Comparing genotype and phenotype in wax-less Barley mutant

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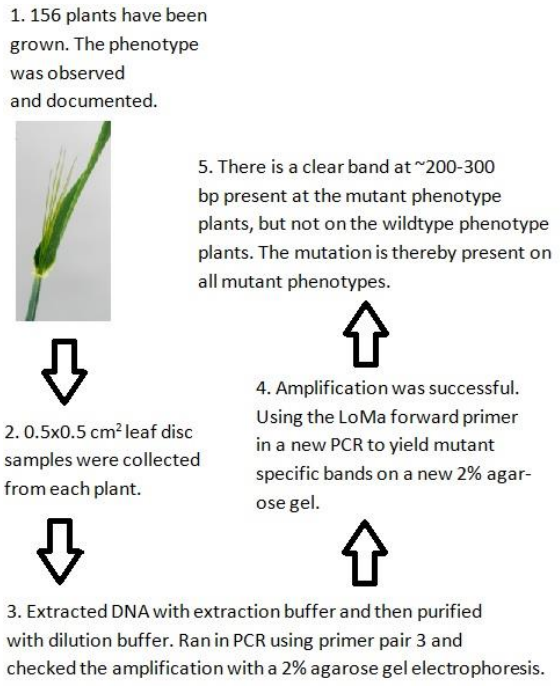
Abstract:

Wax production is essential for the survivability of Barley (*Hordeum vulgare*) plants. They use it to fend off bacteria, viruses and helps rinse the cuticular surface from dirt. Much research has been done regarding the genetic regulation and the *cer-cqu* genes are proven to account for the biosynthesis of wax. A point mutation has been induced within the *cer-u* region which gives rise to waxless plant offspring. By using the point mutation as a landmark for primer recognition when running polymerase chain reactions it is possible to verify that the point mutation disrupts mechanisms of the protein the *cer-cqu* genes code for. The results can then be visualized by gel electrophoresis. Our results show that the mutation in the *cer-u* region renders wax production impossible and that the point mutation is present in all waxless population plants. By learning how to control the production of wax one can use that information to optimize the plants growing, one example is constructing more drought-tolerant plants.

Graphical Abstract:



Introduction:

Barley is one of the four legumes used in food- and brewing industries. In e.g. agriculture it would be interesting to study the defensive mechanisms that barley use for optimization of growth, one of them being the production of wax. It has long been known that barley produce wax to protect themselves against pathogens (virus, bacteria and fungi), UV-radiation and also to remove dirt from the cuticular surfaces (von Wettstein-Knowles *and* Søgaard, 1980). This project regards the genes that are involved in the biosynthesis of wax.

Previous research by von Wettstein-Knowles et al. (1980) has identified that some the genes that accounts for the production of wax are the eceriferum (*cer*) genes. The *cer-cqu (cer-c, cer-q* and *cer-u)* genes are involved in different steps in a pathway to produce β-diketones, hydroxi-β-diketones and alkan-2-ols, which stands for more than half the contents of wax. It has been discovered that mutations in these regions disable various steps in this pathway and leads to a lack of wax production on certain

surfaces (von Wettstein-Knowles, 1972). The *cer-cqu* genes are also in very close proximity to eachother (von Wettstein-Knowles, 1976), 0.0025 cM, which suggests that the genes are often inherited together and code for a multifunctional protein (von Wettstein-Knowles and Søgaard, 1980).

Our focus in this research lies in the *cer-u* part of the *cer-cqu* gene. This specific allele is responsible for converting β-diketones to hydroxi-β-diketones (von Wettstein-Knowles, 1979; von Wettstein *and Søgaard* 1980).

Point mutations were originally induced within barley seeds by different radiation techniques (Lundqvist, U. 1992). The seed used for this research was originally induced in 1955 with a gamma-ray treatment (*Franckowiak, J. D., and U. Lundqvist, 2012*) to yield a point mutation in the *cer-u* region. Seeds from the treated plant were ordered and planted; when the plant sprouted and appeared to be waxless it was crossed with a commercial wild type called Quench. This yielded an F_1-generation of plants with both phenotypes (has wax or is waxless). A leaf sample was collected from both a mutant and a wildtype plant and sent for sequencing (Eurofins Genomics, Germany) (Lohmann and Klein, 2014). This showed the point mutation (A → C) within the *cer-u* region in the sequenced mutant of the F_1-generation. The sequenced wild type contig and the mutant contig are listed in figure 8.

The sequenced *cer-u* parent was then crossed with Quench to result in an F_2-mapping population that is composed of both waxless mutants and wild type phenotypic barley plants.

The goal is to verify that all the barley plants that are phenotypically waxless in the F_2-population include this A → C mutation and thus we can verify that this mutation is indeed the one causing the plants to become waxless.

One way to determine this is by using a specially designed primer that is complementary to either the wild type or the mutant, based on the mutation. If the last base of the primer is complementary to the template base and then running a PCR (polymerase chain reaction) (Rumsby, 2006) only the genotype in which the primer can bind will give rise to amplified DNA. The genotype which doesn't match the primer will have a nick at the last base and prevent the polymerase from binding in and replicate the DNA. The product can then be run in an agarose gel electrophoresis (Lee et al. 2013) to determine which of the genotypes include bands at the appropriate length.

Materials and methods:

156 Barley plants were grown in a greenhouse that has either the waxless (mutant) or wild type phenotype by breeding a waxless plant with the Quench plant. These plants were given an identification number (1.1, 1.2 up to 1.12, and then 2.1, 2.2 etc.) and their phenotypes were listed. Refer to table 1 and 2 for the full list of phenotypes. Using 96-hole plates a leaf sample

(roughly 0.5 x 0.5 cm^2) was cut off from each of the plants and put in a hole each.

By adding 80µl Extraction buffer to each of the leaf disc extracts and incubating in 95ºC for 10 minutes the cells should be lysed and DNA is free in the sample (leaf disc extract). 80µl of Dilution buffer is then added to neutralize substances that potentially inhibit enzymes. The samples are then run in a PCR using 10µl REDExtract-N-Amp PCR ReadyMix (Sigma-Aldrich Sweden AB, Stockholm) (Zakhrabekova et al. 2015), 6µl ddH_2O, 1µl forward primer (3-1), 1µl reverse primer (3-2) and 2µl leaf disc extract (total volume 20µl). The program used in the PCR was an annealing temperature of 58.8ºC and 34 cycles. Figure 8 shows the sequences of all the primers used.

Using these primers the PCR product should yield a clear band at 559 base pairs for all the samples. If the band is present that means the extraction of DNA has worked. The samples are then run in a 2% agarose gel using the GelGreen™ Nucleid Acid Stain (Valdés et al. 2013), 6µl stain per 60ml Agarose gel for 10 minutes at 100V. The ladder used is the GeneRuler 1kb Plus (fig. 3). For all agarose gels 3µl PCR product and 2µl ladder is loaded in their respective wells. The next step is to distinguish between the wild type and mutant genotypes. For that we have constructed a 25 basepair primer that is complementary to the wild type sequence but not the mutant sequence (fig. 1 and 2).

```
CLUSTAL O(1.2.1) multiple sequence alignment


LoMaPrimer            CGCCCTCGGCAAGTTGAAGCTGGTA 25
WildtypeSequence      CGCCCTCGGCAAGTTGAAGCTGGTA 25
                      *************************
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Fig. 1. Clustal Omega comparison showing the similarity between the LoMa primer and the wild type sequence.

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CLUSTAL O(1.2.1) multiple sequence alignment


LoMaPrimer            CGCCCTCGGCAAGTTGAAGCTGGTA   25
MutantSequence        CGCCCTCGGCAAGTTGAAGCTGGTC   25
                      ************************
```

Fig. 2. Alignment between the LoMa primer and the mutant sequence. Done in Clustal Omega.

Notable is the last adenosine in the primer sequence that is only complementary to the 3' antisense strand of the template in the wild type, and not the mutant which contains a G (instead of T) at that position. With the new forward primer we can run a PCR similar to the previous one, 10µl REDExtract-N-Amp PCR ReadyMix, 6µl ddH_2O, 1µl forward primer (LoMa), 1µl reverse primer (3-2) and 2µl leaf disc extract. The reverse primer used is the same as in the previous PCR reaction. The annealing temperature used in the PCR was 58.8ºC, 34 cycles. The 159 PCR products are then loaded to two gels (figure 4 and 5) and run in an electrophoresis (2% agarose, GelGreen™ Nucleic Acid Stain, 10 minutes, 100V). This concludes the methods that were used. A Dark Reader DR89X Transilluminator (Clare Chemical Research, Inc., CO) was used for visualization of the gels.

Results:

The results will be composed of four agarose gel electrophoresis pictures. Two from the DNA extraction test PCR (fig. 4 and 5) and two for the LoMa primer PCR assay (fig. 6 and 7) The GeneRuler™ 1 kb Plus DNA ladder will also be present (fig. 3).

On fig. 4 a clear band can be seen on most of the samples slightly above the 500 bp marker on the ladder. The primer pair bands are also visible, but vague. It is hard to see the ladder on fig. 5 because of the intensity, but there is an intense band on all samples except on sample six and eight on the first row. In comparison to figure 4 the bands seem to be at the same size compared to the vertical axis on the ladder, at roughly 500 base pairs.

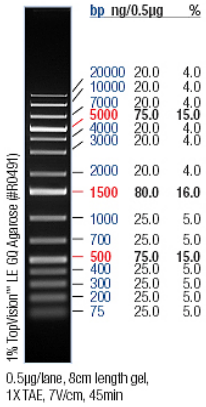

Fig. 3 - The GeneRuler™ 1 kb Plus DNA ladder used in all Agarose gel electrophoreses.

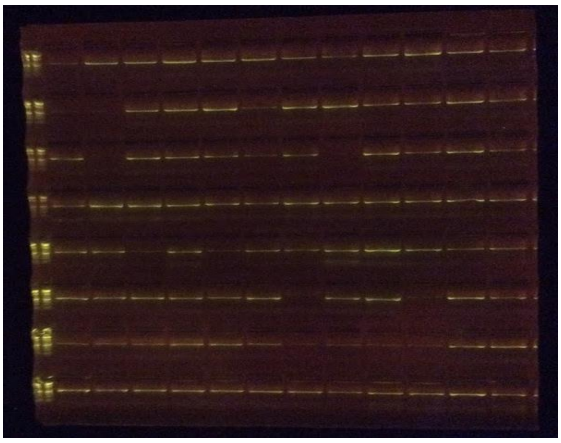

Fig. 4 - Contains the amplified DNA samples from 1.1 to 1.12 (left to right). The second row, from left to right, is 2.1 to 2.12 and so on. The ladders are placed at the very left.

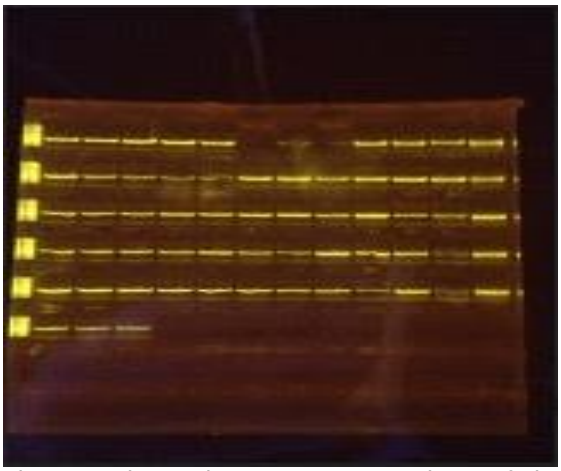

Fig. 5 - Shows the *cer-u* PCR products of the second 96-hole plate. Follows the same loading system as figure 1. The first row shows from left to right 9.1 to 9.12, second row 10.1 to 10.12, and so on.

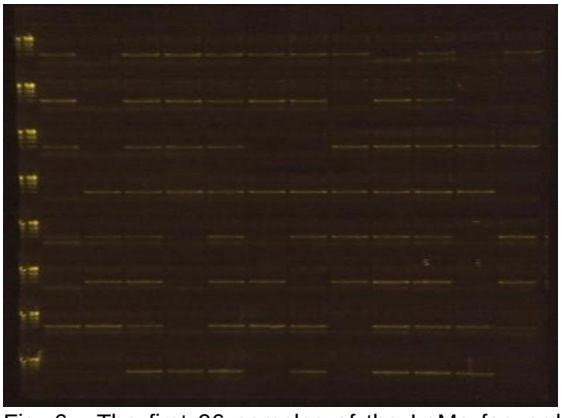

Fig. 6 - The first 96 samples of the LoMa forward primer assay. The ladder is on the left hand side. First sample loaded in the first lane is cer-u 1.1 to 1.12 on the last lane on the same row. Second row has from left to right 2.1 to 2.12 and so on.

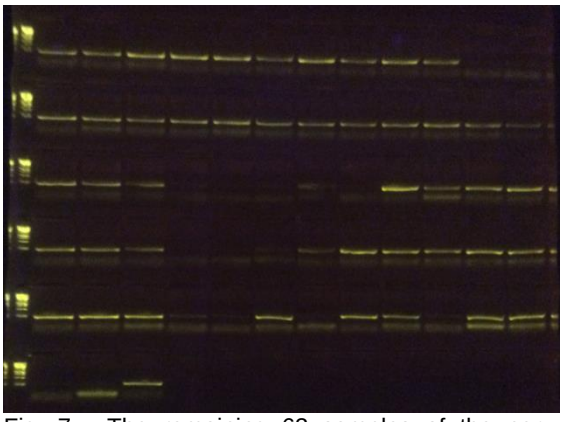

Fig. 7 - The remaining 63 samples of the *cer-u* samples run with the LoMa forward primer. Samples follow the same order as in fig. 4, but starts from 9.1 to 9.12 on the first row and 10.1 to 10.12 on the second row.

Figure 6 and 7 contains the PCR products received using the LoMa forward primer and the 3.2 reverse primer (Fig. 8). On fig. 6 there are clear bands on most of the samples around the 300 bp marker. Same applies for fig. 7, only that they seem to be slightly below the 300 bp reference. A minority of the wells does not have a band at the size of interest.

38/154 plants are mutants. It equals to 0.24675 = 24.7%.

Using a **χ^2-test with the general formula:**

$$\chi^2 = \sum_{i=1}^{n} \frac{(O_i - E_i)^2}{E_i}$$

(Olsson et al. 2012, p 208)

Observed mutant phenotypes: 38

Expected mutant phenotypes:

$154 \cdot 0.25 = 38.5$

Observed wild type phenotypes: 116

Expected wild type phenotypes:

$154 \cdot 0{,}75 = 115.5$

$$\frac{(38 - 38.5)^2}{38.5} + \frac{(116 - 115.5)^2}{115.5} = 0.00865$$

Discussion:

As mentioned before, when the F_1-mutant was sequenced it showed a point mutation (A → C) within the border region (Fig. 6) and the goal is to verify that the same mutation is present in all the F_2-mutants.

The first thing we did was to extract the DNA and amplify the border region with a PCR to make sure that we have DNA sample in the extracted leaf discs. This would be verified if the first agarose gels contained a band at 559 base pairs. If we look at figure 4, we can see that the first clear band on the ladder corresponds to the 500 base pair milestone. Bands are present slightly above that, which most likely corresponds to the mentioned 559 base pair band. This shows that the extraction of DNA was indeed successful. The gel on figure 5 the ladder especially, is hard to read because of its intensity. This is most likely because of pipetting error or calibration not being optimal. There are also some wells on figure 4 and 5 that doesn't have a band, 3.2 and 6.7 (fig 4) are examples, these are dead plants that never grew (Table 1).

When we knew the extraction has been done correctly we tried doing another PCR with a different forward primer that does not complement to the point mutation. The logic behind this

method is that when the primer binds the 3' complement of the template we want to amplify it can only fully bind to the sequence that has a matching last base. When it binds to the mutant DNA that last base will fail to bind and form a nick at the last base which prevents the taq-polymerase from binding in and inserting nucleotides. When run in a gel electrophoresis we should only see one band present on the samples representing wild types that is 234 basepairs, and no bands on the mutant samples. If we look at figure 4, there is a band around the 300 milestone on the ladder which is only close. It is possible that if the gels were run a little longer (15-20 minutes) and on separate gels to allow more space, it would be easier to see exactly where it is. What this would imply is that on figure 4, for example, samples 1.1, 1.3 and 1.4 would be wild type genotype and thereby also wild type phenotype, whilst samples 1.2, 1.9 and 1.11 would be mutant genotype and phenotype. Looking at the chart with the phenotypes (table 1), 1.1, 1.3 and 1.4 are wild type phenotypes while 1.2, 1.9 and 1.11 indeed are mutant phenotypes. In fact, all plants that has been marked with the phenotype mutant has either no visible band or a very vague band around the 300 bp marker. If there is a vague band perhaps that can be explained by that the method is not a hundred percent, and that the polymerase can still bind the nick with very low affinity, but it could also be explained by the fact that some plants may actually have a different phenotype, but the wax is too hard to see because the amount is very low.

If we look at the second plate, fig. 5, 12.4 and 12.5 are examples where there is no visible band at ~200-300 base pairs. If we look at the chart for phenotypes and genotypes (Table 2) they are listed as the phenotype mutant.

For the reason that all phenotypes (Table 1 and 2) matches the genotypes (Fig. 6 and 7) we can draw the conclusion that the point mutation A → C is present in all the mutants, and has an effect on the production of wax.

The same method could in theory be used to determine whether or not point mutations in other parts of the cer-cqu region are relevant for the wax production. This would one of the steps to take to gain further knowledge of the mutations and how they affect the production of wax.

What is also interesting is that the mutation follows the mendelian laws of inheritance acting as an autosomal recessive trait. 38 out of 154 plants (not counting the parents or the two dead plants) have the phenotype of mutant. This equals to ~24.7% of the mapping population. Seeing there is no way to divide the ~75.3% wild type phenotypes into homozygotes and heterozygotes, they will be pooled into one group. As calculated in the results section, this yielded a χ^2-value of 0.00865 for two degrees of freedom.

H_0: Observed = Expected

H_1: Observed ≠ Expected

According to the sheet for the χ^2-distribution function (Olsson et al. 2012, p. 282) it is within 99.95%

certainty that the trait acts as an autosomal recessive trait according to mendelian inheritance. H_0 cannot be discarded. It is safe to say that 25% of the plants are homozygote for the mutant allele while 75% are either homozygote or heterozygote for the wild type allele.

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Appendix:

cer-u.21

Primer 3-1 -CGGACCTGGCAACTCGACAA-

Primer 3-2 -ACGATGATAACGATACCGCTGGT-

LoMa primer -CGCCCTCGGCAAGTTGAAGCTGGTA-

Morex_contig_1562667 around primer pair 3:

AAAAGGACTGTGCAGGAGACCCATAGAGTATAAATGTGAGCAGAATGATTTGAACCATGGTTAGT
CGAGTCCTGCCCCCAAAACTGCACCGGACCAAGAGGTTGTTCTCAAAGAGTTCGATCAATACAATG
GTAATGTGAAAATAATTTTGATTAATTCTAGGTACTTGCCGACATCTCGCAACCGTCGGACCTGGC
AACTCGACAAGCTTGTAAGAACCAAGATTTCGGAGATCATAAAGGCGCGGCATGCTACCGGTGTCT
ACGGTGACGACCTGCTCGGGCAGATGTTGTGGCTCCAGAGGTCGGGTGCTGGCGCCACTGCCGAGA
CCCTGAGCACCGAGGAGATGGTCGGCGAGTGCAGGACCTTCTTCATGGCTGGGTACGAAACCAGC
GCCAACCTTATTACCTGGGCCATGTTCCTGCTCGCCAGGTACCCACGTTGGCAGGAGATGGTTAGG
GACGAGGTCGTCCGGGAGTACCCTGCTCACCAGCCACCATTCGGTGACGCCCTCGGCAAGTTGAAG
CTGGTATGATACGCATATAGTATGGATGGACATTAAACCTCATCCATACAACTTTGATATATCGATC
CTTGTCCATAAAAGAATTAATGAATGCCATGCTCACTTGCAGCTTAACATGTTACTCTTGGAGACAC
TGAGGCTCTATGGCCCCCTATCATTCCTGCAGAGGAAGACGGCCTCAGACACAATCCTCGCACACG
TGAAGGTGCCGAAAGGAACGATGATAACGATACCGCTGGTGATGTTGCACCGGGACAAAGAGGTT
GGGGACCCGACGCCGACGAGTTTAACCCGATGAGGTTCCAGAATGGCTTCTCAAGAGCCGC

Sequenced cer-u.21:

AGATCATAGAGGCGCGGCATGCTACCGGTGTCTACGGTAACGACCTGCTCGGGCAGATGTTGTGGC
TCCAGAGGCCGGGTGCTGGCGCCACCGCCGAGACCCTGAGCACCGAGGAGATGGTCGGCGAGTGC
AGGACCTTCTTCATGGCTGGGTACGAAACCAGCGCCAACCTTATTACCTGGGCCATGTTCCTGCTCG
CCAGGTACCCACGTTGGCAGGAGATGGTTAGGGACGAGGTCGTCCGGGAGTACCCTGCTCACCAG
CCACCATTAGGTGACGCCCTCGGCAAGTTGAAGCTGGTCTGCTACGCATATAGTATGGATGGACAT
TAAACCTCATCCATACAACTTTGATATATCCGTGTCCATAAAACAATTAATGAATGCCATGCTCACT
TGCAGCTTAACATGTTACTCTTGGAGACACTGAGGCTCTATGGCCCCCTATCATTCCTGCAGAGGA
AGACGGCCTCAGACACAATCCTCGCACACGTGAAGGTGCCGAAAGGAACGATGATAACGTACCCG
CTG

Fig. 8 - the sequence of the wild type (morex_contig_1562667) with primer binding sites highlighted in their representing colours. Shows the point mutations of interest location highlighted in the colour red. Primer 3-1 (forward) and 3-2 (reverse) were the ones used during the extraction and dilution of DNA from the leaf extracts. The LoMa primer (forward) and the 3-2 primer were used during the genotype determining PCR.

Plant	Phenotype	Genotype
Cer-u.21.1.1	WT	WT
Cer-u.21.1.2	Mut	Mut
Cer-u.21.1.3	WT	WT
Cer-u.21.1.4	WT	WT
Cer-u.21.1.5	WT	WT
Cer-u.21.1.6	WT	WT
Cer-u.21.1.7	WT	WT
Cer-u.21.1.8	WT	WT
Cer-u.21.1.9	Mut	Mut
Cer-u.21.1.10	WT	WT
Cer-u.21.1.11	Mut	Mut
Cer-u.21.1.12	WT	WT
Cer-u.21.2.1	WT	WT
Cer-u.21.2.2	Mut	Mut
Cer-u.21.2.3	WT	WT
Cer-u.21.2.4	WT	WT
Cer-u.21.2.5	WT	WT
Cer-u.21.2.6	WT	WT
Cer-u.21.2.7	WT	WT
Cer-u.21.2.8	Mut	Mut
Cer-u.21.2.9	WT	WT
Cer-u.21.2.10	WT	WT
Cer-u.21.2.11	Mut	Mut
Cer-u.21.2.12	Mut	Mut
Cer-u.21.3.1	WT	WT
Cer-u.21.3.2	Dead	Dead
Cer-u.21.3.3	WT	WT
Cer-u.21.3.4	WT	WT
Cer-u.21.3.5	WT	WT
Cer-u.21.3.6	Mut	Mut
Cer-u.21.3.7	Mut	Mut
Cer-u.21.3.8	WT	WT
Cer-u.21.3.9	WT	WT
Cer-u.21.3.10	WT	WT
Cer-u.21.3.11	WT	WT
Cer-u.21.3.12	WT	WT
Cer-u.21.4.1	Mut	Mut
Cer-u.21.4.2	WT	WT
Cer-u.21.4.3	WT	WT
Cer-u.21.4.4	WT	WT
Cer-u.21.4.5	WT	WT
Cer-u.21.4.6	WT	WT
Cer-u.21.4.7	WT	WT
Cer-u.21.4.8	WT	WT
Cer-u.21.4.9	WT	WT
Cer-u.21.4.10	WT	WT
Cer-u.21.4.11	WT	WT
Cer-u.21.4.12	Mut	Mut
Cer-u.21.5.1	WT	WT
Cer-u.21.5.2	WT	WT
Cer-u.21.5.3	WT	WT
Cer-u.21.5.4	Mut	Mut
Cer-u.21.5.5	WT	WT
Cer-u.21.5.6	Mut	Mut
Cer-u.21.5.7	WT	WT
Cer-u.21.5.8	WT	WT
Cer-u.21.5.9	WT	WT
Cer-u.21.5.10	WT	WT
Cer-u.21.5.11	Mut	Mut
Cer-u.21.5.12	WT	WT
Cer-u.21.6.1	Mut	Mut
Cer-u.21.6.2	WT	WT
Cer-u.21.6.3	WT	WT
Cer-u.21.6.4	Mut	Mut
Cer-u.21.6.5	WT	WT
Cer-u.21.6.6	WT	WT
Cer-u.21.6.7	Dead	Dead
Cer-u.21.6.8	WT	WT
Cer-u.21.6.9	WT	WT
Cer-u.21.6.10	WT	WT
Cer-u.21.6.11	Mut	Mut
Cer-u.21.6.12	WT	WT
Cer-u.21. 7.1	WT	WT
Cer-u.21. 7.2	WT	WT
Cer-u.21. 7.3	WT	WT
Cer-u.21. 7.4	Mut	Mut
Cer-u.21. 7.5	WT	WT
Cer-u.21. 7.6	WT	WT
Cer-u.21. 7.7	WT	WT
Cer-u.21. 7.8	Mut	Mut
Cer-u.21. 7.9	WT	WT
Cer-u.21. 7.10	WT	WT
Cer-u.21. 7.11	WT	WT
Cer-u.21. 7.12	Mut	Mut
Cer-u.21. 8.1	Mut	Mut
Cer-u.21. 8.2	Mut	Mut
Cer-u.21. 8.3	WT	WT
Cer-u.21. 8.4	WT	WT
Cer-u.21. 8.5	WT	WT
Cer-u.21. 8.6	Mut	Mut
Cer-u.21. 8.7	WT	WT
Cer-u.21. 8.8	Mut	Mut
Cer-u.21. 8.9	WT	WT
Cer-u.21. 8.10	WT	WT
Cer-u.21. 8.11	WT	WT
Cer-u.21. 8.12	Mut	Mut

Table 1 - Excel sheet for the first 96 samples of the *cer-u* plant phenotypes and genotypes (fig. 4). Plant number listed in the first first cell of each row and then phenotype and wild type in the next cells.

Plant	Phenotype	Genotype
Cer-u.21. 9.1	WT	WT
Cer-u.21. 9.2	WT	WT
Cer-u.21. 9.3	WT	WT
Cer-u.21. 9.4	WT	WT
Cer-u.21. 9.5	WT	WT
Cer-u.21. 9.6	WT	WT
Cer-u.21. 9.7	WT	WT
Cer-u.21. 9.8	WT	WT
Cer-u.21. 9.9	WT	WT
Cer-u.21. 9.10	WT	WT
Cer-u.21. 9.11	Mut	Mut
Cer-u.21. 9.12	Mut	Mut
Cer-u.21. 10.1	WT	WT
Cer-u.21. 10.2	WT	WT
Cer-u.21. 10.3	WT	WT
Cer-u.21. 10.4	WT	WT
Cer-u.21. 10.5	WT	WT
Cer-u.21. 10.6	WT	WT
Cer-u.21. 10.7	WT	WT
Cer-u.21. 10.8	WT	WT
Cer-u.21. 10.9	WT	WT
Cer-u.21. 10.10	WT	WT
Cer-u.21. 10.11	WT	WT
Cer-u.21. 10.12	WT	WT
Cer-u.21. 11.1	WT	WT
Cer-u.21. 11.2	WT	WT
Cer-u.21. 11.3	WT	WT
Cer-u.21. 11.4	Mut	Mut
Cer-u.21. 11.5	Mut	Mut
Cer-u.21. 11.6	Mut	Mut
Cer-u.21. 11.7	WT	WT
Cer-u.21. 11.8	Mut	Mut
Cer-u.21. 11.9	WT	WT
Cer-u.21. 11.10	WT	WT
Cer-u.21. 11.11	WT	WT
Cer-u.21. 11.12	WT	WT
Cer-u.21. 12.1	WT	WT
Cer-u.21. 12.2	WT	WT
Cer-u.21. 12.3	WT	WT
Cer-u.21. 12.4	Mut	Mut
Cer-u.21. 12.5	Mut	Mut
Cer-u.21. 12.6	WT	WT
Cer-u.21. 12.7	WT	WT
Cer-u.21. 12.8	WT	WT
Cer-u.21. 12.9	WT	WT
Cer-u.21. 12.10	WT	WT
Cer-u.21. 12.11	WT	WT
Cer-u.21. 12.12	WT	WT
Cer-u.21. 13.1	WT	WT
Cer-u.21. 13.2	WT	WT
Cer-u.21. 13.3	WT	WT
Cer-u.21. 13.4	Mut	Mut
Cer-u.21. 13.5	Mut	Mut
Cer-u.21. 13.6	WT	WT
Cer-u.21. 13.7	Mut	Mut
Cer-u.21. 13.8	WT	WT
Cer-u.21. 13.9	WT	WT
Cer-u.21. 13.10	WT	WT
Cer-u.21. 13.11	WT	WT
Cer-u.21. 13.12	WT	WT
Cer-u.21 A	Mut	Mut
Cer-u.21 B	Mut	Mut
Quench B	WT	WT

Table 2 - Follows the same logic as table 1. Filled with the remaining 60 plants including the 3 parental plants (fig. 5).