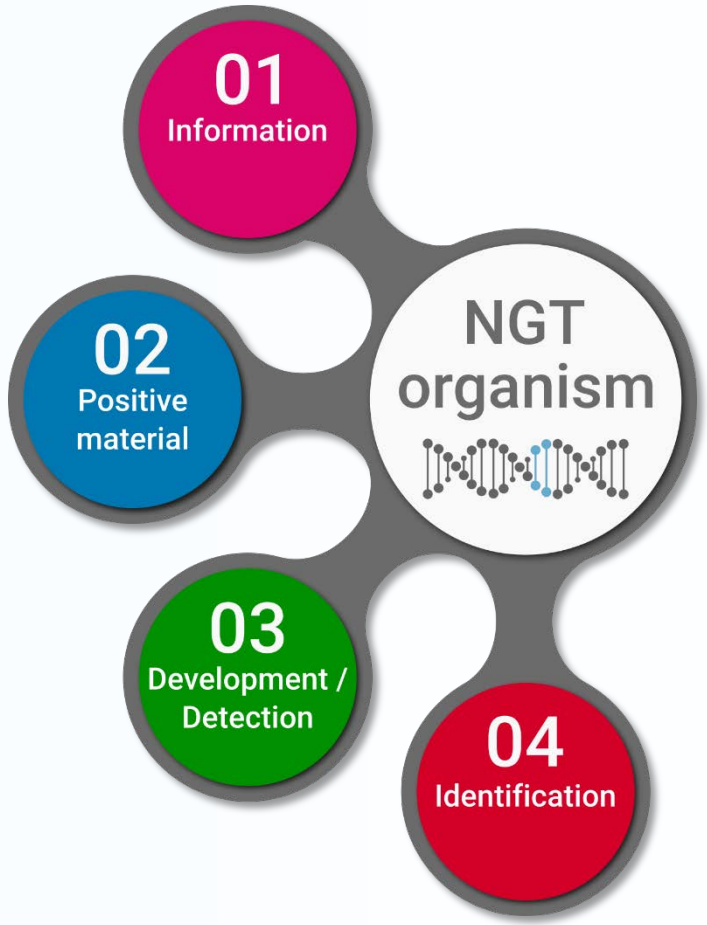
Corn (Corteva Agriscience)
- ➔ method verification / optimization

01 Information | 02 Positive material | 03 Development / Detection | 04 Identification

Tomato (Sanatech Seed)
- ➔ NGS pipeline development
- ➔ qPCR/dPCR methods under consideration

01 Information | 02 Positive material | 03 Development / Detection

Screening for guide RNA scaffold coding sequence
- ➔ ongoing interlaboratory trial



Summary (2)

Significance of an analytical result for the detection / identification of a NGT organism

- decision on a case-by-case basis
  - probability of the occurrence of an identical SNV/InDel obtained through spontaneous or breeding-induced mutation
  - position of the mutation (e. g. essential or non-essential)
  - selection pressure on gene/locus (e. g. extensive use of herbicides)

- combination of detection methods for intended mutation(s) and unintended mutation(s)

  ⇨ multi-target approaches

- when unintended/spontaneous mutations are targeted, these must be
  - accurately characterized
  - stably linked to the locus of the intended mutation(s)
  - maintained through breeding

  ⇨ sequence information needed

Thank you for your attention

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