Development of asymmetric somatic hybridization technology in industrial chicory (*Cichorium intybus* L.)

Dieter Deryckere

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whenever i'm alone with you
you make me feel like i am home again
like i am whole again
like i am young again
like i am free again

however far away

i will always love you

(R.J. Smith, The Cure)

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Merci

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Merci

Dieter

Table of contents

Abbreviations and acronyms

AC	alternating current
ADP	adenosine diphosphate
AFLP	amplification fragment length polymorphism
AP	alkaline phosphatase
ATP	adenosine triphosphate
BAP	benzylaminopurine
BCIP	5-bromo-4-chloro-3'-indolyphosphate
BER	base excision repair
CAPS	cleaved amplified polymorphic sequence
CFW	Calcofluor White M2R
CGMS	cytoplasmic genetic male sterility
CHS	chalcone synthase
Ci	centromeric index
CMS	cytoplasmic male sterility
cp	chloroplast
CPD	cyclo-butane pyrimidine dimer
cpDNA	chloroplast DNA
DAPI	4',6-diamidino-2-phenylindole
DC	direct current
DIG	digoxigenin
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DP	degree of polymerization
DP_{av}	average degree of polymerization
dsb	double strand break
dsDNA	double stranded DNA
dUTP	deoxyuridine triphosphate

EDTA	ethylenediaminotetra-acetate
EtBr	ethidium bromide
FDA	fluorescein diacetate
FeNa-EDTA	iron sodium ethylenediaminotetra-acetate
FISH	fluorescence *in situ* hybridization
FITC	fluorescein isothiocyanate
GFP	green fluorescent protein
GGO	genetisch gemodificeerd organisme
GISH	genomic *in situ* hybridization
GMO	genetically modified organism
GMS	genetic male sterility
HPLC	high-performance liquid chromatography
HRM	high resolution melting
IAA	indole-3-acetic acid
ILVO	Institute for Agricultural and Fisheries Research
INDEL	insertion/deletion
IOA	iodoacetamide
IRAP	inter-retrotransposon amplified polymorphism
ISH	in situ hybridization
ISSR	inter-simple sequence repeat
ITS	internal transcribed spacer
LMPA	low melting point agarose
M	metacentric
MMF	massive mitochondrial fusion
MMR	mismatch repair
MPP	microprotoplasts
MS	Murashige and Skoog
MSAP	methylation-sensitive amplification polymorphism

mt	mitochondrial
mtDNA	mitochondrial DNA
MW	molecular weight
NAA	naphthaleneacetic acid
NBT	nitro-blue-tetrazolium
NER	nucleotide excision repair
NOR	nucleolar organizing region
ORF	open reading frame
PAL	phenylalanine ammonia lyase
PAT	phosphinothricin acetyltransferase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PFGE	pulse field gel electrophoresis
PI	propidium iodide
PP	pyrimidone dimer
PS II	photosystem II
RAPD	random amplification of polymorphic DNA
rDNA	ribosomal DNA
REMAP	retrotransposon-microsatellite amplified polymorphism
RER	rough endoplasmic reticulum
RFLP	restriction fragment length polymorphism
Rho	rhodamine β isothiocyanate
RNA	ribonucleic acid
ROS	reactive oxygen species
rRNA	ribosomal RNA
RT	room temperature
SCGE	single cell gel electrophoresis
SDS	sodium dodecyl sulphate

SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SE	standard error
SI	self-incompatibility
SL	sesquiterpene lactones
SM	submetacentric
SNE	single nucleotide extension
SNP	single nucleotide polymorphisms
SSC	saline sodium citrate
ssDNA	single stranded DNA
SSR	simple sequence repeats
TAE	tris-acetate-EDTA
TDZ	thidiazuron
T_m	melting temperature
tRNA	transfer RNA
Tyr-FISH	tyramid-FISH
UV	ultraviolet

Chapter 1 - Introduction on *Cichorium* and somatic hybridization

Parts of this chapter are based on a publication to be submitted.

1.1 *Cichorium*

Industrial chicory (*Cichorium intybus* L., 2n = 2x = 18) belongs to the subfamily Cichorioideae, which is a part of the Asteraceae family, containing approximately 23,000 species. Within the *Cichorium* genus there are 2 important cultivated species: *C. intybus* and *C. endivia*. *C. endivia* is an annual, self-compatible crop, whereas *C. intybus* is annual or biannual and self-incompatible. Different taxonomic trees of *Cichorium* can be found in literature. Lucchin et al. (2008) classifies the species according to their application (Table 1-1); two main *Cichorium* types (with different breeding objectives) can be distinguished: the salad-type genotypes such as *C. endivia* and *C. intybus* var. *foliosum* and the root chicory genotypes (*C. intybus* var. *sativum*) mainly used for the inulin extraction. This classification is in accordance with Kiers et al. (2000), who showed the existence of four cultivar groups within *C. intybus*: (1) the root cultivars, (2) the witloof cultivars, (3) the sugarloaf cultivars and (4) the Radicchio cultivars. The endives (*C. endivia*) are generally divided in three groups: (1) the broad-leaved Scarole group, (2) the crispy and curly narrow-leaved Frisé group endives and (3) the ancient endive cultivars with narrow, incised leaves (Ryder, 1999; Kiers et al., 2000) (Fig. 1-1).

Table 1-1 Chicory and endive: European species of *Cichorium*, cultivar groups and use (Lucchin et al., 2008)

Taxonomic determination	Cultivar group	Use
C. endivia		
subsp. *endivia*	wild	
var. latifolium	Endive	Salads
var. crispum	Crispum	Salads
subsp. *pumilum*	wild	
C. intybus		
subsp. *intybus*	wild	
var. foliosum	Witloof chicory	Cooked/salad
	Pain de sucre	Cooked/salad
	Radicchio	Salad
	Catalogne	Cooked
var. sativum	Root chicory (roasted)	Coffee substitute
	Root chicory (industrial)	Inulin extraction
	Root chicory	Cooked
subsp. *glabratum*	wild	
C. spinosum	wild	

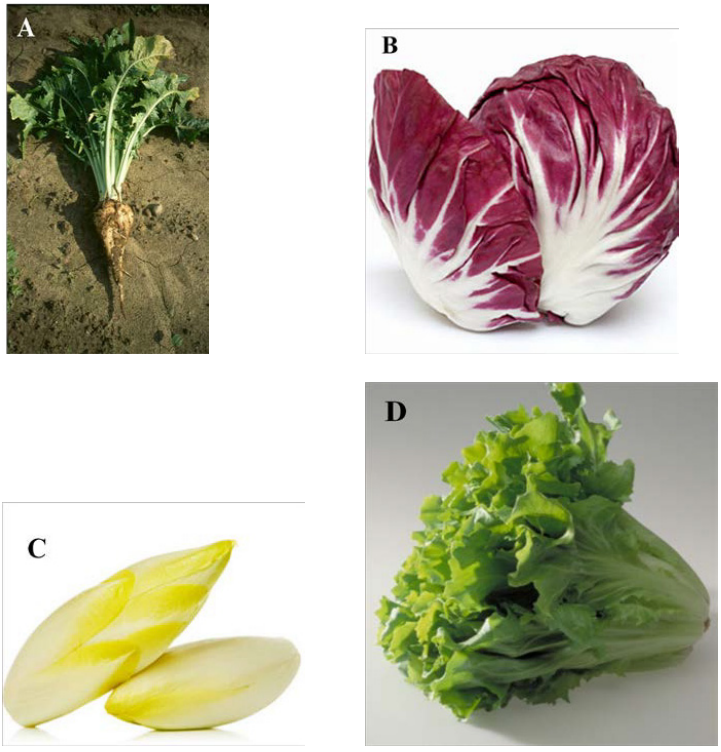


Fig. 1-1 (A) Industrial chicory *C. intybus* (B) *C. intybus* 'Radicchio' types, (C) *C. intybus* 'Witloof' types and (D) Endive *C. endivia*

The first written facts on chicory were mentioned in the Egyptian era; the ancient Egyptians grew chicory as a medicinal plant and vegetable, and for animal forage. Later on, Greeks and Romans proceeded the cultivation of chicory as a vegetable crop (Grieve, 1971). Gradually, chicory and endive (*C. endivia* L.) became two traditional European crops and through time these two species developed in a variety of cultivated types. At the time of Napoleon, in the 19th century, the so called 'Magdeburg' chicory (*C. intybus*) was introduced as a coffee substitute since Napoleon banished the coffee import through a trade embargo with England. At the end of the 19th century about 13,000 ha of chicory to be used as coffee surrogate was grown in Belgium. Nowadays, there is still coffee chicory production in the north of France, India and South Africa (Lucchin et al., 2008). Also the leaf vegetable *C. intybus* var. *foliosum* 'Witloof', or Belgian endive, is considered to be a derivative of the 'Magdeburg' chicory and is suggested to be introduced by a Belgian farmer around 1870 (Lucchin et al., 2008). The Belgian endive has cream-yellowish, packed leaves which have been forced from roots, kept on soil or hydroponic trays, in darkness and warmth inhibiting chlorophyll development

(Vanstreels et al., 2002; Hertog et al., 2007). According to 2002 US Department of Commerce tariff and trade data, the US imported over 2.3 million kilograms of Belgian endive and 1.9 million kilograms roasted chicory roots for coffee (Schmidt et al., 2007). Belgium is the main exporter of the witloof heads (19,000 ton) (In: Proeftuinnieuws Oktober 2010). Next to witloof, the Italian red headed 'Radicchio' types and the Pain de Sucre types (*C. intybus* var. *foliosum*) are used as salads. The Italian red chicory is mainly grown in the north eastern regions of Italy, but is also grown in Flanders (Belgium). The Pain de Sucre types are mainly cultivated in northwestern Europe (Kiers et al., 2000; Lucchin et al., 2008). From 1870 onwards, cultivars of the Belgian endive (witloof) were developed by mass selection. Several breeding goals became important, including uniform tight heads, commercial uniform size and shape, tolerance to internal browning, resistance to premature bolting and reduced bitterness (Ryder, 1999). Also new variants were developed; the 'roodloof' cultivar combined the features of an Italian red chicory and witloof types (Bannerot and de Coninck, 1976).

From the 1970s onwards, new broad leaf varieties of chicory were bred, the so called forage chicory. In 1986, the world's first forage cultivar of chicory, *C. intybus* 'Grasslands Puna' was released in New Zealand. This cultivar has a wide range of adaptation and is nowadays being grown worldwide, including Canada, US, Mexico and China (Hume et al., 1995; Wang and Cui, 2011).

1.2 Industrial chicory

1.2.1 Cultivation

Since 1990, root chicory (*C. intybus* var. *sativum*) has become more important for the extraction of inulin and its hydrolysis products such as oligofructose and fructose, whereas earlier it had only been used as a coffee surrogate. The presence of inulin, a soluble dietary fiber is the reason for the revival of chicory breeding (Baert and Van Bockstaele, 1993; Baert, 1997; Velayutham et al., 2006). The cultivation of the root chicory is situated in the traditional production areas in North-Western Europe (Belgium, The Netherlands and the north of France). At present, in The Netherlands about 4,000 ha and in France about 2,000 ha of industrial chicory are cultivated. In Belgium, industrial chicory was produced on 8,126 ha in 2010, www.statbel.fgov.be. The evolution of the chicory acreage in Belgium from 1990 till 2010 is shown in Fig. 1-2. The first statistics available showed a 13,000 ha chicory acreage in Belgium in 1895. This number dropped to less than 1,000 ha until 1990, as chicory was mainly produced as coffee surrogate. From 1990 onwards, chicory was grown for its inulin and fructose and the acreage increased to 15,000 ha in 2005. Due to the reformation of the European sugar policy in 2005, EU member states were asked to abandon part of their production quotas to reduce the total EU sugar production by approximately 6 million tons. Therefore, the industry agreed to stop the production of fructose from chicory. Consequently, the chicory acreage in Belgium dropped to 8,000 ha since then.

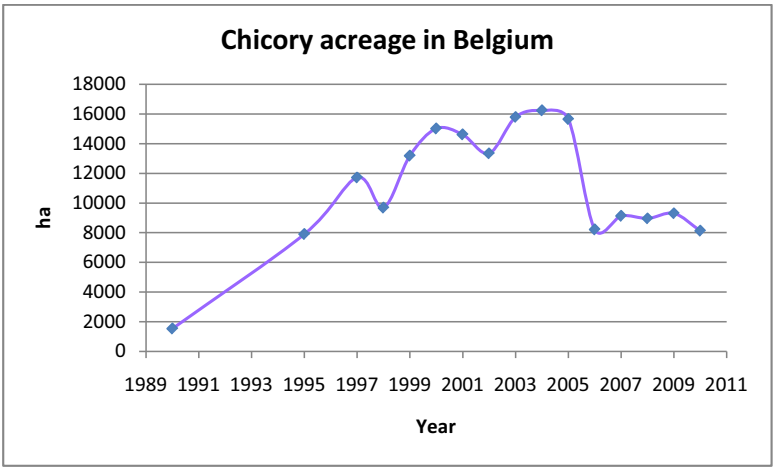


Fig. 1-2 Chicory acreage in Belgium (ha) from 1990 till 2010

Industrial chicory is a biannual plant; in the first year a vegetative state is maintained, including the growth of a tap root and leaves forming a rosette. Overwintering or cold treatments (< 5°C) are needed for the vernalization of the plants to induce seed stalk growth. Consequently, in the second season, the plant becomes generative, developing a stem bearing blueish flowers. The flowers exhibit the features of typical Asteraceae ligulate flowers, including the inflorescence structure containing 15 to 25 single androgynous flowers (Ryder, 1999; Lucchin et al., 2008).

1.2.2 Use of industrial chicory

1.2.2.1 Inulin

Chicory is one of the two plants, next to Jerusalem artichoke (*Helianthus tuberosus*), that is industrially exploited for the extraction of inulin-type fructans (Chi et al., 2011). High levels of fructans are found in the chicory roots, while chicory leaves only contain low fructan concentrations (Ernst et al., 1995). Fructans are linear polydisperse carbohydrates consisting of β (2→1) fructosyl-fructose linkages, with or without a glucose unit at the reducing end (Monti et al., 2005; Roberfroid, 2005) (Fig. 1-3).

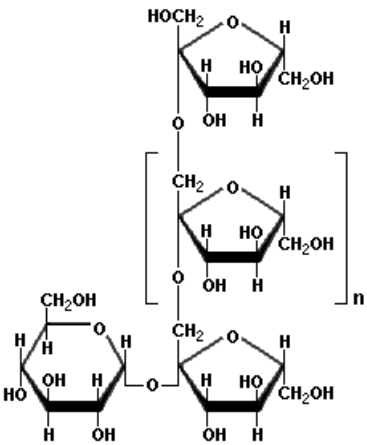


Fig. 1-3 Molecular composition of inulin consisting of n β (2→1) fructosyl-fructose lineages, with or without a glucose unit at the reducing end (after Bais and Ravishankar, 2001).

Chicory inulin is stored in the taproot as a spare carbohydrate (Bais and Ravishankar, 2001) and has a degree of polymerization (DP) of 2 to 60 (DP_{av} = 12). Almost 10% of the fructans in crude chicory inulin extracts has a DP ranging between 2 and 5. During shoot formation,

the accumulated inulin is partially hydrolyzed through the enzyme inulase and oligofructose is formed (DP 2 to 8, DP_{av} = 4) (Bais and Ravishankar, 2001; Roberfroid, 2005). The β (2→1) bonds of the inulin-type fructans are not digested by animal intestinal enzymes and are, therefore, low caloric dietary fibers. Fermentation of the undigested inulin takes place by beneficial bacteria *Lactobacillus* and *Bifidus* in the colon. Consequently, a preferentially stimulated growth of these bacteria causes a drastic change in the gut microflora, increasing the amount of health-promoting bacteria and decreasing harmful bacteria. Inulin also promotes the absorption of calcium by lowering the pH of the colon. Besides these prebiotic features of the long inulin chains, its short chain hydrolysis product, oligofructose, has a sweetening feature. The shorter the oligofructose chain, the sweeter the taste and the higher the caloric content. A reduction in total cholesterol level and a decrease of triglyceridaemia is ascribed to the use of inulin-type fructans (Roberfroid and Delzenne, 1998). Moreover, inulin can be used to optimize food and feed textures: adding water to inulin results in a structure that can be used as a fat replacer, with the same mouthfeel and texture as fat. A high DP inulin facilitates gel formation at lower concentrations (Koch et al., 1999). Nowadays, the production of health promoting nutrition based on inulin is marketed. Oligofructose-enriched inulin, which is produced by mixing low- and high-molecular weight inulin chains, can be found in several nutrition drinks and food (Niness, 1999; Roberfroid, 2005). Next to the medicinal and nutritive aspects, inulin is a promising alternative for expensive and rare raw materials. Inulin is a renewable, inexpensive and abundant compound that can be used as the source for ethanol fermentation and single-cell protein, single-cell oil, citric acid, inulooligosaccharides and other chemicals production. Inulin is also found in cosmetic products (Chi et al., 2011).

Sucrose plays a major role in inulin metabolism by promoting polymerization and inhibiting depolymerization. Sucrose is translocated from the leaves to the root and split in fructose and glucose units, which are used to build up fructans. When shooting is started, the sucrose transport from leaves to root decreases and consequently, inulin chains are broken down to fulfill the need for energy supplies. The economically optimal harvest date is met when the highest yield of extractable inulin is achieved in the chicory root (Baert and Van Bockstaele, 1993).

Harvest date, plant density and temperature all influence the fructan yield and quality (DP, fructan chain length) of chicory. Water regime and nitrogen supply did not change the distribution of the DP classes of chicory (Koch et al., 1999; Monti et al., 2005). Baert et al. (1997) tested the effect of sowing and harvest date on inulin chain length with three chicory

varieties for two years (1992 & 1993) in Belgium. Inulin yield increased with 30 % in both years when early sowing (before mid-April) was performed. The average inulin chain length was two fructose units longer when the roots were harvested around mid-September, compared to 2 months later. In contrast, the carbohydrate yield was only 10% lower at this early harvest date than at the late harvest date. However, the inulin breakdown was more influenced by the different chicory varieties. Koch et al., 1999 described similar results for six chicory cultivars grown in Sweden. The highest DP_{av} values were obtained when harvesting in mid- and late-October. The DP_{av} values dropped when the weather turned colder, in mid-November. Also cultivar differences were observed as four cultivars showed significantly higher DP_{av} values. Ernst et al., 1995 noted the decrease of inulin-type fructans during cooler fall temperatures and especially during storage. However, fructans containing no glucose, the so-called inulonoses, accumulated at storage time. The inulonoses can be regarded as a breakdown product of inulin and thus can be a good indicator of inulin mobilization.

1.2.2.2 Other extracts

Next to inulin, in ethanolic and methanolic extracts, the sesquiterpene glycosides are found in chicory. They reduce cholesterol (Kim, 2000) and glucose uptake (Kim and Shin, 1996) in rats, affect the lipid and fatty acid concentrations (Kim and Shin, 1998) and intestinal morphology in rats (Kim, 2002) and affect tumor development in mice (Hazra et al., 2002), they also prevent immunotoxicity (Kim et al., 2002) and are showing anti-inflammatory properties (Cavin et al., 2005; Schmidt et al., 2007).

Other compounds found in roots and heads of chicory are the sesquiterpene lactones (SL) responsible for plant bitterness (Bais and Ravishankar, 2001). SLs are a group of C_{15} terpenoid compounds including more than 500 members, characteristic for the Asteraceae family (Ferioli and D'Antuono, 2012). Chicory SLs are mainly lactucin, lactucopicrin or their derivatives (Kisiel and Zielinska, 2001; Ferioli and D'Antuono, 2012). SLs are believed to have biological and pharmaceutical activities including anti-tumour, anti-leukaemic, cytotoxic, antimicrobial and allergenic properties (Ferioli and D'Antuono, 2012). Their clinical use was also described for inflammatory conditions by the use of SLs of *Tanacetum parthenium* for the treatment of migraines (Palevitch et al., 1997) and SL containing *Arnica montana* gel for treating arthritis (Knuesel et al., 2002). Due to their contribution of the bitterness of the chicory plant, SLs play a major role in appetite and digestion in humans (Kisiel and Zielinska, 2001).

1.3 Breeding of industrial chicory

1.3.1 Classical breeding program

Today, industrial chicory is mainly cultivated for the extraction of inulin. A high root yield, a high inulin content in the root and high-quality long inulin chains are major characteristics in chicory breeding. Besides genetically, these features are also influenced by cultivation methods, including sowing date and harvest date (Baert, 1997). Early sowing prolongs the growth season, contributing to a higher inulin yield. A fast early leaf growth may even increase this yield (Baert, 1997). Industrial chicory seeds are usually sown between mid-April and mid-May. However, early sowing increases the risk of bolting (becoming generative) due to the exposure to low vernalization temperatures (Devacht et al., 2011). Because of the high heritability of bolting resistance, this may be overcome by breeding (Baert and Van Bockstaele, 1993). Besides bolting, also low temperatures negatively affect the early growth of the chicory plants. The optimal harvest date is defined as the moment the maximum yield of long chain inulin per ha is achieved (Baert and Van Bockstaele, 1993). After a cold period (harvest period), the inulin chains are depolymerized and the content of free fructose and sucrose increases with harvest time, while the content of free glucose and inulin diminishes. Varieties showing a slow breakdown of the inulin chains are preferred (Baert, 1997). The pollination of the allogamous industrial chicory occurs by flying insects.

The classical breeding program for the production of industrial chicory varieties at the Institute for Agricultural and Fisheries Research (ILVO) in cooperation with the COSUCRA-Groupe Warcoing S.A. division Chicoline is shown in Fig. 1-4. The varieties are developed from clones or their halfsib progeny. Selection is based on the root shape, weight, inulin content and chain length. The selected roots are vegetatively propagated. The clones are selected on their bolting and disease resistance. Selected clones are then used in polycrosses. Seeds are harvested on each genotype. These halfsib families are tested in early-sown bolting tests and normally-sown yield trials. Bolting resistance, leaf development and disease, root yield and shape, inulin content and DP are determined. Four to 10 clones or remnant seed with the best performing progenies form the components of a synthetic candidate race. The progeny tests provide roots for the breeding populations. By recurrent selection, the frequency of desired genes in the selected roots is upgraded. The candidate varieties are tested in official trials in Belgium, The Netherlands or France. Table 1-2 gives the actual industrial chicory variety list of 2012 for Belgium with their agronomical performances.

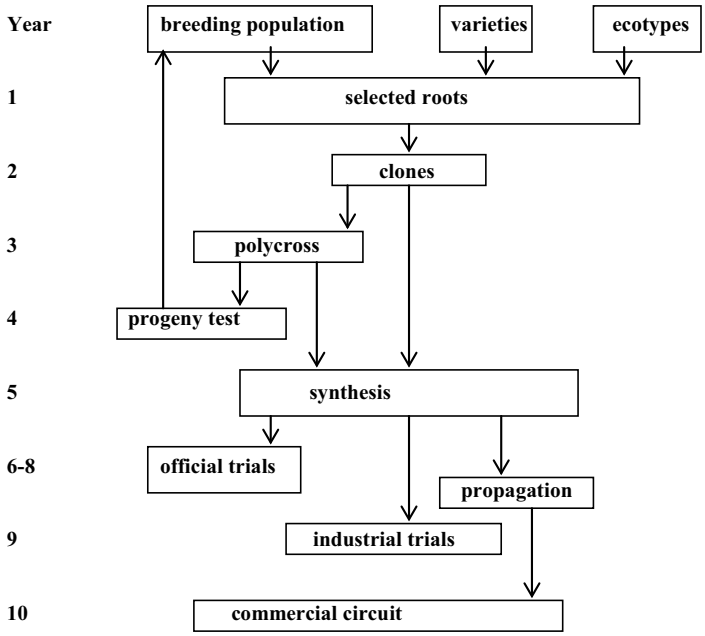


Fig. 1-4 The classical breeding program for the production of industrial chicory varieties at ILVO

Tabel 1-2 Belgian list 2012 of industrial chicory varieties with their agronomical features (Pannecoucque et al., 2012)

Features	Yield (relative) fresh *	Yield (relative) carbohydrates	Total carbohydrate content **	DP ***	Tarra (%)
Canzona	98.8	102.0	20.3	10.8	10.2
Continuo	96.9	97.9	20.1	10.3	11.1
Crescendo	99.0	98.1	19.5	10.0	11.5
Diesis	102.4	101.4	19.6	10.4	11.8
Enigme	96.4	98.5	20.1	10.5	11.7
Gong	100.6	102.2	20.0	10.4	10.8
Hera	103.3	99.8	19.0	9.8	11.8
Maurane	100.9	98.3	19.2	10.1	11.9
Melci	102.5	101.2	19.4	10.0	11.4
Zingaro	99.2	100.5	19.9	10.4	11.7
Mean	60.0 t/ha	11.8 t/ha	19.7	10.3	11.4

* 100 = mean of all races
**Total carbohydrate content = (% fructose + % glucose, after hydrolysis) / 1.1 (1.1 is used as a correction for the molecular weight of water used for hydrolysis)
*** DP = (fructose / glucose, after hydrolysis) + 1

1.3.2 Hybrid breeding

As in many flowering plant species, maintenance of the genetic variability is the key to the further adaptation in a changing environment. One strategy for increasing genetic variability is limiting inbreeding due to self-fertilization. Self-incompatibility (SI) can be described as a cell-cell recognition mechanism by which the pistil discriminates self-pollen from genetically distinct pollen (Dzelzkalns et al., 1992; Varotto et al., 1995). Thus, not only the germination of self-pollen is inhibited, but also the pollen of identical incompatible phenotypes. Another form of SI is pseudoselfincompatibility, characterized by a variable degree of selfcompatibility and a phenomenon of pollen competition favouring the growth of allopollen tubes to selfpollen tubes within the style. *C. intybus* is self-incompatible. Two SI types can be distinguished. Gametophytic SI is determined by the haploid genome of the pollen. Sphorophytic SI is determined by the diploid genome of the pollen parent plant, by the pollen coat formation during microgametogenesis (Dzelzkalns et al., 1992; Lucchin et al., 2008). Observations in Italian red chicory (Varotto et al., 1995) and 'Witloof' chicory (Eenink, 1981) suggested the presence of sphorophytic incompatibility in *C. intybus*. Regardless of the SI system, still selfing at a low rate can occur in chicory. As stated by Lucchin et al., 2008 SI chicory plants might produce 1 or 2 fertile seeds per flowerhead by selfing. Due to the high number of flowerheads produced during a generative period, the amount of seeds formed can be high. In contrast with *C. intybus*, *C. endivia* is a self-pollinating species with less than 1% spontaneous cross fertilization (Rick, 1953). Chicory and endive are genetically close (Rick, 1953). Therefore, interspecific crosses could take place between the two species. Due to the SI system in chicory and the selfing of the endives, the easiest cross type is *C. intybus* x *C. endivia*. This cross leads to the formation of diploid, hybrid plants containing 50% of the nuclear information of both parents and the cytoplasm of *C. intybus*. As we are interested in *C. intybus* plants containing the *C. endivia* cytoplasm, this cross is undesired and the *C. endivia* x *C. intybus* cross is needed.

Commercial breeding in chicory has traditionally been based on intercrossing a number of phenotypically superior parents selected for several commercial traits (Lucchin et al., 2008). Hybrid vigour or heterosis can exploit the superior performance of the heterozygous hybrids in comparison to the parents. Moreover, their uniformity offers great opportunities for modern growing and harvesting techniques. In chicory, breeders observed heterosis effects in progeny obtained from crosses between distant genotypes, indicating that F_1 hybrids can contribute to the development of chicory (Bannerot and Deconinck, 1965; Bannerot and Deconinck, 1970). Hybrid production requires a good pollination control where selfing of the female line is

inhibited (Perez-Prat and van Lookeren Campagne, 2002; Nizampatnam et al., 2009). However, since the SI system is unstable under certain environmental conditions and is not 100% reliable (Baert and Van Bockstaele, 1993), difficulties arise in setting up a reliable hybrid seed production scheme. RAPD analysis of F_1 hybrid seed samples of *C. intybus* revealed only 71.25% true hybrids (Bellamy et al., 1996). Manual emasculation of the female plant is an option but this technique is labour-intensive, time-consuming and thus, expensive. Male sterility can contribute to the creation of 100% true hybrids without the use of manual labour.

1.4 Cytoplasmic male sterility

1.4.1 Male sterility types

Three types of male sterility are present: Genetic male sterility (GMS), cytoplasmic male sterility (CMS) and cytoplasmic genetic male sterility (CGMS). GMS is based on a recessive mutation (ms) that inhibits the normal functioning of nuclear genes involved in pollen production (Lucchin et al., 2008). Development of male sterile lines through genetic engineering has opened new ways of hybrid breeding. A chimaeric ribonuclease gene, *barnase* from *Bacillus amyloliquifaciens*, cloned under a tapetum-specific promoter TA29, was expressed in the anthers of transformed tobacco and oilseed rape plants. The expression of this gene destroyed the tapetal cell layer surrounding the pollen sac and prevented pollen formation, leading to nuclear male sterility (Mariani et al., 1990). Similar experiments were performed on *Brassica napus* (Denis et al., 1993) and cauliflower and chicory (Reynaerts et al., 1993). Expression of a ribonuclease inhibitor gene, *barstar*, in the tapetal cells, restored fertility in the male sterile plants by binding to the ribonuclease active site, preventing *barnase* from damaging the cell's RNA (Mariani et al., 1992). Another gene encodes for phosphinothricin acetyltransferase (PAT) enzyme which inactivates phosphinothricin, the active component in the broad-spectrum, contact herbicide glufosinate. Although these lines are tolerant to the herbicide, their hybrid progeny may or may not be tolerant to the herbicide. Selection of male sterile plants in breeding programs could be detected using this herbicide.

CGMS results from the interaction of a sterile cytoplasm (S) and the homozygous recessive alleles (rf/rf) present at one (or more) nuclear restorer locus (loci). This sterility type, caused by a cytoplasmic gene dysfunction, can be restored in its progeny by crossing with another plant containing dominant nuclear restorer genes (Rf). These Rf genes interact through different mechanisms to restore the malfunctioning of the mitochondria: mitochondrial transcript processing, posttranscriptional functions, possible modes of biochemical detoxification and alteration of mitochondrial genome organization (Leon et al., 1998). CMS, which is closely related to CGMS, is characterized by mutations or rearrangements in the mitochondrial genome, leading to alterations in transcription and/or translation (Kohler et al., 1991). CMS by rearrangements in the mtDNA was already observed in 150 plant species including maize (Dewey et al., 1986; Dewey et al., 1991), sunflower (Kohler et al., 1991), *Beta vulgaris* (Saumitou-Laprade et al., 1993), rice (Akagi et al., 1995), *Brassica* (Bellaoui et al., 1998), tobacco (Bergman et al., 1995), petunia (Rasmussen and Hanson, 1989), bean

(Janska and Mackenzie, 1993), radish (Makaroff and Palmer, 1988), tomato (Stoeva-Popova et al., 2007) and sorghum (Bailey-Serres et al., 1986). CMS is inherited as a dominant, maternally transmitted trait (Perez-Prat and van Lookeren Campagne, 2002). Alloplasmic CMS is a result of inter-, intraspecific or intergeneric crosses. Due to incompatibilities between the nuclear genome and the mitochondrial genome or both mitochondrial genomes of the different parents, mutations, recombinations or deletions can occur which lead to disturbances in mitochondrial gene expression or signal transduction. These errors are no longer suppressed by the original parental nuclear restorer genes, thus causing CMS (Stoeva-Popova et al., 2007). Moreover, when no mutations or recombinations occur, CMS can also be obtained due to incompatible nuclear-cytoplasmic interactions between the nucleus and the alien cytoplasm (Hanson and Conde, 1985).

Several techniques can be used to introduce CMS in plant species. Inter-, intraspecific or intergeneric crosses contribute to an alloplasmic CMS. However, intergeneric crosses might negatively influence agronomically important features. Therefore, inter- and intraspecific crosses are more likely to succeed. Alloplasmic CMS was observed in *Brassica* (Shinada et al., 2006) and *Gossypium* (Galau and Wilkins, 1989). As in any other breeding program, the introduction of new features into a crop is restricted by reproductive barriers and the genetic structure of the different populations. It is also a time-consuming approach, including the need for backcrosses to eliminate undesired features. To address these possible limitations in conventional commercial breeding programs, somatic protoplast hybridization can be useful to circumvent sexual incompatibility and to enable the direct transfer of both nuclear and cytoplasmic genome features into the plant cells (Deryckere et al., 2012). Another technique to obtain CMS is through mutagen application. In sunflower, mitomycin and streptomycin induced mutations in cytoplasm DNA, leading to CMS (Jan and Rutger, 1988). In petunia, chemical treatment with ethylnitroso urea induced CMS in 27 plants (Harten et al., 1985).

1.4.2 Male gametophyte development and molecular basis of CMS

Anthers of *C. intybus* are tetrasporangiate. The primary sporogenous cells in the anther develop into pollen mother cells. Microspores, originated from the pollen mother cell, form microspore tetrads after meiosis. Callosic walls are formed around the tetrad and between each monad, leading to microspores. The nucleus of the microsporocytes will undergo mitosis and form two unequal nuclei, a large vegetative and a small generative. *Cichorium* pollen is spherical, tricolpate and bi-cellular (Chehregani et al., 2011). A three layered, dicotyledonous-

type anther wall is formed from differentiating archesporial cells. The three layers consist of an epidermis, endothecia and a tapetum layer. A general remark is that mitochondria are vital elements in pollen development (Chehregani et al., 2011).

In plant populations, a disturbed floral development, mainly exhibited by male sterility is mainly due to a dysfunctioning nucleus-mitochondria connection. Next to the natural occurrence of CMS in plant species, CMS has been established as a result of crossing a nuclear genome in an alien cytoplasmic background, called alloplasmic male sterility. Several reasons are mentioned, including aberrant cytoplasmic genes that are no longer restored by their original nuclear genes and hybridization-induced disturbances in cytoplasmic genome configurations (Hanson and Bentolila, 2004).

Due to its maternal inheritance, it became obvious that CMS was the result of errors in either the mt or cp genome. Several strategies have been used to identify molecular markers associated with CMS. The most simple strategy was the comparison of cytoplasmic genomes of CMS and a fertile line. However, when there is no recently emerged CMS in those plants, differences in cytoplasmic genomes can be contributed to evolution. Another strategy was the use of somatic hybrids containing a recombinant cytoplasmic genome. When these recombinant genomes induce CMS, molecular markers could be identified. Through this technique, it was shown that fertility was not segregating with cpDNA (Hanson and Bentolila, 2004).

The first indications of the involvement of the mitochondrial genome in CMS were described in maize. Male-sterility conferring (cms-T) maize mitochondria contained a recombined T-urf2H3 fragment and novel ORFs. These novel ORFs were suggested to be involved in maize CMS (Dewey et al., 1986). In *Petunia*, a mtDNA arrangement unique to the CMS parent was found in all stable sterile somatic hybrids, but in none of the stable fertile somatic hybrids, suggesting that this fragment segregates with CMS in somatic hybrids (Boeshore et al., 1985). Hanson and Bentolila (2004) described 12 mtDNA regions associated with CMS in *Brassica*, radish, rice, sorghum, wheat, sunflower, maize and *Petunia*. In as much examples ATP synthase subunits sequences were involved. Male sterile tobacco lines displayed abnormal mitochondrial atp1 transcript accumulation and reduced floral ATP/ADP ratios. A novel ORF, located upstream of atp1 produced transcripts only detected in the male sterile lines (Bergman et al., 2000). Also, a lower activity of the enzym ATP synthase in seedling tissue of male sterile sunflower was observed. Indeed, during microspore development, the demand for energy and mitochondrial substrates can be so high that a dysfunctioning leads to fatal pollen development (Sabar et al., 2003). Several other hypotheses were noted on the mechanisms of

the CMS-associated genes. The T-urf2H3 protein in CMS-T maize formed pores in the inner mitochondrial membrane in the presence of a fungal toxin. This maize T-urf2H3 protein is suggested to cause pores in anthers, leading to pollen abortion. Similar mitochondrial membrane disruptions were associated with chimeric genes conducting oxidase activity in *Petunia* and *Nicotiana* (Hanson and Bentolila, 2004). In *Phaseolus*, the CMS-associated ORF239 protein inhibited the cell wall synthesis of developing microspores (Schnable and Wise, 1998). Some mt proteins were only expressed in regenerative tissues of *Phaseolus*. Other mt proteins reached an elevated level in tapetal or sporogenous tissues due to an increased expression or an increased number of mitochondria in these tissues. (Hanson and Bentolila, 2004).

Fertility restorer alleles undo the effects of CMS-associated genes. Like SI, also restoration systems can be sporophytic or gametophytic. Sporophytic restorers act prior to meiosis, gametophytic restorers act after meiosis in microspores or pollen. Fertility restoration can be contributed to one or few restorer loci (Schnable and Wise, 1998). The restorer alleles influence the (post)transcript profile, the protein accumulation or both. In CMS-sunflower, an increased polyadenylation and elevated degradation of the atpA-orf522 transcript, correlated with CMS in sunflower, is observed in restored lines. The polyadenylation can be developmentally regulated by nuclear genes (Gagliardi and Leaver, 1999). The *Rf2* locus in maize functions as an aldehyde dehydrogenase. *Rf2* can therefore reduce the amount of toxic aldehyde produced by the CMS-associated T-urf2H3 fragment (Liu and Schnable, 2002). As mentioned before, a peculiar nuclear-controlled fertility restoration was observed in common bean. The nuclear *Fr* locus resulted in the loss of a CMS-associated mtDNA region *pvs* (Janska et al., 1998). *Fr* lowered the RNA expression by reducing the DNA amount.

1.4.3 CMS in *Cichorium*

In chicory, CMS doesn't occur naturally. However, up to now, different approaches were undertaken to obtain male sterility in chicory. Tetraploid chicory was developed to allow the production of triploids after crossing with diploids. The triploid plants were completely sterile and had a higher sugar reserve than diploids (Rambaud et al., 1992). As mentioned before, Reynaerts et al. (1993) induced GMS in the witloof chicory (*C. intybus* 'Hollandse Middelvroege') through genetic engineering. GMS lines were produced by expressing the *RnaseT1* from *Aspergillus oryzae* or *barnase* from *Bacillus amyloliquifaciens*, cloned under a

tapetum-specific promoter TA29, isolated from tobacco. To restore fertility, a ribonuclease inhibitor gene, *barstar*, was expressed simultaneously with barnase in the same tapetal cells.

Alloplasmic CMS chicory plants were obtained by intergeneric protoplast fusions of chicory mesophyll protoplasts and hypocotyl protoplasts of CMS sunflower plants. *C. intybus* 'Magdeburg' varieties (Rambaud et al., 1993) and red chicory genotypes (Varotto et al., 2001) were used as chicory fusion partners. As stated by Varotto et al. (2001), the observed male sterility in the cybrids might be attributed to the introgression of CMS sunflower cytoplasm or to the incompatibility between the chicory nucleus and the alien sunflower cytoplasm. Rambaud et al. (1993) noted that in the cybrids large parts of sunflower mtDNA were incorporated in the chicory mtDNA. These rearrangements affected mtDNA transcription and abnormalities were observed both in fertility and vigour of the plant. Only one plant showed yields similar to the parental plants. The mitochondrial fragment, *orf522*, responsible for CMS in sunflower, was not responsible for CMS in chicory. *Orf522* was absent in one male sterile cybrid (Dubreucq et al., 1999) and present in restored, fertile cybrids (Rambaud et al., 1997). Therefore, CMS was believed to result from a dysfunction between the chicory nucleus and the recombined, hybrid mitochondrial genome (Dubreucq et al., 1999). The mtDNA of the progeny of plants derived from CMS chicory cybrids and different chicory pollinators was analyzed. After four generations, sunflower mtDNA was still present in cybrid mtDNA. However, the mitochondrial genome was not stably inherited in the sexual progeny. It seemed that the recombined mitochondrial genome was pursuing stability by eliminating particular parts of its mtDNA (Rambaud et al., 1997). The use of different pollinators revealed that fertility was restored when using a certain pollinator, although a recombined mitochondrial genome containing sunflower fragments was present (Dubreucq et al., 1999). The nuclear genotype is believed to be a determining factor for fertility restoring through its involvement in the mitochondrial genome structure (Rambaud et al., 1997). In CMS common bean, restoration of pollen fertility is controlled by a single dominant gene, *Fr*. Remarkably, no sterility segregation was noticed for fully restored fertile F_2 plants. The fertility restoration was a permanent condition that could not be reversed by segregation at the *Fr* locus. Moreover, the presence of the *Fr* gene directly altered the configuration of the mitochondrial genome by the loss of a 25 kb mitochondrial fragment (Mackenzie and Chase, 1990).

Cappelle et al. (2007) performed interspecific protopast fusions between *C. intybus* and *C. endivia* and produced one symmetric (tetraploid) somatic hybrid exhibiting male sterility out of 192 hybrids. Male sterility was maintained in the progeny after backcrossing with

tetraploid fertile *C. intybus* genotypes. The chloroplast genome was identical to that of *C. intybus*, while the mitochondrial genome showed fragments from both partners and recombined, unique fragments. The presence of only one male sterile plant among the hybrids of *C. intybus* and *C. endivia* was low in comparison to the higher frequence of sterile plants in a sunflower/chicory fusion experiment (Rambaud et al., 1993). This could be due to the smaller genetic distance between endive and chicory compared to sunflower and chicory. A greater genetic distance between species enhances mitochondrial rearrangements, leading to male sterility (Cappelle et al., 2007).

1.4.4 Nucleo-plastome incompatibility

Next to nucleo-mitochondrial, also nucleo-plastome incompatibilities can arise. The development of chloroplasts is co-ordinated by chloroplast and nuclear genes encoding chloroplast proteins. A nucleo-plastome incompatibility can lead to a failure in chloroplast development, inducing a hampered chloroplast activity and albinism (Leon et al., 1998). This was observed in cybrids of *Nicotiana tabacum* and *Hyoscyamus aureus* (Zubko et al., 2002) and interspecific sexual hybrids in *Zantedeschia* (Yao and Cohen, 2000). The cybrids of *N. tabacum* and *H. aureus* were generated by fusion of untreated tobacco protoplasts with γ-irradiated *H. aureus* protoplasts The cybrids contained a plastome of *H. aureus* and a recombined mtDNA. The nucleo-plastome incompatibility was expressed as a chlorophyll deficiency of cotyledonary and true leaves at early stages of development. The normal green coloration, however, was restored during later development. These cybrids were fertile and didn't show other incompatibilities (Zubko et al., 2002). Also the plastome-genome incompatibility in the interspecific hybrids in *Zantedeschia* didn't cause a decrease of the male fertility (Yao and Cohen, 2000). This suggests that nucleo-plastome incompatibility doesn't involve CMS in these cybrids.

1.5 Plant mitochondria

The main activity of plant mitochondria is the production of ATP through respiratory oxidation of fats, carbohydrates and proteins. Moreover, they produce primary and secondary metabolites, stress-related reactive oxygen species (ROS), synthetize nucleotides, vitamins and cofactors, metabolize lipids and amino acids, participate in the photorespiration and export organic acid intermediates for wider cellular use (Millar et al., 2005). The mitochondrial (mt) genome is much larger and highly variable in plants (high rates of rearrangements, duplication, genome growth and shrinkage) in comparison to the mt genome of other eukaryotes. Their size varies from 200 kb in *Brassica* species to 2500 kb in melon (Nair, 1993; Wolstenholme and Fauron, 1995). In contrast to their size, the coding capacity of the mt genome is remarkably low. Sequencing of the total mt genome of *Arabidopsis* identified 57 genes encoding components of complexes I to V, cytochrome c biogenesis, rRNAs, tRNAs and ribosomal proteins (Unseld et al., 1997). However, typically 90% of the total sequence can be attributed to introns and repeated elements (Galtier, 2011). The majority of mitochondrial proteins are encoded in the nucleus. The plant mt genome consists of a large circular molecule containing the whole genome and a couple of long repeats. Homologous recombination of these repeats produces subgenomic circles of highly variable size (Backert et al., 1996). In addition to these large circular molecules, the presence of linear and circular DNA plasmids and ds RNAs have been reported in many higher plant mitochondria (Nair, 1993). The high rate of size variation can be contributed to the high coding redundancy. The variable mt genome structure is due to a active recombination system and extraneous DNA integration. DNA sequence homologies have been detected between nuclear and chloroplast DNA and mtDNA (Nair, 1993; Mackenzie and McIntosh, 1999). The nucleotide substitution rate, however, is very low, even lower than for nuclear DNA. The existence of an efficient DNA repair activity to undo the DNA damage caused by ectopic recombinations between repeated elements is the main reason for this low substitution rate. This is in contrast with animal mtDNA, which is devoid of repeated elements and ectopic recombinations, where no efficient DNA repair system is required, implementing a higher point mutation rate (Galtier, 2011). The plant mt genome is multipartite containing different, redundant subgenomic molecules which are recombinationally active repeated sequences. Next to these molecules, the presence of smaller repeated sequences can induce recombinations causing novel open reading frames (ORFs) (Vedel et al., 1994). The subgenomic molecules can be held at a low copy number, even fewer than one per cell. Subdividing the genome by a differential

distinction of these unique forms gives the mitochondria a clear advantage in creating variation. A sudden increase in the copy number of the subgenomic molecules can cause genomic rearrangements. Otherwise, a decrease in copy number can give rise to the silencing of certain encoded genes (Janska et al., 1998). Not much is known on the mode of replication of chromosomal mtDNA. However, a rolling-circle mode of replication in the mitochondria of *Chenopodium album* was observed (Backert et al., 1996). Observations in other plant species suggest the rolling-circle model as a common phenomenon in higher plants (Backert et al., 1996). The copy number and general regulation of the mt genome is under nuclear control. This suggestion is supported by findings of a *CHM* locus in *Arabidopsis* (Martinez-Zapater et al., 1992) and *Fr* gene in common bean (Mackenzie and Chase, 1990). Moreover, an ongoing process of mitochondrial genes tranfer to the nucleus is described. In angiosperms, the transfer of mt ribosomal protein genes to the nucleus was observed (Palmer et al., 2000). Transcription of plant mt genes is regulated by at least one nuclear-encoded RNA polymerase. Mackenzie and McIntosh (1999) suggested that different types of promotors might be responsible for particular genes, indicating that these promotors need their own specific (nuclear) transcription factors. Next to the direct nuclear-mediated mitochondrial transcription, nuclear-mediated transcript processing is an effective tool of gene regulation in plant mitochondria (Gray et al., 1992). In CMS-T maize, two nuclear loci are believed to promote mitochondrial transcript splicing (Laughnan and Gabay-Laughnan, 1983). Another indirect nuclear-mediated regulation is the phosphorylation of mitochondrial proteins.

1.6 Somatic hybridization

1.6.1 Historical overview

Genetic improvement of cultivated species has been extensively investigated by breeders in their efforts towards a successful crop production. However, in conventional breeding programs, the transfer of desirable traits is restricted to sexually compatible species. A somatic hybridization protoplast-based approach may be an alternative for sexual interspecific hybridization, that is often confronted with prezygotic or postzygotic barriers (Eeckhaut et al., 2006). Plant cells from which the cell wall has been enzymatically or mechanically removed are called protoplasts. Theoretically, protoplasts are totipotent, meaning that after their isolation and subsequent culture they have the capability to dedifferentiate, re-enter the cell cycle, go through repeated mitotic divisions and then proliferate or regenerate into various organs. In other words, applying the correct physical and chemical stimuli would suffice to regenerate fertile plants through tissue culture practices. This provides a multitude of opportunities for crop improvement, including a system for protoplast fusion (somatic hybridization), somaclonal variation, and plant transformation. For foreign gene introduction, somatic fusion is superior to plant gene transformation in some aspects as antibiotic resistance markers can be avoided and complex agronomic traits controlled by polygenes can be introgressed (Xia, 2009).

Although both mechanical isolation (Klercker, 1892) and fusion (Küster, 1909) were originally described more than a century ago, protoplast related research was only well initiated after the first enzymatic digestion (Cocking, 1960). Some years later, mass production of protoplasts thanks to the availability of commercial macerating enzymes enabled worldwide protoplast research on a wide array of plant species. The first report on the full plantlet regeneration from protoplasts was described in tobacco mesophyll cells (Takebe et al., 1971). Tobacco was also the first crop in which successful interspecific somatic hybridization was reported through symmetric protoplast fusion (Carlson et al., 1972). Protoplast culture at low densities became possible for more species after the publication of the Kao & Michayluk medium composition (Kao and Michayluk, 1975). The first application of irradiation to obtain asymmetric hybrids was performed in parsley (Dudits et al., 1980). Since then, the 1970s and 1980s onwards, much progress has been reported on protoplast regeneration and fusion. Those were compiled in reviews by Melchers and Labib (1974), Davey & Kumar (1983), Gleba and Sytnik (1984), Bravo and Evans (1985) and Davey and

Power (1988). In spite of regeneration problems, protoplast fusion became a common technique for the introduction of novelties in commercial crops (Brown and Thorpe, 1995). However, during the 1990s protoplast based technologies for gene transfer were overshadowed by recombinant DNA technologies. Partly due to public antagonism towards these technologies, interest in protoplast research was renewed, although at that time only a limited number of plant families produced regenerative protoplasts (Waara and Glimelius, 1995).

A somatic breeding protocol can typically be subdivided into the following steps: isolation, fragmentation (in case of asymmetric hybridization), fusion, regeneration and selection (Liu et al., 2005). Protoplast isolation is typically performed through a 1-step-procedure in which a pectinase and a cellulase type enzyme are jointly added to an osmotically corrected solution to respectively separate the cells from their pectin rich matrix and to dissolve the cellulose rich cell wall. Several agents can be used to create an osmotic equilibrium between the protoplasts and their environment to prevent protoplast bursting: metabolically inactive sugar alcohols such as mannitol and sorbitol are most frequently applied but also glucose, sucrose or salts can be used (Razdan, 2003). Carlson et al., 1972 described the first protoplast fusion. However, this fusion between tobacco species was spontaneous. Fusions can also be induced through mechanical pushing, $NaNO_3$ treatment, or high pH/Ca^{2+} treatment (Razdan, 2003). Currently, fusion is nearly exclusively performed through polyethylene glycol (PEG) (Kao et al., 1974) or electrofusion (Zimmerman and Scheurich, 1981). Chemical fusogens cause the isolated protoplast to adhere to each other and lead to tight agglutination. By adding a high pH/Ca^{2+} solution, cell membranes will be disrupted and neighbouring protoplasts will fuse. Chemofusion is non-specific and inexpensive but can be cytotoxic. For electrofusion, protoplasts are first aligned between electrodes in a low strength electric field generated by alternating current, upon which fusion is induced by application of one or a few high-voltage direct current pulses. The electroporation induced by these pulses enables cell fusion. Electrofusion is less cytotoxic than chemical fusion, but more expensive. After fusion, different types of homokaryons or heterokaryons can be created, as well alloplasmic hybrids (cybrids) (Liu et al., 2005). Fusion of divergent parental protoplasts leads to the formation of symmetric hybrids, combining both nuclear genomes. Symmetric fusion events, however, lead to the incorporation of total genomes, which can disturb the regeneration capacity, the development or the fertility of the somatic hybrid. By reducing the amount of transmitted nuclear information, these problems can be overcome. Asymmetric fusions enable us to only transfer partial genomes. Several techniques can be used for fragmentation of the so-called

'donor' genome, such as X or gamma rays, ultraviolet (UV) irradiation (Hall et al., 1992) or microprotoplasts (Yemets and Blume, 2009). Moreover, by introgressing fewer genes than after sexual crossing or symmetric somatic fusion, the number of backcrosses could be significantly reduced. Also, cytoplasmic genomes can be recombined with nuclear genomes for applications like CMS introduction (Liu et al., 2005). Although much investigation was already performed on fragmentation, chromosome elimination is random and unpredictable. Generally, irradiation causes the reduction of the amount of donor DNA, but this may vary from a few traits, one or a few chromosomes to a big part of the donor genome (Waara and Glimelius, 1995). Protoplast regeneration is often the bottleneck in somatic hybridization breeding programs. Evidently, regeneration within a single species is strongly genotype related. In a given crop however, lots of parameters are usually optimized to achieve an optimal efficiency. In Razdan (2003), Davey et al. (2005) and Veilleux et al. (2005), the importance of parameters as protoplast source, protoplast density in culture, chemical media composition, physical culture method, refreshment rates and plant hormones are discussed. Regeneration problems have forced researchers to come up with more innovative approaches, such as electrical stimulation, non-ionic surfactants and artificial gas carriers. A complete overview of regeneration related parameters is presented by Davey et al. (2005). All types of fusion products can be found after protoplast fusion; heterokaryotes, homokaryotes and unfused parental protoplasts. Identifying the hybrids in a population requires a stringent selection system. Usually, screening is performed during or after *in vitro* regeneration. Apart from morphological markers, many tools were developed (Liu et al., 2005); flow cytometry, *in situ* hybridization, isoenzymes and molecular markers. A thorough screening can be complicated by genotype instability such as chromosome loss or by hybrid growth vigor as in sexual hybrids (Eeckhaut et al., 2006). Tables 1-3, 1-4 and 1-5 give a extended overview of protoplast research during the last decade. Special attention is given to protoplast regeneration, (a)symmetric hybridizations and hybrid screening methods.

1.6.2 Protoplast regeneration

Protoplast regeneration is often the bottleneck in somatic hybridizations. Therefore, and due to the increasing interest in protoplast-related research, nowadays, much is invested in protoplast regeneration protocols. Table 1-3 gives an overview of recent progress of protoplast regeneration in spermatophytes. For many species complete protoplast regeneration has been realized for the first time including for *Echinacea purpurea*, *Gossypium hirsutum*,

Gossypium davidsonii, *Ipomoea cairica*, *Lilium japonicum*, *Solanum virginianum*, *Zingiber officinale*, *Cyclamen alpinum*, *Cyclamen graecum*, *Cyclamen mirabile* and *Cyclamen coum* (Table 1-3). In the case of *Kalanchoë blossfeldiana*, protoplast regeneration was achieved for the first time in the Crassulaceae family (Castelblanque et al., 2010). In recent years, regeneration protocols for plants that could already be regenerated were redefined and optimized for their use in a later protoplast-related research. Variable parameters for an increased efficiency of protoplast regeneration were investigated. An analysis of the most successful methods during the last decade on protoplast regeneration gives us following summary:

1.6.2.1 Protoplast source

The most efficient protoplast sources are mesophyll cells and suspension cells; other protoplast suppliers are callus, hypocotyls somatic embryos and, in a single case, cotyledons. Donor material type has often been decisive for successful regeneration. *Ipomoea* white soft callus is not regenerative, as opposed to compact callus (Guo et al., 2006). In citrus, calli donor material is suspected to result in slower colony growth rates (Takami et al., 2005). In the case of *Kalanchoë blossfeldiana*, leaves were used, and leaf preculture can lead up to isolation of more dense protoplast types, probably due to dedifferentiation during the preculture (Castelblanque et al., 2010). Dedifferentiation requires that somatic cells reprogram and enter the cell division cycle. *Arabidopsis thaliana* mesophyll protoplasts were used as a representation of dedifferentiated cells in which the vacuolar architecture could be monitored during repair mechanisms (Sheahan et al., 2007). Before dedifferentiated plant cells entered cell division, the vacuole developed a complex architecture which is actinomyosin dependent and contributes to nuclear positioning and an enhanced cellular metabolism, both required for division. Embryogenic suspension cells were particularly used as protoplast source in monocot species (Chabane et al., 2007). Suspension cells are more dedifferentiated than callus, which is a possible reason for their better regeneration upon protoplast isolation. Like callus and unlike mesophyll cells, suspension cells contain more mitochondria, suggesting a better energy supply to dividing protoplasts (Moreira et al., 2000). When mesophyll cells were used to create somatic hybrids, their lack of calli differentiation capacity might decrease hybrid regeneration capacity (Szczerbakowa et al., 2005). Mesophyll cells were rarely used in monocots. Although cell suspensions theoretically were the best starting material they were often hard to accomplish in cereals (Li et al., 2004). Another drawback is the possible introduction of cytological aberrations or mutations (Grosser et al., 2007b). For instance,

standard citrus fusions are performed between cell suspension protoplasts and mesophyll protoplasts, because complementation from the leaf parent probably allows the fusion products to overcome cytological mutations, built up over time during callus or cell suspension culture (Guo and Grosser, 2005). As opposed to this, Wu et al. (2005) used callus as protoplast source for both partners, yielding 90% hybrids among the regenerants, probably due to heterosis effects.

1.6.2.2 Culture types

Table 1-4 shows that about half of the culture types mentioned were liquid based, whereas the other half was mostly based on culture in alginate or agarose embedding. Mostly these embedding agents were used for culture in beads or layers in liquid medium. Only four reports mentioned culture in solid medium as the optimal strategy. Recent results have led to a better understanding of the importance of culture systems. A nurse layer of tuber mustard cells significantly increased regeneration of cauliflower (Sheng et al., 2011) and red cabbage protoplasts (Chen et al., 2004). It was the first time red cabbage protoplast culture was successful; without a nurse layer microcalli did not form. Also the sustained division of banana protoplasts exclusively occured when a feeder system was implemented (Xiao et al., 2007); possibly the feeder layer had a signaling function on top of a nutrient providing function. Cell suspensions were often used as feeder layers (He et al., 2006), and their efficiency was determined by their culture time, possibly because a more vigorous growth coincided with the release of more stimulatory substances in the medium that can initiate divisions in the more recalcitrant protoplasts. As not any cell suspension was a suitable source for a feeder layer, as well genotype, pretreatment and medium of the original callus could affect the final efficiency of the protocol. Liquid medium rarely yielded better protoplast division (Castelblanque et al., 2010). The lower colony formation in liquid medium was assumed to be caused by a shortage of aeration and light (Azad et al., 2006) or a release of toxic components (Duquenne et al., 2007). Shrestha et al. (2007) proposed that the reasons for efficient division of cell suspension *Phalaenopsis* protoplasts were the better dilution of inhibitory substances and the better distribution of nutrients. Niedz (2006) regenerated somatic embryos through culture of citrus protoplasts on semi-permeable membranes which enabled a better oxygen supply to the cells. Microfluidic devices were beneficial for culture of small cell populations and continuous supplementation with no need of large culture media volumes. *Nicotiana tabacum* protoplasts were cultured in microfluidic polydimethylsiloxane channels with microtubes for continuous medium supply and successfully developed into

microcolonies within four weeks (Ko et al., 2006). A general finding was the better performance of protoplasts when embedded in alginate or agarose. In the genus *Cichorium*, regeneration of a wide variety of species and genotypes could be accomplished by agarose bead culture (Deryckere et al., 2012). For other crops, as well beads, discs, layers, thin layers or extra thin films were used. A major advantage of embedding systems is the easier handling of the cultures which permits replacement of the culture media without disturbing the development of the microcolonies and may prevent microbial contamination. When discs were used, protoplasts divided at a higher rate at the edge (Rakosy-Tican et al., 2007). The thinner the matrix was, the higher plating efficiencies were (Pati et al., 2005). For carrot protoplast culture, layer thickness was minimized by circular rotation of the protoplast/alginate suspension during application and before polymerization (Grzebelus et al., 2012a). Also the embedding agent type affects the final outcome, possibly by interacting with genotype, osmolarity, temperature, culture system or aeration (Prange et al., 2010; Kielkowska and Adamus, 2012). This is in accordance with earlier postulations on the positive effect of embedding by membrane stabilization through lipid peroxidase inhibition, the prevention of leakage of cell wall precursors or other metabolites, and lower ethylene levels (Bajaj, 1989). Moreover, protoplast aggregation leading up to poor oxygen supply and browning was avoided (Pati et al., 2008; Lian et al., 2011). Also, the osmotic pressure changed steadily instead of stepwise (Kanwar et al., 2009). Briere et al. (2004) studied the organization of actin microfilaments during sunflower protoplast culture in an agarose matrix. Removal of the cell wall completely disrupted the actin cytoskeleton, which became progressively reorganized into cortical microfilament arrays and actin cables during protoplast culture. Treatment of protoplasts with arginine-glycine-aspartic acid (Arg-Gly-Asp) motif-containing peptides, to inhibit putative cell contacts with the agarose matrix, strongly affected this repair process: microfilament elongation and cable formation were inhibited and the connectivity between the cortical network and the perinuclear basket was lost. Furthermore, embryoid formation induced by agarose embedding was reduced. Similar effects were observed with a short treatment with latrunculin B, known to disrupt actin microfilaments. These results indicate that the actin network is involved in the signaling process that leads to polarity acquisition and embryoid determination in agarose-embedded protoplasts. It is thus likely that agarose embedding of protoplasts allows the formation of transmembrane adhesion complexes that anchor the protoplast to this extracellular matrix and stabilize the cytoskeleton network. This will help to determine and/or fix the polarity of the cell, leading to asymmetric division and embryoid development. Another important parameter for optimal protoplast

culture are initial protoplast density. Typically, protoplast densities were 100,000-200,000 ml^{-1}, with a minimum of 10,000 for *Ipomoea cairica* (Guo et al., 2006) and a maximum of 1,000,000 for *Solanum virginianum* (Borgato et al., 2007), *Phoenix dactylifera* (Chabane et al., 2007) and *Musa* species (Dai et al., 2010).

1.6.2.3 Medium composition

Chemical modifications have recently contributed to regeneration of some afore recalcitrant species or materials. A contribution on the optimization of mineral composition with respect to the type of callus development in *Lupinus* was published (Sonntag et al., 2009), stating that B5 mineral composition, established by Gamborg et al. (1968), resulted in more compact callus development than MS medium (Murashige and Skoog, 1962) or any other composition attempted. In 1984, Okamura et al. published the detrimental effect of ammonium on regeneration of Asteraceae plants. Recent publications however clarified that this is not restricted to Asteraceae (Umate et al., 2005; Fiuk and Rybczynski, 2007; Guo et al., 2007; Kanwar et al., 2009). In *Beta vulgaris*, protoplast regeneration recalcitrance was problematic. The plating efficiency of mesophyll cells however drastically increased after adding 100 nM phytosulfokine, a peptide growth factor, that has antioxidant properties but possibly also generates a nurse cell effect (Grzebelus et al., 2012b). Furthermore, through extensively investigating the identification of genotype related factors like cell wall polysaccharides responsible for plant regeneration from single cells, the supply of exogenous arabinogalactan protein-rich extracts significantly improved the protoplast derived callus organogenesis (Wisniewska and Majewska-Sawka, 2007; Wisniewska and Majewska-Sawka, 2008). Galactoglucomannan-derived oligosaccharides in very low concentrations acted as regulatory/signaling molecules in plant cells elongation, differentiation and development. They evoked inhibition of elongation growth induced by auxins probably associated with cell wall modifications catalyzed by peroxidases. Combined with NAA they positively influenced not only the viability, but also the protoplast regeneration and division. They influenced both quality and quantity of extracellular proteins in regenerating protoplasts. They probably fulfilled a protective role in this process of spruce protoplast regeneration (Kakoniova et al., 2010). Sotiriou et al. (2007) have presented the first report on the presence of glucuromannan in cell walls regenerated from protoplasts; simultaneously oligosaccharides and arabinogalactans were released. In Brassicaceae, iodoacetamide (IOA) was added to prevent division of unfused protoplasts, who subsequently could nurse the fused cells (Chen et al., 2005; Tu et al., 2008). Plant hormones remain among the best studied parameters for

protoplast regeneration. Auxin 1-NAA addition to protoplasts isolated from leaves of 6-day-old wheat seedlings induced an increase in the cytosolic Ca concentration within 5–10 s, while the physiologically non-active analogue, 2-NAA, did not (Shishova and Lindberg, 2004). A complicated mechanism of auxin-induced rise in cytosolic Ca was suggested. A 2,4-D shock was found to be indispensable for *Helianthus* protoplast regeneration (Taski-Ajdukovic et al., 2006). The cytokinin TDZ act as a regulator of morphogenetic responses, including somatic embryogenesis, micro-propagation, regeneration and shoot formation, probably through modulation of auxin levels (Xiao et al., 2007; Thomas, 2009); for shoot induction, the cytokinin type may be decisive (Borgato et al., 2007). Also, as well interactions of gibberellic acid (Yang et al., 2007a; An et al., 2008) as ethylene inhibitors (Guo et al., 2007) with regeneration were studied. Endogenous hormones can interact with exogenously applied plant growth regulators, as demonstrated by the different reaction of multiple explants types on phytohormone treatments (Sun et al., 2005). Polyamines are known to be involved in a variety of growth and developmental processes in higher plants, as well as in adaptation to stresses. The isolation process contributed to increased putrescine levels, which were higher in non-totipotent tobacco protoplasts than in totipotent tobacco protoplasts (Papadakis et al., 2005). During culture, putrescin predominated over other polyamines, and the highest accumulation was found in totipotent protoplasts. The authors suggested that the levels and metabolism of the intracellular polyamines could be related to the expression of totipotency of plant protoplasts. Rakosy-Tican et al (2007) found that a combination of spermidine and haemoglobin increased plating efficiency but was unable to provoke full regeneration. They proposed spermidine to stimulate mitosis and to reduce stress impacts.

Table 1-3 Progress on protoplast regeneration in different plant families from 2004 onward.

Plant family and species	Protoplast source[y]	Culture method	Optimal density (10^4 pp/ml)	Best result[z]	Reference
Alstroemeriaceae					
Alstroemeria spp.	C	Liquid	10	P	Kim et al., 2005
Apiaceae					
Daucus carota	H	Thin alginate layer	40	P	Grzebelus et al., 2012a
Araceae					
Anthurium scherzerianum	SE	Agarose beads	10	MCO	Duquenne et al., 2007
Anubias nana	M	Agarose beads	50	MCO	Pongchawee et al., 2006
Cryptocoryne wendtii	M	Liquid	50	MCO	Pongchawee et al., 2007
Spathiphyllum wallisii	SE	Agarose beads	10	MCO	Duquenne et al., 2007
Arecaceae					
Phoenix dactylifera	C	Liquid, feeder layer	100	C	Chabane et al., 2007
Phoenix dactylifera	C	Feeder layer	100	C	Rizkalla et al., 2007
Asclepidaceae					
Tylophora indica	M	Liquid	50	P	Thomas, 2009
Asteraceae					
Chrysanthemum spp.	M	Agarose solid plating, co-culture	10	MCO	Zhou et al., 2005
Chrysanthemum indicum	M	Liquid	10	C	Eeckhaut and Van Huylenbroeck, 2011
Cichorium intybus	M	Agarose embedded	5	P	Deryckere et al., 2012
Echinacea purpurea	M	Alginate block/liquid	10	P	Pan et al., 2004
Helianthus annuus	H	Alginate discs	80	P	Rakosy-Tican et al., 2007
Brassicaceae					
Brassica oleracea	H	Agarose embedded, co-culture	10	P	Chen et al., 2004a
Brassica oleracea	H	Agarose embedded, co-culture	10	P	Sheng et al., 2011
Brassica oleracea	H	Alginate layer	40	P	Kielkowska and Adamus, 2012
Caryophyllaceae					
Dianthus acicularis	M, SC	Solid (Gelrite)	10	P	Shiba and Mii, 2005
Chenopodiaceae					
Beta vulgaris	M	Thin alginate layer	40	MC	Grzebelus et al., 2012b
Convolvulaceae					
Ipomoea cairica	M	Liquid	1-2	P	Guo et al., 2006
Crassulaceae					
Kalanchoë blossfeldiana	M	Liquid	10	P	Castelblanque et al., 2010
Cucurbitaceae					
Cucumis anguria	M	Liquid	20	C	Gajdova et al., 2007
Cucumis melo	M	Liquid	20	C	
Cucumis metuliferus	M	Liquid	20	C	
Cucumis sativus	M	Liquid	20	C	
Fabaceae					
Astragalus melilotoides	C	Liquid	40-50	P	Hou and Jia, 2004
Ceratonia siliqua	H	Liquid	25-40	MC	Sotiriou et al., 2007
Lupinus luteus	H	Liquid	20	MCO	Wiszniewska and Pindel, 2009
Robinia pseudoacacia	C	Liquid	20-40	P	Kanwar et al., 2009
Gentianaceae					
Gentiana kurroo	SC (CO)	Agarose bead cultures	20	P	Fiuk and Rybczynski, 2007
Goodeniaceae					
Scaevola aemula	M	Agarose droplets	10	E	Wang, 2011
Hypericaceae					
Hypericum perforatum	HC	Alginate blocks	20	P	Pan et al., 2005
Iridaceae					
Iris fulva	SC	Agarose block	10	P	Inoue et al., 2004
Lauraceae					
Cinnamomum camphora	SC	Liquid	10	P	Du and Bao, 2005

Liliaceae					
Lilium japonicum	SC	Agarose embedded, nurse cells	10	P	Komai et al., 2006
Muscari neglectum	C	Alginate beads with nurse cells	10	P	Karamian and Ranjbar, 2010
Malvaceae					
Gossypium hirsutum	SE, SC	Liquid	20-100	P	Sun et al., 2005c
Gossypium hirsutum	SC	Liquid	20	P	Wang et al., 2008a
Gossypium klotzschianum	SE, SC	Liquid	20-100	P	Sun et al., 2005b
Gossypium davidsonii	SC	Liquid over solid	20-100	P	Yang et al., 2007a
Moraceae					
Morus indica	M	Liquid	10	P	Umate et al., 2005
Musaceae					
Musa acuminata	SC	Liquid, feeder layer	100	P	Xiao et al., 2007
Musa paradisiacal	SC	Liquid, feeder layer	100	P	Dai et al., 2010
Myrsinaceae					
Cyclamen coum	SC	Agarose or alginate embedded	15	P	Prange et al., 2010b
Cyclamen alpinum	SC	Agarose or alginate embedded	15	P	Prange et al., 2010a
Cyclamen graecum	SC	Agarose or alginate embedded	15	P	Prange et al., 2010a
Cyclamen mirabile	SC	Agarose or alginate embedded	15	P	Prange et al., 2010a
Cyclamen persicum	SC	Alginate films	15	P	Winkelmann et al., 2006
Nelumbonaceae					
Lotus corniculatus	CO	Extra thin alginate film	20	P	Pati et al., 2005
Orchidaceae					
Phalaenopsis sp	SC	Solid, gellan gum	10	P	Shrestha et al., 2007
Poaceae					
Zea mays	SC	Solid/feeder/liquid	20-40	P	He et al., 2006
Rutaceae					
Citrus sinensis	C	Alginate beads	25	E	Niedz, 2006
Phellodendron amurense	M	Solid, gellan gum	40	P	Azad et al., 2006
Solanaceae					
Calibrachoa spp.	M	Liquid or alginate embedded	15	S	Meyer et al., 2009
Petunia spp.	M	Liquid	15	S	Meyer et al., 2009
Nicotiana tabacum	M	Extra thin alginate film	10	P	Pati et al., 2005
Solanum virginianum	M	Thin alginate layers in liquid	100	P	Borgato et al., 2007b
Ulmaceae					
Ulmus minor	M	Agarose droplets	20	MC	Conde and Santos, 2006
Zingiberaceae					
Zingiber officinale	SC	Liquid	10-50	P	Guo et al., 2007b

[y]C: callus; CO: cotyledon; H: in vitro hypocotyls; HC: hypocotyls derived callus; M: mesophyll cells from in vitro leaves; SC: suspension cells; SE: somatic embryos

[z]C: callus; E: embryos; MC: microcalli; MCO: microcolonies; P: plants; S:shoots

1.6.3 Symmetric hybridization

In Table 1-4, publications of the past 10 years on symmetric hybridization are listed. The best studied plant families when analyzing symmetric fusions, were Brassicaceae, Rutaceae and Solanaceae. Compared to earlier publications (before 2004), innovation was mainly achieved by making new fusion combinations. In symmetric hybridization experiments, the use of either chemical fusion mediation or electrofusion largely depended on the plant family. PEG-mediated fusion was standard in Brassicaceae, whereas electrofusion was routineously applied in Solanaceae and Malvaceae; for Rutaceae both fusions methods were used. Over all families, the most widely used tissue types were leaf mesophyll cells, cell suspension cells, callus and hypocotyls (mainly in Brassicaceae and Fabaceae). Somatic embryos, cotyledons, pollen and petioles were only occasionally used. The final objectives of the different projects were quite diverse. The most important driver was breeding for biotic resistance or tolerance, especially in the genus *Solanum*. Gene pool enlargement of a commercial crop through fusion with a wild species and the creation of hybrids to monitor hybrid creation and to develop tools for hybrid screening was another important aim. Of course, this was primarily performed in well studied families as Rutaceae, Brassicaceae and Solanaceae. Of relatively lesser importance were breeding for abiotic resistance or tolerance, production of secondary metabolites, rootstock breeding in Citrus, hybridization, altered morphology and ploidy breeding. Biomass production, introduction of cytoplasmic male sterility, N_2 fixation and formation of storage roots were rare objectives (Table 1-4).

1.6.4 Asymmetric hybridization

Asymmetric fusion techniques have been widely applied over the last decade and several new asymmetric hybrids were obtained (Table 1-5). Genome fragmentation of the donor parent encourages the elimination of much of its redundant genetic material in the somatic hybrid. Moreover, most karyotype instability causing donor genes are elimated during the first post-fusion mitoses, as opposed to symmetrical fusions after which eliminations can occur up to the first sexually derived generation (Cui et al., 2009). The most studied families were Brassicacae and Poaceae followed by Rutaceae. In asymmetric hybridization experiments, a 4-fold more chemical fusions were performed than electrical fusions. The Apiaceae species *Bupleurum scorzonerifolium* was used as acceptor as well as donor. Recently, a lot of new combinations to produce somatic hybrids were produced. For the first time, an asymmetric

hybrid was reported in banana (Xiao et al., 2009). Interfamilial asymmetric hybrids have been produced for the first time between the dicot *Arabidopsis thaliana* and the monocot common wheat (Deng et al., 2007). Fusion between phylogenetically remote tall fescue, Italian ryegrass and common wheat was achieved (Cheng and Xia, 2004; Ge et al., 2006; Cai et al., 2007). In cotton, asymmetric hybrids were obtained as an alternative for symmetric hybrids (Yang et al., 2007b). New somatic hybrids were obtained between monocot *Festuca arundinacea* Schreb. and dicot *B. scorzonerifolium* through symmetric as well as asymmetric fusions (Wang et al., 2011b). The first successful somatic hybrid regeneration between *Oryza sativa* L. *japonica* and *O.meyerina* L. was reported (Yan et al., 2004). Scholze et al. (2010) produced the first *Raphanus-Brassica* somatic hybrids with fungal and virus disease resistance. Cybrids were produced between chloroplast transformant tobacco and petunia (Sigeno et al., 2009). Using UV irradiated asymmetric hybrids a radiation hybrid panel was established for *Lolium multiflorum* (Cheng et al., 2006). Taski-Adjukovic et al. (2006) regenerated an asymmetric hybrid between sunflower and *Helianthus maximiliani* for the first time. Acceptor protoplast sources for asymmetric hybridization existed mainly of suspension cell cultures, mesophyll, callus and hypocotyls. The donor protoplast source rarely differed from the one for the acceptor. Brassicaceae and Asteraceae hypocotyl acceptor protoplasts were combined with mesophyll donor protoplasts (Taski-Ajdukovic et al., 2006; Scholze et al., 2010; Wang et al., 2011a). Biotic resistance introduction, genetic variation, hybrid analysis, fragmentation technology development and secondary metabolite production were recently the most important aims for asymmetric hybridization. Other motives were plastome and/or CMS transfer or a modification of agronomic traits such as protein content or seedless fruits. Abiotic resistance introduction, hybridization, genome mapping and the establishment of chromosome addition lines were rare objectives (Table 1-5).

1.6.5 Fragmentation techniques - Irradiation

1.6.5.1 Historical overview

Symmetric protoplast fusion combines complete genomes and cytoplasms of different parents. Fusion of intact cells leads to hybrids with desirable as well as undesirable traits, disturbing the regeneration capacity, the development or the fertility of the somatic hybrid. By reducing the amount of transmitted nuclear information, these problems can possibly be overcome. By introgressing fewer genes than after sexual crossing or symmetric somatic fusion, the number of backcrosses could be significantly reduced. Asymmetric protoplast

fusion enables the selective transfer of one or few donor chromosomes or fragments. To create cybrids, only cytoplasmic related features are transmitted after complete fragmentation of the donor nucleus. Several techniques can be used for donor genome fragmentation, such as X or gamma rays, UV irradiation (Hall et al., 1992) or microprotoplasts (MPP) (Yemets and Blume, 2009). The technique of MPP requires donor cells with one or few chromosomes through synchronisation, micro-nucleation and MPP isolation from plant cells (Zhang et al., 2006; Famelaer et al., 2007). However, the contamination of MPP by a low percentage of non-fragmented protoplasts can cause problems in further protoplast screening (Hall et al., 1992). The first application of irradiation (X-ray) to obtain asymmetric hybrids was performed in parsley (Dudits et al., 1980). UV-light used to create asymmetric hybrids was used for the first time on *Nicotiana* donor protoplasts (Dimanov and Atanassov, 1989). Asymmetric fusion products were also noticed without fragmentation treatment. Due to chromosome rearrangements during meiosis, chromosomes and chromosome fragments of different parents could be lost (Rambaud et al., 1993). The production of these asymmetric hybrids decreased possible long term irradiation effects on hybrid growth and development (Li et al., 2004). Whereas earlier, X or gamma rays were more frequently used for the fragmentation of the donor protoplasts, UV treated protoplasts are currently more widely applied. However, within a single species susceptibility towards both radiation types can strongly differ (Wang et al., 2012). Hall et al. (1992) investigated whether UV radiation (a non ionizing radiation tool) could be used as an alternative for ionizing radiation techniques (X or gamma rays). Fragmentation through ionizing radiation resulted in less genomic elimination than desired (Famelaer et al., 1990). The extent of DNA loss did not clearly correlate with the ionizing radiation dose. UV had a detrimental effect on sugar beet protoplasts: resynthesis of a cell wall, cell growth and cell division were partially or totally eliminated. Protoplast viability had not decreased after 6 days culture, but after 14 days, the UV-treated cells died. Another advantage of UV radiation over ionizing radiation was its easy application and high reproducibility (Hall et al., 1992). Both UV and X ray irradiation efficiently induced asymmetry in somatic hybrids between *Brassica napus* and *Arabidopsis thaliana*, in a dose-dependent manner (Forsberg et al., 1998). A restriction enzyme treatment, creating DNA double strand breaks, didn't affect the frequency of hybrid asymmetry.

1.6.5.2 Effect of UV irradiation on plant systems

The reduction of the ozone layer, because of man's activities, has opened the discussion on plant response mechanisms towards solar UV radiation. UV radiation, about 7-9% of the solar

radiation plants are exposed to, is generally classified into three classes: UVA (315-400 nm), UVB (280-315 nm) and UVC (200-280 nm) (Navratilova et al., 2008; Sarghein et al., 2011). UVC rays, containing the highest radiation energy, are mainly absorbed by the ozone layer and don't reach the earth's surface. However, all types of UV radiation can influence plant processes in different ways; by DNA damaging, causing heritable mutations, or by altering physiological processes (Stapleton, 1992). UV photons are effeciently absorbed by biological macromolecules, chromophores, resulting in photochemical reactions. Among those chromophores, nucleic acids absorb most and are thus more sensitive to the destructive action of UV. Other plant chromophores are proteins, flavoproteins and lipids. Any changes of UV conditions might lead to physiological alterations in the plant. The effects of UV radiation differs between species and varieties (Teramura, 1983), and can be primary or secondary. UV-induced primary effects, like mutations, in DNA and/or RNA can cause secondary effects including an altered protein transcription/translation. Lipid components of plant membranes absorb UV rays effectively, causing changes in membrane characteristics, leading to alterations in permeability, ionic balance, photosynthesis and respiration (Caldwell, 1981). Also plant disease susceptibility, plant growth and development, photosynthesis and reproduction can be affected (Teramura and Sullivan, 1994; Jansen et al., 1998). Increased UVB irradiation results in structural changes like epidermal deformation, altered cuticular composition and increased flavonoid levels (Stapleton, 1992) and ultrastructural changes including dysfunctioning of chloroplasts and peroxisomes, auxin inactivation and ATPase destruction (Sarghein et al., 2011). Moreover, environmental conditions, including temperature, soil and air nutrient concentrations and moisture, might affect the UV sensitivity of plants (Sarghein et al., 2011).

As stated before, UV is efficiently absorbed by nucleic acids. The consequences of UV absorption are the highest at the DNA level. Much higher UV doses are needed to inactivate RNA and proteins, due to the relative cellular abundance of these compounds compared to DNA (Caldwell, 1981). The best studied UVC-induced DNA lesions are dimers of pyrimidine bases such as cyclobutane-type (CPDs) and pyrimidone-type (PPs) dimers. CPDs are subjected to conversion of CC to TT tandem substitution. UVC is therefore frequently used for mutagenesis studies. Mismatch repair (MMR) prevents the formation of these UV-induced tandem mutations by repairing mismatched bases and small insertions/deletions (Skinner et al., 2008). Next to mutations, UV irradiation induces the formation of single strand breaks (ssbs) through base or nucleotide excision repair (BER or NER). Double strand breaks (dsbs) are observed when CPDs or PPs are not excised before DNA replication (Abas et al., 2007).

DNA photoproducts, protein crosslinks and ROS damage are other common UVC-induced cytotoxins (Peak and Peak, 1986). DNA damage can be repaired by photoreactivation, excision repair and recombinational repair, however, the last mechanism has not yet been reported in plants (Stapleton, 1992). Photoreactivation is an enzymatic photoreversal which induces the monomerization of pyrimidine dimers by photolyases. It is driven by UVA and blue light. Excision repair is non light-driven and not limited to pyrimidine dimers. This mechanism involves the identification and excision of the lesion from the strand and the synthesis of a replacement patch using the complementary strand as a template (Caldwell, 1981; Stapleton, 1992). A non molecular repair system is photoprotection: organisms, subjected to longwave UV or visible radiation in an earlier stage, are less influenced when submitted to high energetic radiation. Photoprotection will lead to a delayed cell division, allowing more time for cell repair systems. As a consequence of photoprotection, plant competitiveness is reduced leading to a delayed plant growth or leaf expansion.

In most UV photoinhibition studies, UV, at shorter wavelengths, reduces plant photosynthesis. However, chlorophyll concentration only decreased when applying large UVB doses. Moreover, some studies suggest an increased photosynthesis through the stimulative effect of UV at longer wavelengths (Caldwell, 1981). The UVB-induced photosynthesis reduction is mainly due to the UV damage of Photosystem II (PS II), directly or indirectly through photoreceptors quinones, leading to a decrease in the oxidative capacity. An altered chloroplast ultrastructure and reduced Rubisco activity are other consequences of UVB radiation. Plants have developed several responses to circumvent UV damage, for instance anatomical changes such as alterations in epidermal layers and epicuticular waxes, an increased leaf thickness or weight and changes in leaf ultrastructures (Teramura and Sullivan, 1994). Another strategy is the accumulation of UV-absorbing compounds, such as flavonoids and anthocyanins, in the leaf epidermis, reducing UVB radiation transmittance. Flavonoids accumulation is regulated by enzymes in the flavonoid biosynthetic pathway. The production of these enzymes, chalcone synthase (CHS) and phenylalanine ammonia lyase (PAL) is induced by UV radiation. However, these strategies are leading indirectly to lower photosynthesis by also hindering the penetration of visible, photosynthetic radiation (Stapleton, 1992; Teramura and Sullivan, 1994).

1.6.5.3 Fragmentation in asymmetric fusions for the last decade

UV irradiation was the most common fragmentation tool as shown in Table 1-5: in 21 of the 35 asymmetric protoplast fusions reported, UV irradiation was the preferred nuclear

fragmentation tool. On several occcasions, UV treated donor cells were fused with iodoacetamide (IOA) treated recipients. Gamma (γ) irradiation was reported in 6 studies. In citrus, two fusion combinations were made between IOA-treated recipient protoplasts and γ-irradiated donors (de Bona et al., 2009a). Microprotoplast isolation through ultracentrifugation was also attempted, either alone (Xu et al., 2006) or combined with γ irradiation (de Bona et al., 2009b). X-rays were used as a fragmentation tool by Yan et al., (2004), Ge et al., (2006) and Scholze et al., (2010), combined with IOA acceptor inactivation in the Poaceae fusions. For combining *N. tabacum* and *N. repanda*, Sun et al. (2005) inactivated the acceptor cytoplasm by rhodamin 6G and fused directly with the receptor. A general problem is the quantification of DNA damage after an irradiation treatment. Abas et al. (2007) presented Comet assay single cell gel electrophoresis as a reliable tool to observe single and double strand breaks in mesophyll protoplasts of *Nicotiana plumbaginifolia*, and Xu et al. (2007) revealed extensive DNA fragmentation in UV irradiated *Citrus unshiu* protoplasts with the terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling assay. UV treatment significantly enhanced the levels of single and double strand breaks in light and dark cultured protoplasts. In cucumber, the negative effect of UVC irradiation on cell wall regeneration, protoplast viability and the intensity of the nuclei after DAPI staining is significant (Navratilova et al., 2008). Therefore, the use of micronuclei and MPPs has been considered as a method for partial genome transfer. In *Beta vulgaris* suspension cells, synchronisation and micronucleation were the first steps towards an efficient MPP production (Famelaer et al., 2007). After *Citrus unshiu* suspension cells were treated with a spindle toxin to obtain micronuclei, the micronucleated protoplasts were isolated, followed by ultracentrifugation to obtain MPPs (Zhang et al., 2006). Xu et al. (2006) isolated cytoplasts through protoplasts ultracentrifugation and produced diploid alloplasmic hybrids.

1.6.6 Fragmentation techniques - cytoplasmic inactivation

The use of metabolic inhibitors, as iodoacetamide (IOA), in plant somatic hybridizations was initiated in 1978 (Nehls, 1978). IOA can inhibit protoplast division and thus reduce the survival rate of protoplasts, while division is recovered after fusion with untreated protoplasts. These features make IOA a suitable inhibitor for the selection of somatic hybrids in plant cells (Iriawati et al., 1996). Moreover, the metabolic inhibitors were not only used as selection markers, but also to perform asymmetric fusions. Fusion of IOA-treated recipient parental

protoplasts with irradiated donor protoplasts could produce (totally) asymmetric fusion products.

Iodoacetate and IOA have approximately the same spectrum, but IOA tends to penetrate the cells more easily because of being uncharged at neutral pH (Wright, 1978). Therefore, IOA is nowadays more frequently used. IOA is an alkylating reagent for cysteine residues. By reaction with cysteine, it is an irreversible inhibitor of enzymes with cysteine at the active site (Sigma-Aldrich). The exact function of IOA has not yet properly been described. IOA has already been noted as a glyceraldehydes 3-phosphate dehydrogenase inhibitor, thus blocking glycolysis, causing a significant reduction in ATP levels and a loss of viability (Epstein et al., 1981; Hilf et al., 1986). Another report described IOA as an inhibitor of the mitochondrial oxidative phosphorylation, also causing reduced ATP levels (Minqin et al., 2005). The effects of IOA, observed in bean protoplasts, included viability reduction after 3 days of culture, absence of cell wall regeneration and cell division, overformation of rough endoplasmic reticulum (RER), formation of circular Golgi complexes, increased mitochondria size, overaccumulation of lipid bodies and swelling of the thylakoid structure in chloroplasts (Iriawati et al., 1996). Varotto et al. (2001) noted that IOA acts as an irreversible inhibitor of the mitotic-spindle assembly at the prophase of mitosis, thus impeding the cell division.

From 2004 onward, incubation with iodoacetate and IOA was the preferred acceptor metabolic inhibitor in 8 of the 9 asymmetric protoplast fusions where the cytoplasm of the acceptor is inactivated. The other chemical used was rhodamine 6-G (Table 1-5).

1.6.7 Screening techniques

Somatic hybrids containing complete nuclear complements of both parents are generally rare (Liu et al., 2005). Nevertheless, somatic hybrids with complete chromosome addition have recently been produced in various plant families (Takami et al., 2005; Sarkar et al., 2011). The possible reason for this cytological phenomenon could be that the chromosomes are highly homologous and chromosomal rearrangement occurred during fusion and regeneration. However, most regenerated fusion products don't have an additive genome. Different causes of elimination have been proposed by Wang et al. (2008b): (1) a different cell cycle of the remote parents, (2) smaller centromeres of eliminated chromosomes, (3) DNA methylation of genes involved in centromere function and (4) existence of secondary metabolites in the cytoplasm fusion partners. Whole chromosome block elimination and fragment loss upon genomic rearrangements are other possible explanations (Guo et al., 2010). During recent

years, a wide array of screening methods has been applied for hybrid analysis during or after protoplast fusion (Table 1-4 and 1-5). They can be subdivided into 5 types. A first type of tools was used to monitor or direct the fusion event and thus to optimize the entire process. This was done by labeling of heterokaryons with different fluorescing agents, magnetic cell sorting using antibiotin microbeads or proces monitoring using GFP transformed genotypes as fusion partners. When using fluorescing agents to label parental protoplasts, however, cytotoxic side-effects could disable regeneration (Duquenne et al., 2007). The four other types of tools were used after fusion products had been partly or fully regenerated. The second group of tools were the cytogenetic ones. Information on ploidy level was obtained directly, through chromosome counting and/or indirectly, through flow cytometry. Genomic/Fluorescence in situ hybridization (GISH/FISH) for hybrid genome determination and meiotic analyses were also described. A third type of tools was the most popular one, namely molecular markers. In a majority of the publications reporting on complete plant regeneration, the genomic constitution of the alleged hybrids was looked into with molecular tools. DNA markers were sometimes complemented with isozyme analysis, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) or sequence analysis. The most frequently employed molecular markers were Random Amplification of Polymorphic DNA (RAPD), Simple Sequence Repeats (SSR), Amplification Fragment Length Polymorphism (AFLP), Restriction Fragment Length Polymorphism (RFLP) and Cleaved Amplified Polymorphic Sequence (CAPS). PCR-RFLP and CAPS analysis using mitochondrial or chloroplast universal primer pairs have proven to be efficient and reliable methods for characterizing the cytoplasmic genome. This technique was applied for both chloroplast and mitochondria screening, whereas SSR was only used once for chloroplast evaluation. Southern blotting for cpDNA and mtDNA were employed to screen cytoplasmic DNA, whereas Northern blotting was used once for chloroplast evaluation. Compared to RFLP with labeled probes, CAPS is simpler, more rapid and less expensive (Guo et al., 2004). Chloroplast SSR is even more convenient and efficient since enzyme cutting following PCR reaction is not needed (Cheng et al., 2005). Also **s**equencing of common bands and searching for restriction endonuclease sites could be cheaper and more convenient than actual CAPS analysis (though after sequencing CAPS could be used to confirm the results). Sequence analysis yielded SNPs that were used for restriction mapping and CAPS marker development. This is a very robust, reproducible and codominant, but monogenic inheritance (Rode et al., 2010). Somewhat less conventional techniques were occasionally used, such as Inter-Retrotransposon Amplified Polymorphism (IRAP)/ Retrotransposon-microsatellite Amplified

Polymorphism (REMAP) (Patel et al., 2011), microsatellite anchored fragment polymorphism, semi-quantitative real time PCR (Wang et al., 2011b), Inter-simple Sequence Repeat (ISSR) (Borgato et al., 2007a; Bidami et al., 2007), Internal Transcribed Spacer (ITS) (Ovcharenko et al., 2011) or DNA methylation-sensitive amplification polymorphism (MSAP) technologies (Cai et al., 2007). Spectrometrical methods like High-performance Liquid Chromatography (HPLC) (Han et al., 2009), constituted the fourth group of screening tools, but were not used very often. Finally, tools were created to determine the practical value of the screening products, such as biotests for abiotic and biotic tolerance or resistance, pollen fertility testing, fatty acid analysis or determination of diverse agronomical traits.

1.6.7.1 Fluorescence in situ hybridization (FISH)

Karyological analyses have been performed to characterize cultivars, to develop genetic and physical maps or to detect the origin of hybrids in numerous crops (Jiang and Gill, 2006). An additive cytogenetic tool for individual plant chromosome characterization is fluorescence *in situ* hybridization (FISH). Single-stranded DNA probes, labeled with fluorescent dyes, hybridize on complementary target sequences. The site specific hybridization is visualized by fluorescence detection. The introduction of i*n situ* hybridization (ISH) in the late 1960s enabled the accurate location of specific DNA and RNA sequences in mammalian tissues and cells (Chevalier et al., 1997). ISH was introduced in plants in 1985 (Jiang and Gill, 1994). Its sensitivity depends on (1) probe construction and the related hybridization conditions, (2) the type of probe labeling, (3) the method for signal detection and (4) the sample preparation, which must allow the hybridization (Chevalier et al., 1997). The first DNA/RNA probes were radioactively labeled. Despite of the high sensitivity of radioactively labeled probes, safety problems, reduced stability and slow visualization nursed the search for alternatives. Direct fluorochrome-labeled DNA/RNA showed relatively low sensitivity (Chevalier et al., 1997). The development of the biotin-avidin system enabled FISH. Biotin, a small vitamin molecule, binds with high affinity to avidin and streptavidin. Avidin, an egg white protein, and streptavidin, originated from *Streptomyces avidini*, can be conjugated with fluorescent dyes or other detection markers. The production of biotin-labeled UTPs enabled the production of biotinylated nucleic acids. As a biotin alternative, digoxigenin, a steroid isolate from *Digitalis purpurea*, can be used for labeling probes. Compared to radioactive ISH, which uses the amplificatory effect of autoradiography, FISH using biotin/digoxigenin requires 10- to 50-fold higher concentration of probes (Chevalier et al., 1997).

ISH and FISH techniques have known a remarkable evolution and have obtained a key role in modern molecular cytogenetics. One major disadvantage of FISH probes are the low detection rates, in particular, for small DNA fragments. Most of the reports stated a lower limit for 1-3 kb fragments (Jiang and Gill, 2006). However, through tyramid-FISH (Tyr-FISH) the signal amplification was significantly improved, leading to a 10-100 times higher sensitivity. Probes smaller than 1 kb can be visualized using Tyr-FISH (Khrustaleva and Kik, 2001). However, the detection of small probes is often not reproducible. For karyotyping, repetitive DNA sequences, e.g. ribosomal genes (rRNA genes) and multicopy gene families can deliver unique FISH patterns, by which chromosomes within a species can be distinguished. Furthermore, the use of multiple repetitive DNA probes for FISH can increase the resolving power between chromosomes. The rDNA segments, e.g. 5S and 45S, are among the most widely used for physical chromosome mapping. Since these DNA fragments are highly conserved, probes originally from wheat can be used in other eukaryotic species. Although the copy number of these fragments can vary among plant species of the same genus (Pedrosa-Harand et al., 2006), the locations of these loci are mostly maintained (Jiang and Gill, 1994). 45S rDNA sequences, composed of three genes coding for 18S, 5.8S and 28S rRNA, two intergenic spacers and an external transcribed spacer, are on the nucleolar organizing region (NOR). Repeats of these units form clusters on one or several chromosome pairs. NOR variations, in the number and location, are studied between or within species (Britton-Davidian et al., 2012). A critical requirement for successful chromosome identification is an efficient chromosome preparation procedure. The most common targets for FISH are mitotic metaphase chromosomes from root tips. Their condensation state is, however, an important factor for probe accessibility and thus, influencing the resolving power of FISH. Minimally 5-10 Mb distance between two DNA probes on barley metaphase chromosomes were necessary to resolve FISH signals (Pedersen and Lindelaursen, 1995). Less condensed prometaphase chromosomes (Cheng et al., 2002) or interphase nuclei (Jiang et al., 1996) can be used, although they contain a high variation in chromatin condensation. The chance of obtaining a satisfactory FISH mapping depends also on the position of the target DNA inside the chromosome (Jiang and Gill, 2006).

1.6.8 Cytoplasmic inheritance

Somatic fusion can yield a combination of cytoplasms from different sources, unlike sexual cross hybridization that leads to maternal inheritance of cytoplasmic genomes (Xu et al., 2005). Nuclear inheritance during cell division is highly ordered, ensuring stringent, unbiased partitioning of chromosomes before cell division. Similarly, cytoplasmic components such as chloroplasts, mitochondria, and the endoplasmic reticulum display distinctive partitioning strategies that ensure unbiased inheritance before dedifferentiating cells enter cell division (Sheahan et al., 2004). Compared to nuclear DNA, cpDNA and mtDNA had relatively complex inheritance modes. Uniparental random chloroplast transmission has been predominantly detected in many citrus protoplast fusion combinations, regardless of whether they were intergeneric or interspecific. In some combinations, suspension parent cpDNA was transmitted to the somatic hybrids while in others leaf parent cpDNA was integrated (Fu et al., 2004; Medina-Urrutia et al., 2004; Takami et al., 2005). The general consensus is that cpDNA is randomly transmitted because of little difference in chloroplast abundance between the leaf and the embryogenic parent or the minor effects of cp on its transmission. For the hybrids obtained by Guo et al. (2008b), the random or selective cpDNA inheritance apparently depended on the ploidy level of the hybrids. As for the mtDNA, nearly all of the somatic hybrids got theirs from the suspension parents (Fu et al., 2004; Medina-Urrutia et al., 2004; Xu et al., 2004; Guo et al., 2004; Takami et al., 2005). The preferential transmission was probably due to the difference in abundance (more mitochondria in embryogenic parent protoplasts) (Xu et al., 2004). However, mtDNA can also be derived from the leaf parent (Olivares-Fuster et al., 2005; Guo et al., 2008). In other citrus hybrids, all cytoplasmic DNA was exclusively inherited from 1 of the partners (Fu et al., 2011). This was also monitored after fusing two callus protoplast partners (Takami et al., 2004). In *Citrus* + *Microcitrus* cybrids, at all times the nuclear genome was inherited from 1 partner and all cytoplasmic DNA was identical to the one of the other partner (Xu et al., 2004). This configuration was likely the most stable and perhaps the only regenerative one. Wang et al. (2010) described a cybrid that combined nucleus and chloroplasts from partner 1 and mitochondria from parent 2. The same nuclear DNA/mt DNA pattern was seen in *Citrus* + *Poncirus* cybrids (Medina-Urrutia et al., 2004). Coexistence of different cytoplasm DNA types within a single hybrid genotype is also possible. In the *Citrus* + (*Citrus* x *Poncirus*) hybrids described by Guo et al. (2007a) the normal mtDNA (embryogenic parent) / cpDNA (random) was observed, except in 1 plant where SSR markers demonstrated coexistence of cpDNA. Whether this coexistence was persistent or just a temporary status due to incomplete sorting out of cpDNA from 1

fusion partner, is still unclear. In *Solanum* somatic hybrids, coexistence of mtDNA, but not of cpDNA, was recorded (Sarkar et al., 2011; Polzerova et al., 2011). Like nuclear genomes, cytoplasmic genomes were not always stable upon fusion. Xu et al. (2005) studied *Citrus* + *Fortunella* somatic hybrids and found that mitochondria were transmitted from the suspension parent, but some fragments had been lost, unlike the cpDNA that was unmodified. Intergenomic chloroplast recombination is a rare event in higher plants in contrast to mitochondrial genomes that show high recombination levels (Trabelsi et al., 2005). MtDNA recombination was proven in *Triticum aestivum* + *Setaria italica* (Xiang et al., 2004), intraspecific *Solanum tuberosum* (Nouri-Ellouz et al., 2006), *Cichorium intybus* + *endivia* (Cappelle et al., 2007), *Solanum bulbocastanum* + *tuberosum* (Iovene et al., 2007), *Solanum tubersosum* + *tarnii* (Thieme et al., 2008) and *Arabidopsis thaliana* + *Brassica oleracea* (Yamagishi et al., 2008). Although more rarely occurring, cpDNA recombination in hybrids has been demonstrated. In *Triticum aestivum* + *Setaria italica* hybrids, cpDNA coexistence as well as recombination occurred (Xiang et al., 2004). It was also observed in *Solanum tuberosum* + *verneï* (Trabelsi et al., 2005), *Solanum berthaultii* + *tuberosum* (Bidani et al., 2007) and *Bupleurum schorzonerifolium* + *Swertia mussottii* (Jiang et al., 2012). An important practical application of new genome/cytoplasmome combinations is the introduction of CMS. Fitter et al. (2005) demonstrated the possibility of introgressing CMS carried by mtDNA from a wild species into the cultivated crop. For mitochondrial interaction after protoplast fusion at the subcellular level, by fusing protoplasts containing either green fluorescent protein or MitoTracker-labelled mitochondria, Sheahan et al. (2005) reported the phenomenon of massive mitochondrial fusion (MMF) which led to near-complete mixing of the mitochondrial population within 24 h. MMF appeared specific to dedifferentiation, since it also occurred in mesophyll protoplasts of *Arabidopsis* and *Medicago* but not in protoplasts from already dedifferentiated cells such as tobacco BY-2 or callus cultures. These results allow a clearer interpretation of how novel mitochondrial genotypes develop following cell fusion. In other investigations, Sytnik et al. (2005) demonstrated that also chloroplasts can be transferred to remote species by protoplast fusion.

Table 1-4 Progress on symmetric protoplast fusion in different plant families and species from 2004 onward.

Plant family and species	Aim	Protoplast Source[u]	Fusion Method[v]	Best Result[w]	Characterization			Reference
					Cytogenetic[x]	DNA-markers	Other methods	
Actinidiaceae								
Actinidia chinensis + *A. kolomikta*	Abiotic resistance	C (CO) + M	PEG	P	CC, FCM	RAPD	Resistance screening	Xiao et al., 2004
Apiaceae								
Daucus carota + *Panax quinquefolius*	Secondary metabolites	SC + C	PEG	C	CC, GISH	RAPD	HPLC, isozyme analysis	Han et al., 2009
Araceae								
Spathiphyllum wallisii + *Anthurium scherzerianum*	Morphology	M + SE	EF	MC			Heterofusion labeling	Duquenne et al., 2007
Asteraceae								
Cichorium intybus + *C. endivia*	CMS	M		P	CC	SSR, CAPS(C)[y]	Mitochondrial DNA specific probes on Southern blots	Cappelle et al., 2007
Dendranthema grandiflorum + *Artemisia sieversiana*	Biotic resistance	M	EF	P	CC	RFLP	Resistance screening, pollen fertility, agronomic traits	Furuta et al., 2004
Brassicaceae								
Arabidopsis thaliana + *Brassica oleracea*	Hybrid analysis	H,M + in vivo M		P			Pollen fertility	Yamagishi et al., 2008
Arabidopsis thaliana + *Bupleurum schorzonerifolium*	Hybrid analysis	SC	PEG	P	CC	RAPD, SSR, SSR(C)	Isozyme analysis	Wang et al., 2008b
Brassica napus + *Camelina sativa*	Secondary metabolites	M	EF	P	FCM	SSR	Pollen fertility, fatty acid analysis	Jiang et al., 2009
Brassica napus + *Isatis indigotica*	Genetic variation, secondary metabolites	M	PEG	P	CC, GISH	AFLP, CAPS(C)	Pollen fertility	Du et al., 2009
Brassica oleracea + *Raphanus sativus*	CMS, hybrid analysis			P		RFLP	Sequencing, chloroplast DNA specific probes on Southern blots	Ohta et al., 2006
Brassica napus + *Raphanus sativus*	Genetic variation	M	PEG	P	GISH	SSR, CAPS	Pollen fertility	Wang et al., 2006
Brassica napus + *Sinapis alba*	Genetic variation	M	EF	P	CC, FCM	SSR, CAPS	Meiosis analysis, pollen fertility, fatty acid analysis	Wang et al., 2005b
Brassica oleracea + *B. juncea*	CMS, genetic variation, ploidy breeding	CO + H	PEG	P	CC, GISH	RAPD		Lian et al., 2011
Brassica oleracea + *B. juncea*	Biotic resistance	H + CO	PEG	P	CC	SDS-PAGE, RAPD	Pollen fertility	Chen et al., 2005
Brassica oleracea botrytis + *B. juncea*	Biotic resistance	H + M	PEG	P		RAPD	Resistance screening	Scholze et al., 2010

Brassica oleracea capitata + *B. carinata*	Biotic resistance	H + M	PEG	P		RAPD	Resistance screening	Scholze et al., 2010
Brassica oleracea italica + *B. rapa pekinensis*	Ploidy breeding	Pollen + M	PEG	C		RAPD		Liu et al., 2007
Brassica oleracea + *Matthiola incana*	Genetic variation	H + M	PEG	P	CC, FCM	RAPD, AFLP		Sheng et al., 2008
Brassica rapa + *B. juncea*	Genetic variation, regeneration studies, CMS	M + CO or H	PEG	P	CC	RAPD	Pollen fertility	Lian et al., 2012
Brassica rapa + *Isatis indigotica*	Secondary metabolites	M	PEG	P	CC, GISH	AFLP		Tu et al., 2008
Raphanus sativus + *Isatis indigotica*	Secondary metabolites	M	PEG	P	CC, GISH	AFLP		Tu et al., 2008
Convolvulaceae								
Ipomoea batatas + *I. carica*	Biotic resistance	M + SC	PEG	P	CC	RAPD	Isozyme analysis	Guo et al., 2006
Ipomoea batatas + *I. triloba*	Storage root; abiotic resistance	SC + petioles	PEG	P	CC, GISH	RAPD, AFLP	Isozyme analysis, resistance screening,	Yang et al., 2009
Fabaceae								
Lupinus angustifolius + *L. subcarnosus*	Genetic variation	M	EF	S		CAPS		Sonntag et al., 2009
Phaseolus vulgaris + *P. coccineus*	Biotic resistance	H + M	PEG, EF	MC			Heterofusion labeling	Geerts et al., 2008
Phaseolus vulgaris + *P. polyanthus*	Biotic resistance	H	PEG, EF	MC			Heterofusion labeling	Geerts et al., 2008
Malvaceae								
Gossypium hirsutum + *G. bickii*	Genetic variation	SC + M	EF	P	CC, FCM	RAPD		Sun et al., 2005d
Gossypium hirsutum + *G. stockii*	Genetic variation	SC + SE	EF	P	CC, FCM	RAPD		Sun et al., 2005d
Gossypium klotzschianum + *G. hirsutum*	Genetic variation	SE + SC	EF	P	CC, FCM	RAPD, SSR		Sun et al., 2011
Myrsinaceae								
Cyclamen persicum + *C. coum*	Morphology	SC	PEG	P	CC, FCM	5.8Sgene based primers	Heterofusion labeling	Prange et al., 2012
Poaceae								
Oryza sativa + *O. punctata*	Genetic variation			P	CC, GISH			Feng et al., 2006
Triticum aestivum + *Agropyron elongatum*	Genetic variation	SC + C		P	GISH	AFLP, SSR, SDS-PAGE		Cui et al., 2009
Triticum aestivum + *Psathyrostachys juncea*	Abiotic resistance, biotic resistance	SC	PEG	P	CC, GISH	RAPD, SSR	Isozyme analysis	Li et al., 2004
Rosaceae								
Rosa damascena + *R. bourboniana*	Secondary metabolites	SC	PEG	C		RAPD	Heterofusion labeling	Pati et al., 2008
Rosa hybrida + *Rosa hybrida*	Biotic resistance	C	PEG	P	CC, FCM	RAPD		Squirrell et al., 2005
Rutaceae								
Citrus amblycarpa + *Poncirus trifoliata*	Rootstock	SC + M	PEG	P	FCM	RAPD, CAPS(C), CAPS(M)[z]		Medina-Urrutia et al., 2004
Citrus aurantium + *Microcitrus papuana*	Abiotic resistance, biotic resistance	SC + M	EF	P	FCM	SSR, CAPS(C), CAPS(M)		Xu et al., 2004
Citrus aurantium + *Poncirus trifoliata*	Rootstock, biotic resistance	SC + M	EF	P	FCM, GISH	AFLP, CAPS(C), CAPS(M)		Fu et al., 2004
(*Citrus nobilis* x *C. deliciosa*) + *C. clementina*	Ploidy breeding	M or C	PEG	P	FCM	SSR		Wu et al., 2005
Citrus reticulata + *Citropsis gabunensis*	Genetic variation	C + M	EF	P	CC, FCM	RAPD, CAPS(C),		Takami et al., 2005

Citrus reticulata + *C. aurantium*	Rootstock	SC + M		P	FCM	SSR, SSRC, CAPS(M)		Guo et al., 2008a
Citrus reticulata + *C. grandis*	Rootstock, biotic resistance	SC + M	PEG	P	FCM	RAPD		Grosser et al., 2007
Citrus reticulata + (*C. sinensis* x *Poncirus trifoliata*)	Rootstock	SC + M	EF	P	CC, FCM	SSR, SSR(C), CAPS(M)	Mitochondrial DNA	Guo et al., 2007a
Citrus reticulata + *Fortunella obovata*	Genetic variation, hybrid analysis	SC + M		P	FCM	SSR, CAPS(C), CAPS(M)		Xu et al., 2005
Citrus sinensis + *C. jambhiri*	Genetic variation			P	FCM	SSR, SSR(C), CAPS(M)	Sequencing	Guo et al., 2008b
Citrus sinensis + *C. micrantha*	Biotic resistance	SC+ M	PEG	P	FCM	RAPD		Khan and Grosser, 2004
Citrus sinensis (GFP transgene) + *C. reticulata*	Genetic variation, hybrid analysis	SC + M	PEG	P	FCM	SSR, SSR(C)	GFP	Guo and Grosser, 2005
Citrus sinensis + *C. unshiu*	Hybridization	C + M	PEG	P	FCM	AFLP		An et al., 2008
Citrus sinensis + *C. unshiu*	Hybridization, ploidy breeding	SC + M	EF	P	FCM	SSR, SSR(C), CAPS(M)		Fu et al., 2011
Citrus sinensis + *Fortunella japonica*	Hybrid analysis	C	EF	P	CC, FCM	RAPD, CAPS, CAPS(C), CAPS(M)		Takami et al., 2004
Citrus unshiu + *C. grandis*	Hybrid analysis, morphology, biotic resistance			P	FCM	SSR, CAPS(M), SSR(C), SDS-PAGE	Mass spectrometry	Wang et al., 2010
Citrus unshiu (GFP transgene) + *C. microcarpa*	Hybrid analysis	SC + M	EF	P	FCM	SSR, CAPS(C), CAPS(M)	GFP	Cai et al., 2006
Salicaceae + Betulaceae								
Populus alba + *Alnus firma*	Biomass production, N_2-fixation	M	EF	P		(none)		Wakita et al., 2005
Populus alba + *Alnus firma*	Hybrid analysis	M	EF	P		RAPD		Sasamoto et al., 2006
Populus alba + *Betula platiphylla*	Biomass production	M	EF	S		(none)		Wakita et al., 2005
Solanaceae								
Nicotiana sanderae + *N. debneyi*	Hybridization, biotic resistance	M	EF	P	CC, GISH	RAPD, IRAP, REMAP, CAPS(C), CAPS(M)	Resistance screening	Patel et al., 2011
Nicotiana tabacum + *Nicotiana glauca*		M		P		RAPD, SCAR		Sun et al., 2007
Nicotiana tabacum + *Nicotiana suaveolens*	Hybrid analysis	M + SC	PEG	P		AFLP, RFLP(C)	Mitochondrial DNA	Fitter et al., 2005
Petunia sp + *Callibrachoa sp*	Hybrid analysis			P		CAPS	Sequencing	Rode et al., 2010
Solanum melongena + *S. marginatum*	Morphology	M	EF	P	CC	ISSR	Magnetic cell sorting, pollen fertility	Borgato et al., 2007a
Solanum tuberosum + *S. berthaultii*	Abiotic resistance, biotic resistance			P	FCM	ISSR, CAPS(C)	Agronomic traits	Bidani et al., 2007
Solanum tuberosum + S. *bulbocastanum*	Biotic resistance, hybrid analysis			P	CC	RAPD		Boltowicz et al., 2005

Solanum tuberosum + S. *bulbocastanum*	Biotic resistance, hybrid analysis		EF	P	CC, GISH	ISSR, CAPS(C), CAPS(M)		Iovene et al., 2007
Solanum tuberosum + S. *bulbocastanum*	Biotic resistance	M	EF	P	FCM	RAPD		Greplova et al., 2008
Solanum tuberosum + S. *bulbocastanum*	Biotic resistance, hybridization			P	CC	ISSR	Meiosis analysis	Iovene et al., 2012
Solanum tuberosum + *S. cardiophyllum*	Biotic resistance	M	EF	P	CC	RAPD		Shi et al., 2006
Solanum tuberosum + *S. cardiophyllum*	Biotic resistance		EF	P	FCM	AFLP, SSR, MFLP	Resistance screening, pollen fertility, agronomic traits	Thieme et al., 2010
Solanum tuberosum + *S. chacoense*	Hybrid analysis, biotic resistance			P	FCM		Meiosis analysis, pollen fertility	Guo et al., 2010
Solanum tuberosum + *S. etuberosum*	Hybrid analysis, biotic resistance	M	EF	P	FCM	RAPD, SSR, CAPS(C), CAPS(M)	Pollen fertility, ELISA	Tiwari et al., 2010
Solanum tuberosum + *S. x michoacanum*	Biotic resistance		PEG	P	CC, chloroplast guard cells	RAPD	Resistance screening	Sczcerbakowa et al., 2010
Solanum tuberosum + *S. nigrum*	Biotic resistance		PEG	P	CC, FCM			Szczerbakowa et al., 2011
Solanum tuberosum + *S. pinnatisectum*	Biotic resistance	M	EF	P	FCM	RAPD		Greplova et al., 2008
Solanum tuberosum + *S. pinnatisectum*	Hybrid analysis, biotic resistance		EF	P		SSR, CAPS(C), CAPS(M)	Resistance screening	Polzerova et al., 2011
Solanum tuberosum + *S. pinnatisectum*	Biotic resistance	M	EF	P	FCM	RAPD, SSR, CAPS(C), CAPS(M)	Pollen fertility, resistance screening	Sarkar et al., 2011
Solanum tuberosum + *S. tarnii*	Biotic resistance	M	EF	P	FCM	AFLP, SSR	ELISA, agronomic traits	Thieme et al., 2008
Solanum tuberosum + *S. tuberosum*	Biotic resistance	M	EF	P	CC	SSR, ISSR	Isozyme analysis, agronomic traits, resistance screening, ELISA	Nouri-Ellouz et al., 2006
Solanum tuberosum + *S. verneï*	Abiotic resistance	M	PEG	P	FCM	RAPD, ISSR, CAPS(C), RFLP(C)	Isozyme analysis	Trabelsi et al., 2005
Zingiberaceae								
Zingiber officinale (intraspecific)	Hybridization	SC	PEG	P	FCM	RAPD		Guan et al., 2010

[u] C: callus; CO: cotyledon; H: in vitro hypocotyls; HC: hypocotyls derived callus; M: mesophyll cells from in vitro leaves; SC: suspension cells; SE: somatic embryos; when not mentioned the protoplast source is described in earlier publications.
[v] EF: electrofusion; PEG: chemical fusion with polyethylene glycol; when not mentioned the fusion method is described in earlier publications.
[w] C: callus; MC: microcalli; P: plants; S:shoots
[x] CC: chromosome counting; FCM: flow cytometry
[y] (C) on chloroplast DNA
[z] (M) on mitochondrial DNA

Table 1-5 Progress is asymmetric protoplast fusion in different plant families and species from 2004 onward.

Plant family and species (acceptor + donor)	Aim	Protoplast source[t]	Fragmentation [u]	Fusion Method[v]	Best Result[w]	Characterization			Reference
						Cytogenetic[x]	DNA-markers	Other methods	
Apiaceae + Gentianaceae									
Bupleurum scorzonerifolium + *Swertia mussottii*	Secondary metabolites	C	UV	PEG	P	GISH	RAPD, SQ RT-PCR	Isozyme analysis, mitochondrial and chloroplast DNA specific probes on Southern blots, HPLC	Wang et al., 2011b
Bupleurum scorzonerifolium + *Swertia tetraptera*	Secondary metabolites	SC + C	UV	PEG	P	CC, GISH	RAPD, SSR(C)	Isozyme analysis, HPLC	Jiang et al., 2012
Apiaceae + Taxaceae									
Bupleurum schorzonerifolium + *Taxus chinensis mairei*	Secondary metabolites	SC	UV	PEG	C	CC	RAPD, SQ RT-PCR	Isozyme analysis, HPLC	Zhang et al., 2011
Asteraceae									
Helianthus annuus + *H. maximiliani*	Biotic resistance	H + M	UV	EF	P		RAPD	Isozyme analysis	Taski-Ajdukovic et al., 2006
Brassicaceae									
Brassica napus + *Isatis indigotica*	Genetic variation, Secondary metabolites	M	IOA(A) + UV	PEG	P	CC, GISH	AFLP, CAPS(C)[y]	Pollen fertility	Du et al., 2009
Brassica napus + *Orychophragmus violaceus*	Chromosome addition lines	M	IOA(A) + UV		P	CC, GISH			Zhao et al., 2008
Brassica oleracea + *B. nigra*	Genetic variation, Biotic resistance	H + M	UV	PEG	P	CC, FCM, GISH	AFLP, CAPS(C), CAPS(M)[z]	Mitochondrial DNA specific probes on Southern blots, resistance screening	Wang et al., 2011a
Brassica oleracea botrytis + *B. carinata* + *B. juncea* + *B.nigra* + *Sinapis alba*	Biotic resistance	H + M	X	PEG	P		RAPD	Resistance screening	Scholze et al., 2010
Brassica oleracea capitata + *Barbarea vulgaris* + *Capsella bursapastoris* + *Diplotaxis tenuifolia* + *Hesperis matronalis* + *Matthiola incana* + *Raphanus sativus* + *Sinapis alba*	Biotic resistance	H + M	X	PEG	P		RAPD	Resistance screening	Scholze et al., 2010

Orychophragmus violaceus + *Lesquerella fendleri* (GFP)	Plastome transfer	M + C	γ	PEG	P		ITS, CAPS(M)	Isozyme analysis, GFP	Ovcharenko et al., 2011
Brassicaceae + Apiaceae									
Arabidopsis thaliana + *Bupleurum schorzonerifolium*	Secondary metabolites	C	IOA(A) + UV	PEG	P	CC, GISH,	RAPD	5S rDNA spacer sequence analysis	Wang et al., 2005a
Arabidopsis thaliana + *Bupleurum schorzonerifolium*	Fragmentation tool		UV	PEG	P	CC,GISH	RAPD, SSR		Wang et al., 2011d
Arabidopsis thaliana + *Bupleurum schorzonerifolium*	Hybrid analysis, fragmentation tool	C + SC	γ	PEG	P	CC	RAPD, SSR	Histology	Wang et al., 2012
Brassicaceae + Poaceae									
Arabidopsis thaliana + *Triticum aestivum*	Hybrid analysis	SC + C	UV	PEG	P	CC, GISH	RAPD, SSR, CAPS(C)	Isozyme analysis	Deng et al., 2007
Malvaceae									
Gossypium hirsutum + *G. klozschianum*	Alternative for symmetric somatic hybridisation	SC	UV	EF	P	CC	RAPD, SSR, CAPS(C)		Yang et al., 2007b
Musaceae									
Musa 'Guoshanxiang' + *M. acuminata*	Biotic resistance	SC	IOA(A) + UV	PEG	P	CC	RAPD, ISSR		Xiao et al., 2009
Poaceae									
Festuca arundinacea + *Triticum aestivum*	Hybrid analysis	SC	UV	PEG	P	CC, GISH	RAPD, SSR(C), MSAP	Isozyme analysis, mitochondrial DNA specific probes on Southern blots	Cai et al., 2007
Oryza sativa japonica + *O. meyeriana*	Biotic resistance	SC	IOA(A) + X	PEG	P	CC	RAPD	Resistance screening	Yan et al., 2004
Triticum aestivum + *Avena sativa*	Hybrid analysis	SC	UV		P	ISH	SSR		Xiang et al., 2010
Triticum aestivum + *Haynaldia villosa*	Biotic resistance, protein content	SC + C	γ	PEG	P	CC, GISH	RFLP(C)	Isozyme analysis, 5S rDNA spacer sequence analysis	Zhou and Xia, 2005
Triticum aestivum + *Lolium multiflorum*	Biotic resistance	SC	UV		P	CC	RAPD, SSR	Isozyme analysis, mitochondrial DNA specific probes on Southern blots	Cheng and Xia, 2004
Triticum aestivum + *Lolium multiflorum*	Agronomic traits, biotic resistance	SC	IOA(A) + X	EF	P	CC	RFLP, AFLP	Isozyme analysis, mitochondrial DNA specific probes on Southern blots	Ge et al., 2006
Triticum aestivum + *Lolium multiflorum*	Radiation hybrid panel / genome mapping		UV		P	GISH	RFLP, SSR	Sequencing	Cheng et al., 2006

Triticum aestivum + *Setaria italica*	Abiotic resistance	(SC+C) +C	UV	PEG	P	GISH, CC	RAPD, RFLP(C), RFLP(M)	Isozyme analysis, 5S rDNA spacer sequence analysis	Xiang et al., 2004
Poaceae + Apiaceae									
Festuca arundinacea + *Bupleurum schorzonerifolium*	Hybrid analysis	SC	UV	PEG	P	CC	RAPD	Isozyme analysis, 5S rDNA spacer sequence analysis	Wang et al., 2011c
Triticum aestivum + *Bupleurum schorzonerifolium*	Genetic variation, genome mapping	C + SC	UV	PEG	P	GISH	CAPS, RAPD, SSR	Isozyme analysis	Zhou et al., 2006
Rutaceae									
Citrus paradisi + *C. sinensis*	Genetic variation	SC	IOA(A) + γ		P	FCM	AFLP		De Bona et al., 2009a
Citrus paradisi + *Swinglea glutinosa*	Fragmentation tool	SC	γ and UC	PEG	C		AFLP		De Bona et al., 2009b
(Citrus reticulata x C. sinensis) + *C.sinensis*	Genetic variation	SC	IOA(A) + γ		P	FCM,	AFLP		De Bona et al., 2009a
(Citrus reticulata x C. sinensis) + *C. unshiu*	Seedless fruits	SC	UC	EF	C	FCM	SSR, CAPS(C)		Xu et al., 2006
(Citrus reticulata x C. sinensis) + *Swinglea glutinosa*	Fragmentation tool	SC	γ and UC	PEG	C		AFLP		De Bona et al., 2009b
Citrus sinensis + *C. unshiu*	Fragmentation tool	SC	UV	EF	P	CC, FCM	RAPD, AFLP, CAPS(C)		Xu et al., 2007
Solanaceae									
Nicotiana tabacum + *N. repanda*	CMS	M	Rhodamin 6G (A)	PEG	P	CC	RAPD, CAPS(M)	Isozyme analysis	Sun et al., 2005a
Petunia hybrid +*Nicotiana tabacum*	Plastome transfer	M	UV	PEG	P		RAPD, CAPS(C)	Mitochondrial DNA specific probes on Southern blots, chloroplast RNA specific probes on Northern blot	Sigeno et al., 2009

[t] C: callus; CO: cotyledon; H: in vitro hypocotyls; M: mesophyll cells from in vitro leaves; SC: suspension cells; when not mentioned the protoplast source is described in earlier publications.
[u] (A): for acceptor; IOA: iodoacetamide; UC: ultracentrifugation; UV: UV-ray irradiation; X: X-ray irradiation; γ: γ-ray irradiation
[v] EF: electrofusion; PEG: chemical fusion with polyethylene glycol; when not mentioned the fusion method is described in earlier publications.
[w] C: callus; P: hybrid plants
[x] CC: chromosome counting; FCM: flow cytometry; (G)ISH: (genomic) in situ hybridization
[y] (C) on chloroplast DNA
[z] (M) on mitochondrial DN

1.7 Objectives and outline

In chicory (*C. intybus* var. *sativum*) cytoplasmic male sterility doesn't occur naturally. However, CMS has been introduced by asymmetric protoplast fusion between chicory and sunflower (Varotto et al. 2001) and by the symmetric fusion between chicory and endive (Cappelle et al. 2007). The asymmetric hybrid (fertile chicory + CMS sunflower) of Varotto is a fusion product between two non crossable species and thus, controversial because of the Genetically Modified Organism (GMO) legislation in Belgium (Europe). Moreover, the use of only a single origin of male sterility in a breeding program is not without risks when analyzing sensitivity to diseases and pathogens. The symmetric hybrid (fertile chicory + fertile endive) of Cappelle is a fusion product between two sexually crossable (fertile) species, resulting in a tetraploid hybrid plant. Tetraploid industrial chicory has shown to exhibit bigger roots and leaves, but no increase in the inulin quantity. Another disadvantage was that these tetraploid plants also contained the undesired genes from endive, requiring time-consuming backcrosses. These facts encouraged us, in collaboration with a Belgian industrial chicory breeding company COSUCRA-Groupe Warcoing S.A. Chicoline division, to search for new CMS sources. Although Cappelle showed the possibility to produce a CMS plant via protoplast fusion of two fertile lines, the real challenge is to create a diploid CMS chicory plant by combining the economically valuable nuclear genome of the industrial chicory with the cytoplasm of other *Cichorium* species to obtain incompatibility between the nucleus and the cytoplasm, theoretically leading to alloplasmic CMS.

Besides the introduction of CMS, also the broadening of the genetic variation in the industrial chicory by means of asymmetric protoplast fusion between protoplasts of different *Cichorium* species was another goal.

The choice of genotypes in our study was based on the genetic distance between putative acceptor and donor genotypes. We expect that a bigger genetic distance between two fusion partners will enhance the occurrence of alloplasmic CMS in the regenerants or will broaden the genetic variation. Microsatellite analysis with 18 markers, provided by COSUCRA-Groupe Warcoing S.A., Chicoline division, yielded a genetic distance tree constructed by Neighbor Joining, based on the DAS (Distance Allele Shared) method. This way, industrial and wild type chicory and endive genotypes were selected for further use in protoplast fusion experiments.

To our knowledge, asymmetric protoplast fusion in chicory has not been exploited so far. However, it offers interesting perspectives for better protoplast regeneration and reduced introduction of undesired chromosome fragments and genes in the fused products and thus decreases the need for backcrosses. Another important advantage of asymmetric protoplast fusion is the ability to transfer only the cytoplasm of donor plants, leading to the formation of cybrids. This is impossible by conventional breeding. Before we perform asymmetric protoplast fusion, we need to fragmentate the donor nucleus and inactivate the acceptor cytoplasm.

The first aim of this thesis was to assess optimal protoplast regeneration conditions for several *Cichorium* species. Simultaneously, fragmentation techniques, based on UV irradiation and iodoacetamide (IOA) incubation were tested to transfer chromosome fragments or even only mitchondria or chloroplasts of the donor plants. The transfer was mediated through both electrical and chemical protoplast fusion. Finally, after fusion, putative hybrid regenerants were tested. Regenerating calli and full grown plantlets were genotypically analyzed.

The specific objectives were:

1) To develop a low melting point agarose-based, universal, protoplast regeneration protocol for different genotypes, as straightforward as possible (§Chapter 2).

2) To develop a reproducible protoplast fusion protocol, based on the two main protoplast fusion techniques, namely electrical and chemical fusion (§Chapter 3).

3) To study the influence of UV irradiation and IOA treatment on the production of asymmetric donor and acceptor genotypes, respectively (§Chapter 4).

4) To initiate karyotype analysis to acquire basic knowledge of the genomic resources of *Cichorium* species (§Chapter 5) and to characterize putative hybrids on a fast and efficient way through high-resolution melting (HRM) analysis (§Chapter 6).

Furthermore, the obtained knowledge was used in asymmetric fusion experiments to acquire hybrid and cybrid *Cichorium* regenerants (§Chapter 7).

Finally, conclusions and future perspectives were expressed in §Chapter 8, focusing on the observations made in the aforementioned chapters.

Chapter 2 - Protoplast regeneration in *Cichorium* species

Based on the published article: Deryckere D, Eeckhaut T, Van Huylenbroeck J, Van Bockstaele E. (2012) Low melting point agarose beads as a standard method for plantlet regeneration from protoplasts within the *Cichorium* genus. Plant Cell Reports 31: 2261-2269.

2.1 Introduction

Somatic hybridization through protoplast fusion and regeneration in *Cichorium intybus* has already been established. Efficient protoplast isolation and regeneration protocols of *Cichorium intybus* var. *foliosum* cultivars have been described by several authors (Crepy et al., 1982; Saksi et al., 1986a; Slabe and Bohanec, 1989). Crepy et al. (1982) noted that protoplasts of witloof type *C. intybus* did not divided in a Murashige and Skoog salts-based medium. By totally replacing nitrate with glutamine at the optimal level of 5 mM, plating efficiencies up to 1% were achieved. Saksi et al. (1986a) found that a lower sucrose concentration (5 g l^{-1}) in the first week of regeneration was beneficial for the division of witloof protoplasts. In the 1990s, protoplast regeneration in *C. intybus* was studied by Rambaud et al. (1990) and Varotto et al. (1997). Both authors reported that the use of a semisolid proliferation medium after a liquid culture phase improved the plating efficiency. Rambaud et al. (1990) studied the protoplast regeneration of *C. intybus* var. Magdebourg. Their observations showed that higher sucrose levels (10 g l^{-1}) and the assocation of nitrates with glutamine gave better results. Moreover, the use of *in vitro* plants allowed a standardized physiological stage of the plants. The youngest plants resulted in higher cell division and bud induction rates. Varotto et al. (1997) studied protoplast regeneration of Italian red chicory types. They noted that one of the most important factors for cell wall regeneration and sustained cell division is the cell density in the starting medium, depending on species and protoplast size. Nenz et al. (2000) demonstrated that the protoplast regeneration cycle could be shortened after embedding the protoplasts in Ca-alginate droplets. This enhanced the division frequency and plating efficiency for Italian red chicory mesophyll protoplasts. More recently, protoplast fusion experiments have illustrated the capacity of somatic hybridisation through protoplast fusion in *Cichorium* species. To obtain male-sterile asymmetric somatic hybrids, iodoacetic acid inactivated mesophyll chicory protoplasts (*C. intybus*) were chemically fused with irradiated hypocotyl sunflower protoplasts (*Helianthus annuus* L.) (Varotto et al., 2001). Cappelle et al. (2007) showed the possibility of regenerating an interspecific protoplast fusion between *C. intybus* and *C. endivia*.

All protoplast research to date has focused on *C. intybus* varieties. However, the published protoplast regeneration protocols were cultivar-specific. Furthermore, no information is available on the regeneration of *C. endivia* protoplasts. Cappelle et al. (2007) mentioned the formation of callus after protoplast regeneration of *C. endivia*, but plants could not be obtained. An efficient plantlet regeneration system is the key to a successful protoplast-based

breeding program not only in chicory, but also in endive. It offers prospects towards the development of new varieties, the introgression of new traits and breeding time reduction compared to the time consuming conventional breeding approach.

The main goal of this chapter was to develop a universal protocol for plantlet regeneration in *Cichorium* species. We therefore evaluated the low melting point agarose bead technique on different *C. intybus* and *C. endivia* genotypes.

2.2 Materials and methods

Plant material

Seeds of two industrial chicory inbred lines, *Cichorium intybus* var. *sativum* ('VL52' and 'L4043') bred at the ILVO and provided by the COSUCRA-Groupe Warcoing S.A. division Chicoline, *in vitro* shoots of two industrial chicory genotypes, *C. intybus* var. *sativum* ('K1729' and 'K1093': clones of heterozygous genotypes), seeds of three accessions of wild type chicory *C. intybus* from Hungary ('Ames22531', 'Ames22532' and 'Pi531291': USDA Plants Database) and seeds of four endive *C. endivia* cultivars (*C. endivia* var. *crispum* 'Wallone Despa', *C. endivia* var. *endivia* 'CICH192', *C. endivia* var. *latifolium* 'nr.5' and *C. endivia* var. *divaricatum* 'CICH50') were used. Seeds of the *in vivo Cichorium* plants were initiated *in vitro*. After rinsing for 1 min in 70% ethanol, seeds were surface sterilized for 20 min in 6.5% NaOCl and germinated in 60 mm petri dishes on solid Murashige and Skoog (1962) medium containing 150 mg l^{-1} casein hydrolysate and 30 g l^{-1} sucrose at pH 5.8. After germination, the plantlets were placed on solid Murashige and Skoog medium containing 20 g l^{-1} sucrose and grown in Meli jars (Meli NV Veurne, Belgium) at 23 ± 2°C under a 16 h/8 h (light/dark) photoperiod at 40 µmol m^{-2} s^{-1} photosynthetic active radiation (fluorescent tube lamps, Sylvania Standaard F40W/33-640/RS Cool White).

Protoplast isolation

Protoplasts were isolated from 2-to-4-week old leaves. Leaves were chopped into small pieces and pre-incubated for 1 h in a 0.5 M mannitol solution P_0 (Table 2-1). Subsequently, the mannitol solution was replaced with an enzymatic mixture containing P_0 medium with 1 mg ml^{-1} cellulase Caylase 345 (Cayla/InvivoGen Ltd., Toulouse, France) and 0.5 mg ml^{-1} pectinase Caylase M2 (Cayla/InvivoGen Ltd., Toulouse, France) at pH 5.5 (Cappelle et al., 2007). Incubation was carried out in darkness at 23 ± 2 °C for 16 h with gentle agitation (25

rpm). After digestion, protoplasts were purified by filtration through a 100 µm pore size sieve (Cell Strainer, BD Falcon™, BD Biosciences) and centrifuged at 100 g for 10 min in a swing-out rotor. Protoplasts were pelleted and the supernatant was removed. Finally, the protoplasts were washed twice with MC_2 medium (Table 2-1) and centrifuged (100 g, 10 min).

Table 2-1 Composition of washing solution and culture media for *Cichorium* protoplasts.

	P_0	**MC_1**	**MC_2**	**MC_3**
Murashige & Skoog macro elements	½ X	½ X (no NH_4NO_3)	½ X (no NH_4NO_3 or KNO_3)	½ X
Heller micro elements	1X	1 X	1 X	1 X
Heller KCl	-	-	1 X (750 mg/l)	-
FeNa-EDTA	18.35 mg/l	18.35 mg/l	18.35 mg/l	18.35 mg/l
Morel & Wetmore vitamines	1X	1 X	1 X	1 X
Inositol	100 mg/l	250 mg/l	100 mg/l	100 mg/l
Glutamine	-	375 mg/l	750 mg/l	-
Sucrose	10 g/l	10 g/l	10 g/l	10 g/l
Mannitol	90 g/l	90 g/l	60 g/l	-
NAA	-	2 mg/l	0.5 mg/l	0.5 mg/l
BAP	-	1 mg/l	0.5 mg/l	0.5 mg/l
Agar	-	-	-	5 g/l
pH	5.5	5.5	5.5	5.5

Microscopical protoplast evaluation

The protoplast yield was determined using a Bürker counting chamber. For viability (% of viable protoplasts) staining, about 100 µl protoplast solution was mixed with 1 µl of a 0.5 % (w/v) fluorescein diacetate (FDA) stock solution (5 mg FDA dissolved in 1 ml acetone), incubated for 10 min at room temperature and observed with a fluorescence microscope. Microscopic detection was carried out using an inverted fluorescence microscope (Leica DMIRB) equipped with a Leica Camera System (Leica DFC320). Cell wall regeneration was studied by using Calcofluor White M2R (CFW) (Sigma-Aldrich). CFW binds strongly to cellulose, carborylated polysaccharides and callose (Hughes and McCully, 1975) and thus can be used as an indicator of cell wall formation in protoplasts. Freshly isolated protoplasts were mixed with a stock solution of CFW resulting in a final dye concentration of 0.01% (w/v). Cell wall formation of the protoplasts was then analyzed. Protoplast diameters were measured by ImageJ Software (NIH, National Institutes of Health).

Protoplast regeneration systems

Experiment 1 was set up to evaluate three culture systems on the regeneration ability of protoplasts of the industrial chicory *C. intybus* var. *sativum* 'VL52', the wild type chicory *C. intybus* 'Pi531291' and the endive *C. endivia* var. *crispum* 'Wallone Despa'. In each culture system, the formation of the four-cell stage (four identical cells after two mitotic divisions of the protoplast), microcolonies (cell clumps of 10 to 20 cells), calli and plants was evaluated for the three genotypes. The media used in this experiment (Table 2-1) were based on Saksi et al. (1986a, 1986b), Cappelle et al. (2007) and Rambaud et al. (1990). Protoplasts were cultured at 23 ± 2 °C in both light and dark conditions (16 h/8 h (light/dark) photoperiod at 40 µmol m^{-2} s^{-1} photosynthetic active radiation).

The first culture system was based on solid medium. In this system, 5 g l^{-1} low melting point agarose (LMPA, Duchefa Biochemie B.V.) was added to both liquid MC_1 and MC_2 medium (Table 2-1). After heating (60 °C) and cooling (35 °C), equal volumes of this liquified MC_1 and MC_2 were mixed with liquid MC_1 and MC_2, containing 1 x 10^5 protoplasts ml^{-1}, respectively. Petri dishes (60 mm) were filled with 5 ml of the protoplast solution (final protoplast concentration was 5 x 10^4 protoplasts ml^{-1}). Because the protoplasts were embedded in this solid matrix, the medium was not refreshed.

The second culture system was based on a liquid medium culture consisting of 5 ml of starting medium (MC_1 or MC_2) containing 5 x 10^4 protoplasts ml^{-1} in a petri dish (60 mm) with gentle shaking (10 rpm). At day 5 of the regeneration process, the medium was fully replaced by fresh medium: MC_1 was replaced by either MC_1 or MC_2; MC_2 was replaced by MC_2 only.

The third culture system was based on low melting point agarose (LMPA) beads. Protoplasts were embedded in LMPA beads and surrounded by liquid media. For culture in LMPA beads, a protoplast suspension (containing 1 x 10^5 protoplasts ml^{-1} liquid MC_2) was mixed with an equal volume of liquified solid MC_2 (containing 5 g LMPA l^{-1} liquid MC_2). Six beads of 50 µl of this mixture were dispensed in a petri dish (60 mm). After solidification of the beads, 5 ml of liquid MC_1 or MC_2 was added. At day 5 of the regeneration process, the liquid medium was fully replaced: MC_1 was replaced by fresh MC_1 or MC_2 and MC_2 was replaced by fresh MC_2.

When the microcolony phase was reached in the liquid culture systems and in the LMPA bead culture system, the liquid medium was refreshed each week. After 4 weeks, the mannitol concentration was stepwise reduced using liquid MC_2, containing 30 g l^{-1} mannitol instead of

60 g l^{-1}. After 6 weeks, protoplast-derived microcalli were transferred onto solid regeneration medium MC_3 (Table 2-1) to induce callus growth.

In experiment 2, the influence of different initial protoplast densities (1, 2, 5, 10 or 20 x 10^4 protoplasts ml^{-1}) in the LMPA beads were tested. All protoplasts were cultured in petri dishes (60 mm diameter) sealed with parafilm. The cultures were kept under 16 h/ 8 h day/night conditions at 23 ± 2 °C. Experiments were repeated 3 to 5 times. Data were collected from 1 to 3 petri dishes per experiment and per treatment.

Experiment 3 was set up to evaluate the impact of different auxins, either 1-naphthaleneacetic acid (NAA) or indole-3-acetic acid (IAA) in combination with cytokinin 6-benzylaminopurine (BAP), on shoot formation. Regenerating calli obtained in experiment 1 were placed on solid MC_3 containing 0.5 mg l^{-1} BAP and 0.1, 0.5 or 1.0 mg l^{-1} NAA or IAA. Subsequently, regenerated shoots were placed on 100 ml solid medium (Murashige and Skoog medium containing 2% sucrose, pH 5.7) in Meli jars for rooting and further growth. Experiments were repeated 3 times. Data were collected from 3 petri dishes per experiment and per treatment.

In experiment 4, the optimal conditions obtained for the model genotypes were tested for the regeneration of other *Cichorium* cultivars: *C. intybus* var. *sativum* ‘K1093’, ‘K1729’ and ‘L4043’, for wild *C. intybus* ‘Ames22531’ and ‘Ames 22532’ and for *C. endivia* var. *endivia* ‘CICH192’, *C. endivia* var. *latifolium* ‘nr.5’ and *C. endivia* var. *divaricatum* ‘CICH50’. Experiments were repeated 3 to 4 times. Data were collected from 2 to 3 petri dishes per experiment and per treatment.

Statistical analysis

One-way analysis of variance (ANOVA) and Tukey’s Post-Hoc test were used to analyze the effect of different media sequences on protoplast regeneration in the LMPA beads, the influence of different initial protoplast densities in the LMPA beads and the effect of the combination of either NAA or IAA with BAP on the shoot formation of regenerating calli. All calculations were obtained using the statistical software package Statistica v.10.

2.3 Results

Protoplast Isolation

On average, 1 x 10^6 highly chloroplast-rich protoplasts of 10-70 µm diameter were isolated per gram of fresh-weight leaves from all of the *Cichorium* genotypes tested. Their viability varied between 85% and 95% (Fig. 2.1).

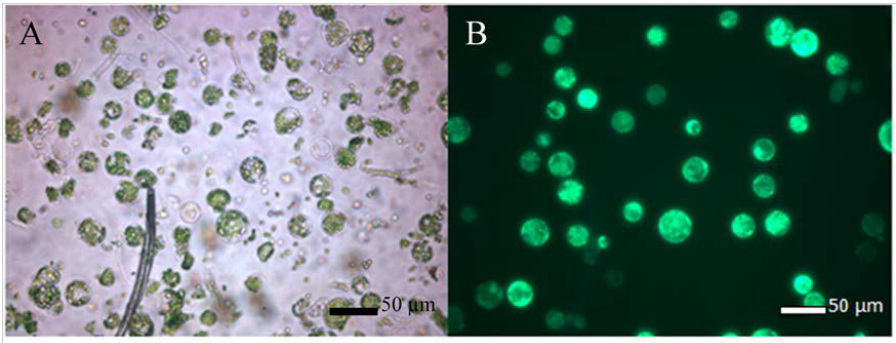


Fig. 2-1 (A) Yield of *C. intybus* var. *sativum* 'VL52' protoplasts after isolation. (B) Viability test with FDA staining: Fluorescent protoplasts are viable.

Protoplast regeneration systems

In experiment 1, the efficiency of the three culture systems with regard to four-cell stage and microcolony formation was tested. In solid medium, the protoplasts did not divide and all genotypes showed a high mortality rate within 5 days after isolation (Table 2-2). The liquid media induced initial divisions in the *C. intybus* 'Pi531291' protoplasts during the first week. Four-cell stages were obtained, but further development was limited. FDA tests on day 5 showed a significantly improved protoplast viability when using MC_1 as the initial medium (56.9 ± 2.8% viable protoplasts) compared to using MC_2 as the initial medium (32.9 ± 6.3% viable protoplasts). In all liquid culture systems, however, anthocyanin production was observed, indicating stress. Monitoring cell wall formation with CFW in the liquid cultures revealed cell wall regeneration one day after protoplast isolation in 15% of the initiated protoplasts on average. After 5 days, up to 40% of the protoplasts had formed a new cell wall, suggesting the possibility of lowering the mannitol concentration after 5 days. However, replacing MC_1 with MC_2 after 5 days of culturing did not enable further divisions. Protoplasts of *C. intybus* var. *sativum* 'VL52' and *C. endivia* var. *crispum* 'Wallone Despa' did not initiate first divisions in the liquid culture system and subsequently died (Table 2-2).

Protoplasts cultured in the LMPA beads obtained the four-cell stage and developed further into microcolonies 14 days after isolation for all genotypes (Table 2-2).

Testing of different media compositions in the LMPA bead technique showed that for both *C. intybus* var. *sativum* 'VL52' and *C. intybus* 'Pi531291' protoplasts, an initial 5-day culture in liquid MC_1 medium produced twice as many four-cell stages compared to the beads in liquid MC_2 medium (Table 2-3). Microcolony development increased significantly when MC_1 was replaced by MC_2 after 5 days of culturing. Refreshing the MC_1 medium with fresh MC_1 limited further development of the four-cell stage. Although an initial incubation of the beads in liquid MC_2 produced a significantly lower number of cells in the four-cell stage than in liquid MC_1, further incubation in fresh MC_2 resulted in a significant higher number of microcolonies in the beads (Table 2-3). Consequently, the highest number of microcolonies was obtained by initial incubation of the beads for 5 days in liquid MC_1 medium and subsequent replacement of MC_1 with MC_2. Moreover, a higher percentage of microcolonies was observed at day 14 than the percentage of four-cell stages after 5 days, suggesting a beneficial effect of the replacement of MC_1 with MC_2. For *C. endivia* var. *crispum* 'Wallone Despa' protoplasts, no microcolonies were obtained under a 16/8 h light/dark photoperiod. However, when culturing the endive protoplasts continuously in the dark, microcolony formation could be observed. The percentage of microcolonies produced was significantly lower compared to *C. intybus* var. *sativum* 'VL52' and *C. intybus* 'Pi531291' (Table 2-3). When transferring the endive microcalli on solid medium for callus growth and shoot induction, standard light conditions were used.

After 4 weeks, the LMPA beads contained a high number of developing microcalli and the mannitol concentration was gradually decreased. After 6 weeks, protoplast-derived microcalli were transferred to solid regeneration medium MC_3 to induce callus growth. For *C. intybus* var. *sativum* 'VL52' and *C. intybus* 'Pi531291' cultures, 4% of the initiated protoplasts on average could be regenerated to calli. For the *C. endivia* var. *crispum* 'Wallone Despa' culture, this was less than 1% (Table 2-3).

Table 2-2 Effect of different culture systems and media composition on the regeneration of *C. intybus* var. *sativum* 'VL52', *C. intybus* 'Pi531291' and *C. endivia* var. *crispum* 'Wallone Despa' protoplasts. Four-cell stage and microcolony formation were analyzed 5 and 14 days after protoplast isolation, respectively.

Culture system	Protoplasts	Medium sequence [z]	5 days (Four-cell stage)	14 days (Microcolony)
Solid	*C. intybus* var. *sativum* 'VL52'	MC1	-	-
		MC2	-	-
	C. intybus 'Pi531291'	MC1	-	-
		MC2	-	-
	C. endivia var. *crispum* 'Wallone Despa'	MC1	-	-
		MC2	-	-
Liquid	*C. intybus* var. *sativum* 'VL52'	$MC_1 \rightarrow MC_1$	-	-
		$MC_1 \rightarrow MC_2$	-	-
		$MC_2 \rightarrow MC_2$	-	-
	C. intybus 'Pi531291'	$MC_1 \rightarrow MC_1$	+	-
		$MC_1 \rightarrow MC_2$	+	-
		$MC_2 \rightarrow MC_2$	-	-
	C. endivia var. *crispum* 'Wallone Despa'	$MC_1 \rightarrow MC_1$	-	-
		$MC_1 \rightarrow MC_2$	-	-
		$MC_2 \rightarrow MC_2$	-	-
LMPA beads	*C. intybus* var. *sativum* 'VL52'	$MC_1 \rightarrow MC_1$	+	-
		$MC_1 \rightarrow MC_2$	+	+
		$MC_2 \rightarrow MC_2$	+	+
	C. intybus 'Pi531291'	$MC_1 \rightarrow MC_1$	+	+
		$MC_1 \rightarrow MC_2$	+	+
		$MC_2 \rightarrow MC_2$	+	+
	C. endivia var. *crispum* 'Wallone Despa'	$MC_1 \rightarrow MC_1$	+	-
		$MC_1 \rightarrow MC_2$	+	+
		$MC_2 \rightarrow MC_2$	-	-

[z] For the liquid and LMPA bead culture: at day 5 of regeneration, MC_1 was replaced by either MC_1 or MC_2. MC_2 was replaced by MC_2 only.

Table 2-3 Effect of different media sequences on *C. intybus* var. *sativum* 'VL52', *C. intybus* 'Pi531291' and *C. endivia* var. *crispum* 'Wallone Despa' protoplast regeneration in LMPA beads (% of the initial number of cultured protoplasts). Four-cell stage and microcolony formation were analyzed after 5 and 14 days, respectively. Callus development was analyzed on solid MC_3 medium.

	Medium sequence[z]	5 days (Four-cell stage)	14 days (Microcolony)[y]	Callus	Plants
C. intybus var. *sativum* 'VL52'	$MC_1 \rightarrow MC_1$	34.5 ± 3.2a[x]	0c	/	/
	$MC_1 \rightarrow MC_2$	39.5 ± 3.9a	47.5 ± 1.0a	4.1 ± 0.2a	+
	$MC_2 \rightarrow MC_2$	18.5 ± 2.2b	26.5 ± 2.5b	2.3 ± 0.2b	+
C. intybus 'Pi531291'	$MC_1 \rightarrow MC_1$	37.5 ± 3.0a	9.0 ± 2.7c	0c	/
	$MC_1 \rightarrow MC_2$	39.5 ± 2.9a	56.5 ± 2.1a	4.4 ± 0.2a	+
	$MC_2 \rightarrow MC_2$	19.5 ± 1.0b	25.5 ± 1.7b	2.0 ± 0.1b	+
C. endivia var. *crispum* 'Wallone Despa'	$MC_1 \rightarrow MC_1$	4.5 ± 0.5*a	0	/	/
	$MC_1 \rightarrow MC_2$	4.5 ± 1.3*a	6.5 ± 2.2*	0.7 ± 0.1	+
	$MC_2 \rightarrow MC_2$	0*b	/	/	/

Data are means ± SE (n = 9, collected from three to five experiments)
[x] a, b, c significant differences based on Tukey's Post Hoc test, $p \leq 0.05$. Results compared within each genotype for the three medium sequences.
[z] At day 5 of regeneration, MC_1 was replaced by either MC_1 or MC_2; MC_2 was replaced by MC_2.
[y] Microcolonies: cell clumps of 10 to 20 cells.
* Regeneration under dark conditions

Experiment 2 showed that for the three genotypes tested, a density of 5 x 10^4 protoplasts ml^{-1} in the agarose beads resulted in the highest plating efficiencies (Figs. 2-2, 2-3). When using lower densities, fewer divisions occurred. The use of higher densities led to high frequency of first mitotic cell divisions, but further development stopped once the four-cell stage was obtained. The protoplast density of 5 x 10^4 protoplasts ml^{-1} was used in further protoplast regeneration experiments with the LMPA beads.

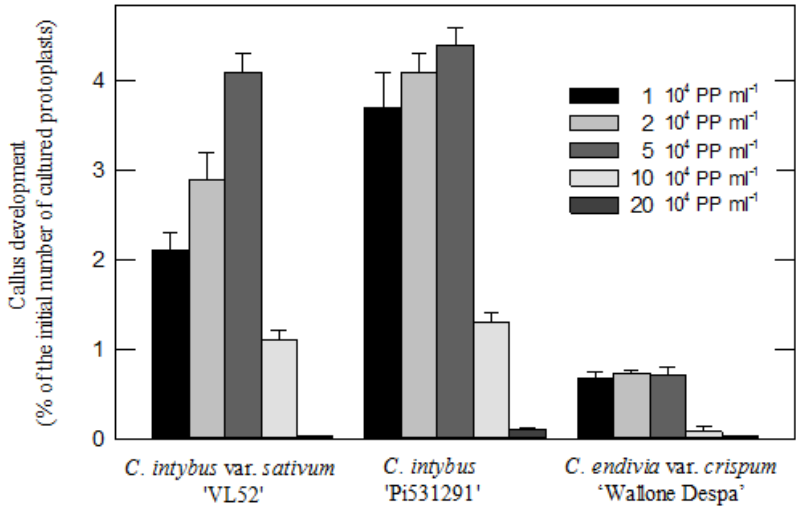


Fig. 2-2 The effect of different initial protoplast densities (1, 2, 5, 10 or 20 x 10^4 protoplasts (PP) ml^{-1}) on callus development in the LMPA beads for three *Cichorium* genotypes (% of the initial number of cultured protoplasts). Data are means ± SE (n = 9, collected from three to five experiments)

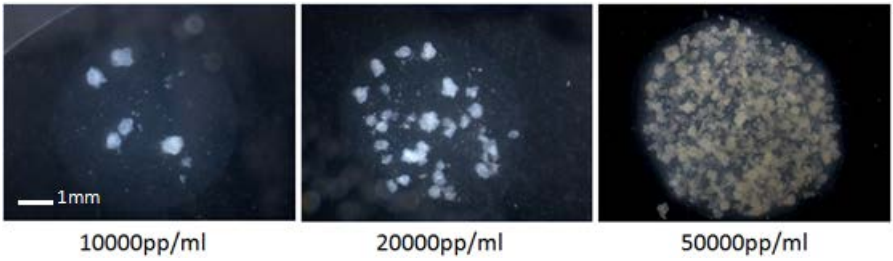


Fig. 2-3 The effect of different initial protoplast densities (1, 2, 5, x 10^4 protoplasts (pp) ml^{-1}) on callus development in the LMPA beads for *C.intybus* var. *sativum* 'VL52'

Experiment 3 demonstrated that shoots were induced more efficiently when callus was cultured in IAA enriched MC_3 medium instead of in NAA enriched MC_3 medium (Fig. 2-4). An IAA concentration of 0.5 mg ml^{-1} combined with 0.5 mg ml^{-1} BAP yielded the highest shoot formation for either *C. intybus* var. *sativum* 'VL52' (67.8 ± 8.8%), *C. intybus* 'Pi531291' (87.5 ± 4.0%) and *C. endivia* var. *crispum* 'Wallone Despa' (26.8 ± 2.6%). At higher IAA concentrations, the shoots were translucent and exhibited hyperhydric symptoms. Those

shoots did not develop further upon isolation. Lower IAA concentrations induced fewer shoots (Fig. 2-4).

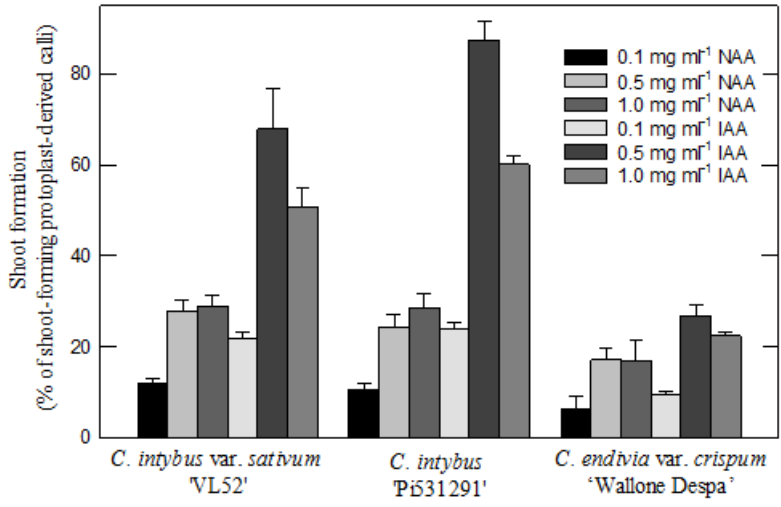


Fig. 2-4 Percentage of shoot-forming *Cichorium* protoplast-derived calli on MC_3 medium supplemented with 0.5 mg l^{-1} BAP and 0.1, 0.5 or 1.0 mg l^{-1} NAA or IAA concentrations. Data are means ± SE (n = 9, collected from three experiments)

In experiment 4, protoplast regeneration was tested on a higher number of *Cichorium* genotypes using the most optimal culture conditions from former experiments. Protoplasts of *Cichorium intybus* var. *sativum* 'K1729' and 'L4043' could be regenerated as efficient as *C. intybus* var. *sativum* 'VL52'. *C. intybus* var. *sativum* 'K1093' yielded even a higher plating efficiency (PE) (Table 2-4). The wild type chicory *C. intybus* 'Ames22532' yielded comparable results as *C. intybus* 'Pi531291'. *C. intybus* 'Ames22531' protoplasts formed as many microcolonies as the model genotype protoplasts. However, the frequency of callus formation was significantly lower compared to the other *C. intybus* cultivars (Table 2-4). The three endive cultivars yielded similar PEs as the endive model cultivar. Continuously dark conditions were needed to observe microcolony formation within the endive cultivars. When transferring the endive microcalli on solid medium for callus growth and shoot induction, standard light conditions were used. However, the endives produced significantly fewer calli than *C. intybus* plants. Full plantlet regeneration of protoplast-derived calli could be obtained for all plants tested.

Table 2-4 Callus formation of several *Cichorium* plants after protoplast regeneration in LMPA beads using the $MC_1 \rightarrow MC_2$ medium sequence (% of the initial number of cultured protoplasts).

Species		Callus (%)
C. intybus var. *sativum*	'VL52'	4.1 ± 0.2bc[x]
	'K1093'	4.9 ± 0.1a
	'K1729'	4.6 ± 0.1ab
	'L4043'	3.9 ± 0.1c
C. intybus	'Pi531291'	4.4 ± 0.2abc
	'Ames22531'	1.7 ± 0.2d
	'Ames22532'	3.9 ± 0.1c
C. endivia var. *crispum*	'Wallone Despa'	0.7 ± 0.1e
C. endivia var. *endivia*	'CICH192'	0.5 ± 0.04e
C. endivia var. *latifolium*	'nr.5'	0.5 ± 0.03e
C. endivia var. *divaricatum*	'CICH50'	0.3 ± 0.05e

Data are means ± SE (n = 8, collected from three to four experiments)
[x] a, b, c, d, e significant differences based on Tukey's Post Hoc test, $p \leq 0.05$. The results for callus formation were compared between each genotype.

2.4 Discussion

Protoplasts were successfully isolated from leaves of four industrial chicory plants, *Cichorium intybus* var. *sativum* 'VL52', 'K1093', 'K1729' and 'L4043', three wild types from Hungary, *C. intybus* 'Ames22531', 'Ames22532' and 'Pi531291', and the endive cultivars *C. endivia* var. *crispum* 'Wallone Despa', *C. endivia* var. *endivia* 'CICH192', *C. endivia* var. *latifolium* 'nr.5' and *C. endivia* var. *divaricatum* 'CICH50' using the method reported in Cappelle et al. 2007. No further optimization of protoplast isolation was needed.

The development of a suitable culture method is a key factor in the final efficiency of a protoplast regeneration protocol (Davey et al., 2005; Eeckhaut and Van Huylenbroeck, 2011). In this study, the regeneration ability of *Cichorium* protoplasts was tested using the gelating agent, low melting point agarose (LMPA). The first gelating agent used for protoplast regeneration was agar. This was successfully performed on tobacco mesophyll protoplasts (Nagata and Takebe, 1971; Davey et al., 2005). However, the use of agarose significantly improved the plating efficiency of regenerating protoplasts in numerous species. Due to its neutral charge and lower degree of chemical complexity, fewer interactions between biomolecular nutrients and agarose occurred. Furthermore, the larger pore size of agarose gels promoted a higher degree of biomolecule exchange within the agarose gel and between the gel and the external environment. The LMPA with a low gelling temperature of 24-30 °C,

used in this study, allowed protoplast mixture with the LMPA solution without exposing the cells to damaging temperatures as the LMPA solution remained fluid at 35 °C and gelation occured below 26 °C (Lorz et al., 1983; Shillito et al., 1983; Lian et al., 2012).

We have evaluated the regeneration efficiency of protoplasts of various *Cichorium* plants in low melting point agarose beads in comparison to liquid and solid cultures.

Culture in exclusively solid medium resulted in no divisions. These protoplasts soon burst and subsequently died. Stress factors typically linked with the use of solid media for protoplasts are the probable cause of this failure. Without the possibility of regular refreshment, the medium dehydrates, and thus lowers the osmotic potential. Moreover, toxic compounds of dying neighbouring protoplasts may accumulate in the medium and inhibit division of other protoplasts (Vanslogteren et al., 1980; Davey et al., 2005; Duquenne et al., 2007).

Liquid medium was not optimal for *Cichorium* protoplast culture either. This was demonstrated by the overall decrease of protoplast viability, the production of anthocyanins (also observed in liquid culture of petunia protoplast (Frearson et al., 1973) and the limited division of protoplasts during the first week of culture. Similar results were obtained in the regeneration of protoplasts of *C. intybus* 'Rosso do Chioggia' in liquid conditions (Nenz et al., 2000). Two possible explanations are the low accessibility of gases at the bottom of the petri dish, where the protoplasts are located (Duquenne et al., 2007), or the exposure of healthy cells to high concentrations of toxic substances of dying neighbouring cell due to the clustering of protoplasts in liquid cultures (Yu et al., 2000).

Regeneration of protoplasts of all the *Cichorium* plants tested was achieved by embedding the protoplasts in LMPA beads surrounded by liquid medium. Compared to the solid medium culture, the LMPA bead technique enabled regular refreshment, preventing medium dehydration and toxic compounds accumulation. Unlike culturing in liquid media, protoplasts in the LMPA beads were homogeneously spread, inhibiting cluster formation. Moreover, due to the low concentration of the low melting point agarose used for the formation of the beads, nutrients and gasses could be exchanged easily between the liquid and solid phase. The use of LMPA in semisolid protoplast regeneration culture systems for chicory has been described previously (Saksi et al., 1986a; Slabe and Bohanec, 1989; Rambaud et al., 1990). Those systems required that the protoplasts first be cultured in liquid medium and subsequently centrifuged before starting culture in a semisolid medium. Protoplast loss can therefore be expected, and clustering can hinder protoplast separation on the semisolid medium. In the Ca-alginate nurse-cultures described by Nenz et al. (2000), protoplasts are embedded in Ca-alginate beads surrounded by liquid medium. This technique requires supplementary steps,

however: at the start of the culture, beads need to be formed through the merger of an alginate solution, in which protoplasts are suspended, as well as a Ca solution. Once microcalli have been formed in the beads, they need to be released by chelating the calcium from the matrix and centrifuging the released colonies, resulting in the aforementioned problems. Our LMPA bead based system has two major advantages compared to these culture systems: (1) it offers a high regeneration capacity for a greater number of genotypes, (2) its simplicity avoids the necessity of supplementary steps such as centrifugation and/or chemical bead breakdown that can be expected to significantly reduce the overall efficiency of the protocol. Developing microcalli can easily be removed from the soft LMPA beads with tweezers and placed on solid medium without an extra centrifugation step. Also, the proliferation of particular cells or colonies can be followed up on a daily basis. Changing or refreshing the medium at any time without disturbing the protoplasts is also possible. This is particularly important when culturing fused protoplasts, because supplementary toxic waste molecules produced by the fusion event must be more rapidly diluted. Our results demonstrate that the LMPA bead technique is a very efficient tool for protoplast regeneration, and makes it possible to change culture media to fit the suitable environmental conditions for many several *Cichorium* types.

After microcalli formation in the LMPA beads and callus development on solid MC_3 medium, shoot development was more efficiently induced when the regenerating calli were cultured in IAA enriched MC_3 medium. Although, previous studies on shoot induction on protoplast-derived calli of *Cichorium* reported the use of a NAA and BAP complemented medium for shoot inducing (Saksi et al., 1986a; Rambaud et al., 1990; Cappelle et al., 2007), we found an IAA and BAP complemented medium to be more effective. This is in accordance with findings in *in vitro* plant regeneration through organogenesis from cotyledon, petiole, leaf and root explants derived calli, which used IAA in favour of NAA in combination with BAP for shoot formation (Park and Lim, 1999; Velayutham et al., 2006; Choi et al., 2009).

When using the LMPA bead technique with the medium sequence $MC_1 \rightarrow MC_2$, followed by an incubation of the protoplast-derived calli on IAA enriched MC_3 medium, total protoplast regeneration was possible within 14 weeks for the *Cichorium* species under study (Fig. 2-5).

In our experiments, a protoplast density of 5×10^4 protoplasts ml^{-1} in the agarose beads resulted in the highest plating efficiencies (Figs. 2-2, 2-3). Compared to previously reported results in chicory protoplast regeneration, which showed the highest plating efficiencies when using 2×10^4 protoplasts ml^{-1} in semisolid culture systems (Rambaud et al., 1990; Varotto et al., 1997) and in alginate culture systems (Nenz et al., 2000), our technique enables regeneration of more protoplasts in a single experiment.

For the first time, plantlets of as well industrial chicory (*C. intybus* var. *sativum*), wild chicory (*C. intybus*) and endive (*C. endivia*) were successfully regenerated from protoplasts, using the LMPA bead technique. To our knowledge, this technique is the only one that induces sustained protoplast division and complete regeneration in such a wide *Cichorium* range. A second innovation presented in this study is the first full plantlet regeneration from endive protoplasts. Consequently, our findings can contribute to the further development of somatic hybridization within *C. endivia* or between different *Cichorium* species. Indeed, the presence of an effective regeneration protocol is indispensable for the development of protoplast-based breeding tools, including both symmetric and asymmetric somatic fusion. The development of the LMPA technique therefore offers significant potential for interspecific *Cichorium* breeding and subsequent genetic variation broadening and introgression of new traits.

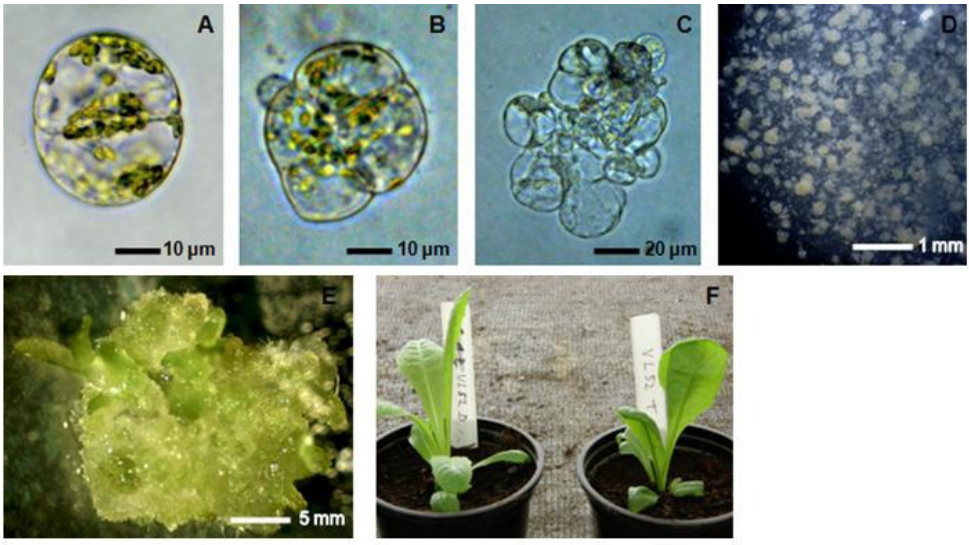


Fig. 2-5 *Cichorium intybus* var. *sativum* 'VL52' protoplast culture: regeneration steps following the LMPA bead technique. First mitotic division (A); Four-cell stage after the second mitotic division (B); microcolony (C); microcalli development (D); shoot development (E); *in vivo* regenerants (F)

2.5 Conclusion

Protoplast regeneration is essential for somatic hybridizations. In this study, a standard method for plantlet regeneration from *Cichorium* protoplasts was developed. We evaluated the effect of the low melting point agarose (LMPA) bead technique on the regeneration capacity of protoplasts of seven *C. intybus* types and four *C. endivia* cultivars. The LMPA bead technique was more efficient than culture in liquid or solid medium and allowed us to obtain plating efficiencies up to 4.9% in *C. intybus* protoplasts and efficiencies of up to 0.7% in *C. endivia* protoplasts. Moreover, the LMPA bead technique offers great advantages over liquid and solid culture systems: the media can be readily refreshed, protoplasts can be monitored separately, and microcalli can easily be removed from the beads. This increased efficiency was observed for all of the 11 *Cichorium* types tested. Shoot formation was induced more efficiently when using 0.5 mg l^{-1} indole-3-acetic acid (IAA) enriched medium (up to 87.5% of the protoplast-derived calli started shoot development) compared to 1-naphthaleneacetic acid (NAA) enriched medium. The LMPA bead technique optimized in this study enabled for the first time the full plantlet regeneration from *C. endivia* protoplasts and increased the protoplast regenerating ability in other *Cichorium* species. This fine-tuned LMPA bead technique can therefore be applied for protoplast regeneration after protoplast fusions of the genus *Cichorium*.

Chapter 3 - Symmetric protoplast fusion in *Cichorium* species

Based on the published article: Deryckere D, Eeckhaut T, Van Huylenbroeck J, Van Bockstaele E. (2012) Optimization of somatic hybridization in *Cichorium* species. Acta Horticulturae (ISHS) 961, 95-102**.** Proceedings of the VII International symposium on *in vitro* culture and horticultural breeding: IVCHB, 'Biotechnological advances in *in vitro* horticultural breeding', September 18-22, 2011, Ghent (Belgium).

3.1 Introduction

Improvement of traits such as yield, biomass production, (a)biotic resistance or tolerance, gene pool enlargement, secondary metabolite production, rootstock or ploidy breeding and altered morphology are of major interest for breeders in many crops. In addition to traditional breeding, protoplast fusion has become an alternative to achieve improved crop performance. The first interspecific hybridization was established between two tobacco species through symmetric protoplast fusion (Carlson et al., 1972). Since the 1980s, protoplast fusion has been commonly attempted in commercial crop breeding. Protoplast fusion transcends the limits of sexual crossing to enable the construction of previously impossible somatic hybrids. Moreover, cytoplasmic features can be transferred as well as traits transcribed from the nuclear genome. The main application of symmetric somatic hybridization is the production of allotetraploid hybrids that combine the complete nuclear genomes of both parents. These allotetraploids can be used as genetic pools, potential rootstock or as male parents in sexual crosses. In the latter case, they can be used to create other tetraploids or, when fused with diploids, they can produce (sterile) triploid cultivars (Rambaud et al., 1992; Grosser et al., 2007a). New (seedless) triploid cultivars can directly be formed by symmetric fusion of haploids with diploids (Olivares-Fuster et al., 2005). The two main fusion tools currently used are electrical and chemical protoplast fusion. Chemical fusion combines the agglutination of neighboring protoplasts through polyethylene glycol (PEG) and the merging of these protoplasts through membrane disruption after adding a high concentration of calcium ions at high pH (Waara and Glimelius, 1995). Electrofusion is initiated by placing a protoplast suspension in a low-conductivity medium between electrodes. Applying an alternating current through the system aligns the protoplasts between the electrodes and forces them into close contact. The merging of the membranes of neighbouring protoplasts is mediated through the application of one (or a few) short pulse(s) of direct current (Waara and Glimelius, 1995). Electrofusion is believed to be less cytotoxic than chemical fusion, because PEG is rather toxic. Moreover, electrofusion allows the fusion conditions to be controlled more easily (Bates et al., 1987). Recent progress in protoplast fusion research showed that Brassicaceae, Rutaceae and Solanaceae are the most studied plant families for symmetric fusions. In general, neither PEG-mediated fusion nor electrofusion is preferred over the other technique. PEG-mediated fusion is more common in Brassicaceae, whereas electrofusion is more routinely used in Solanaceae. For Rutaceae both fusion methods have been used.

Industrial chicory is cultivated for the inulin found in the root. Improving the inulin yield and quality through conventional breeding has reached its limit. Therefore, we aim to broaden the genetic background of industrial chicory through symmetric protoplast fusion with wild chicory or endive. Since a reliable protoplast regeneration system in *Cichorium* has already been established, the use of protoplast fusion techniques is realistic (Crepy et al., 1982; Saksi et al., 1986b; Slabe and Bohanec, 1989; Rambaud et al., 1990; Varotto et al., 1997; Nenz et al., 2000; Deryckere et al., 2012). To date, several chemical symmetric protoplast fusion experiments are used. PEG-mediated chemical fusion of chicory leaf protoplasts has been used to obtain tetraploid plants (Rambaud et al., 1992). Male sterile chicory cybrids were obtained after intergeneric symmetric PEG-mediated fusion between *C. intybus* mesophyll protoplasts and CMS *Helianthus annuus* hypocotyl protoplasts (Rambaud et al., 1993). Interspecific symmetric protoplast fusions between mesophyll protoplasts of *C. intybus* and *C. endivia* have yielded tetraploid somatic hybrids containing the nuclear genomes of both parents (Cappelle et al., 2007).

Rambaud et al. (1990) noted that the growth of *Cichorium* protoplasts is cultivar- and species–dependent, thus requiring refinements and adjustments to the protoplast isolation, regeneration and fusion protocols. The main goal of this study was to develop a universal protocol for symmetric protoplast fusion in *Cichorium* species. We therefore evaluated both electrical and chemical fusion of protoplasts of industrial chicory *C. intybus* var. *sativum* with endive *C. endivia* var. *crispum* and wild type chicory *C. intybus* protoplasts.

3.2 Materials and methods

Plant material

One industrial chicory inbred line *C. intybus* var. *sativum* 'VL52', one wild type chicory *C. intybus* 'Pi531291' from Hungary and one endive cultivar *C. endivia* var. *crispum* 'Wallone Despa' were used. Seeds of *in vivo* plants of the selected *Cichorium* plants were initiated *in vitro*. After rinsing for 1 min in 70% ethanol, seeds were surface sterilized for 20 min in 6.5% NaOCl and germinated in 60 mm petri dishes on solid Murashige and Skoog (1962) medium containing 150 mg l^{-1} casein hydrolysate and 30 g l^{-1} sucrose at pH 5.8. After germination, the plantlets were placed on solid Murashige and Skoog medium containing 20 g l^{-1} sucrose and grown in Meli jars (Meli NV, Veurne Belgium) at 23 ± 2°C under a 16 h/8 h (light/dark) photoperiod at 40 µmol m^{-2} s^{-1} photosynthetic active radiation.

After protoplast isolation (performed according to Deryckere et al., 2012), protoplasts were stained to observe binary heterofusions. One hundred microliters of *C. intybus* var. *sativum* 'VL52' protoplasts were mixed with 1 µl of a 0.5% (w/v) fluorescein diacetate (FDA) stock solution (5 mg FDA dissolved in 1 ml acetone), incubated for 10 min at room temperature and washed by centrifugation. Then 100 µl of *C. endivia* var. *crispum* 'Wallone Despa' or *C. intybus* 'Pi531291' protoplasts were mixed with 1 µl of a 3% (w/v) rhodamine β isothiocyanate (Rho) (Sigma-Aldrich) stock solution (30 mg Rho dissolved in 1 ml acetone), incubated for 10 min at room temperature and washed by centrifugation. Microscopic detection was carried out using an inverted fluorescence microscope (Leica DMIRB) equipped with a Leica Camera System (Leica DFC320).

Protoplast fusion

C. intybus var. *sativum* 'VL52' protoplasts were both chemically and electrically fused with *C. endivia* var. *crispum* 'Wallone Despa' or *C. intybus* 'Pi531291' protoplasts.

Chemical fusion was performed using PEG and a high concentration of calcium ions at high pH. Two PEG types with a different molecular weight (MW) were tested and several incubation times were analyzed by evaluating protoplast viability with FDA and binary heterofusion formation with FDA and Rho. Protoplasts were fused according to a modified protoplast fusion procedure based on Kao (1982). Specifically, protoplasts of the two fusion partners were resuspended at a density of 5 x 10^5 protoplasts ml^{-1} in a washing solution containing one-half strength Murashige and Skoog (MS) macroelements (Murashige and Skoog, 1962) (without NH_4NO_3 and KNO_3), Heller microelements and Heller KCl (Heller, 1953), Morel and Wetmore vitamins (Morel and Wetmore, 1951), 18.35 mg l^{-1} FeNa-EDTA, 100 mg l^{-1} inositol, 750 mg l^{-1} glutamine, 10 g l^{-1} sucrose, 60 g l^{-1} mannitol, 0.5 mg l^{-1} 1-naphthaleneacetic acid (NAA) and 0.5 mg l^{-1} 6-benzylaminopurine (BAP) at pH 5.5. Protoplasts were equally mixed and drops of 150 µl of the mixture were dispensed in 10 petri dishes (5.5 cm diameter). After the protoplasts had settled for 5 min, 100 µl PEG solution [300g l^{-1} PEG MW 3350 or PEG MW 6000 (Sigma-Aldrich), 1500 mg l^{-1} $CaCl_2$ $2H_2O$, 100 mg l^{-1} KH_2PO_4, 10% dimethyl sulfoxide (DMSO), 80 g l^{-1} mannitol, pH 5.5] was added to the protoplast solution in each petri dish and incubated for 1, 2, 5 or 10 min. Subsequently, a Ca-rich solution (80 g l^{-1} mannitol, 7.5 g l^{-1} glycine, 14.7 g l^{-1} $CaCl_2$ $2H_2O$) at high pH (10.5) was added to the fusion mixture in each petri dish. After 5 min, each fusion solution was gently diluted with 3 ml washing medium and incubated for 20 min. After incubation, the contents of the 10 petri dishes were collected, centrifuged (100g, 10 min) and washed twice. The

protoplasts were regenerated according to Deryckere et al. (2012) in low melting point agarose beads at a final density of 5 x 10^4 protoplasts ml^{-1}. After adding the Ca-rich solution and the washing solution, the viability and the binary heterofusion rate were observed microscopically. Experiments were repeated 3 times. Data were collected from 3 petri dishes per experiment and per treatment.

Electrical fusion was performed with an Eppendorf Multiporator. For optimizing the fusion parameters, a micro-fusion chamber (Eppendorf Multiporator) was used to microscopically observe the fusion process. After optimizing the parameters with the micro-fusion chamber, the Eppendorf helix fusion chamber was used for routine applications. Protoplasts of two fusion partners (5 x 10^5 protoplasts ml^{-1}) were equally mixed and resuspended in an iso-osmotic buffer (Eppendorf). Fifty microliters of the mixture were pipetted between the electrodes of the micro-fusion chamber. An alternating current (AC) of 1.5, 1.8 or 2 V was applied for a certain time period (30, 40 or 60 s) during both alignment and post-alignment. Two direct current (DC) pulses of 30, 40, 50, 60, 70 or 80 V for 25 µs were applied to induce membrane fusion. The fusion process was microscopically observed to analyze the alignment process and the fusion rate. Experiments were repeated 3 times. Data were collected from 3 isolations per experiment and per treatment. During fusion, no fluorescent dyes were used to visualize viability and binary heterofusions because the dyes interfere with the iso-osmotic buffer. Protoplast viability was analyzed with FDA directly after fusion. The optimal fusion conditions of the micro-fusion chamber were used in the helix fusion chamber: 200 µl of a 1:1 mixture of the two fusion partners, resuspended in the Eppendorf iso-osmotic buffer, were pipetted in the helix fusion chamber. Ten minutes after fusion, protoplasts were collected, washed and pelleted by centrifugation. The protoplasts were regenerated according to Deryckere et al. (2012) under light conditions. Callus fragments were used for both flow cytometric analysis and further regeneration.

Hybrid Screening

Three independent PEG-mediated fusion experiments between *C. intybus* var. *sativum* 'VL52' and *C. endivia* var. *crispum* 'Wallone Despa' were performed. From each experiment, the ploidy level of 50 regenerating calli was screened using flow cytometry. To this end, nuclei suspensions were prepared following a modification to the protocol described by Galbraith et al. (1983). Each callus (0.5 cm^2) was put in a 55-mm-wide petri dish and 800 µl buffer I (21 g l^{-1} citrate and 5 g l^{-1} Tween dissolved in H_2O) was added. The calli were manually chopped for 1 minute using a razor blade and the total suspension was filtered through a 50 µm filter.

Finally, 400 µl buffer II [40 g l^{-1} dibasic sodium phosphate and 0.0001% (w/v) DAPI] was added. The relative fluorescence of total DNA of single nuclei was analyzed with a Partec Cyflow flow cytometer equipped with a 375 nm UV laser diode. Leaves of *C. intybus* var. *sativum* 'VL52' and *C. endivia* var. *crispum* 'Wallone Despa' were used as internal standard.
The calli were further regenerated to plantlets. Leaf DNA of these plantlets was used for microsatellite marker analysis. This was performed by COSUCRA-Groupe Warcoing S.A., Chicoline division to evaluate the data obtained by flow cytometry: The 18 microsatellite markers (GA118, GA187, GA014, GA019, GA304, GA386, GA495, GA519, GA571, GA395, TGA001, GA007, GA036, GT082, GT082-X, GA355, GA075, GA357; Personal communication COSUCRA-Groupe Warcoing S.A.), are distributed over 7 of the 9 chromosomes of the *Cichorium* species.
Three independent PEG-mediated fusion experiments between *C. intybus* 'VL52' and *C. intybus* 'Pi531291' were performed. From each experiment, 35 regenerating calli were analyzed with the microsatellite markers.

Statistical analysis
One-way analysis of variance (ANOVA) and Tukey's Post-Hoc test were used to analyze the following: 1) the effect of different incubation times in PEG MW 3350 on the viability and fusion properties of protoplasts and 2) the effect of DC pulse voltage on the total fusion rate in electrical fusion experiments. All calculations were obtained using the statistical software package Statistica v.11.

3.3 Results

Protoplast Fusion
When chemically fusing protoplasts, the PEG molecular weight and the incubation time were critical for further protoplast regeneration. Viability tests during chemical fusion showed that PEG MW 6000 was lethal for protoplasts directly after addition, regardless of the incubation time. Consequently, PEG MW 6000 was not used in further experiments. Chemical fusion with PEG MW 3350 enabled protoplast fusion. However, a gradual decrease in protoplast viability was detected as the incubation time in the PEG solution increased (Fig. 3-1). Binary heterofusion was also analyzed (Fig. 3-2). The highest rate of binary heterofusions was observed after 1 min incubation for both *C. intybus* var. *sativum* 'VL52' + *C.endiva* var.

crispum 'Wallone Despa' (8.4 ± 1.3%) and *C. intybus* var. *sativum* 'VL52' + *C. intybus* 'Pi531291' fusions (3.8 ± 0.4%) (Table 3-1). The binary heterofusion rate significantly decreased after 5 min incubation time. The highest total fusion rate was achieved after 1 min incubation for both fusion events. This total fusion rate included, besides the desired binary heterofusions, undesired homokaryon binary fusions and multifusions (Table 3-1).

Electrical fusion experiments were monitored under the microscope using the micro-fusion chamber (Fig. 3-3). Optimal alignment and post-alignment were achieved by applying 1.5 V during 40 s (data not shown). The highest total fusion rate was obtained after two pulses of 40 V for 25 µs. The rates of successful fusion were 47.6 ± 2.6% of the protoplasts of the fusion experiment *C. intybus* var. *sativum* 'VL52' + *C. endivia* var. *crispum* 'Wallone Despa' fused and 51.2 ± 2.3% for the *C. intybus* var. *sativum* 'VL52' + *C. intybus* 'Pi531291' fusion. FDA staining confirmed that after fusion, 85.2 ± 5.6% of the protoplasts were still viable. When pulse voltages of 60 V and higher were used, some protoplasts bursted, resulting in fewer fusion events (Fig. 3-4).

Table 3-1 Effect of different incubation times in PEG MW 3350 solution on the binary heterofusion and total fusion rate of *C. intybus* var. *sativum* 'VL52' and *C. endivia* var. *crispum* 'Wallone Despa' and of *C. intybus* var. *sativum* 'VL52' and *C. intybus* 'Pi531291' protoplasts (% of the initial protoplast number)

Fusion		Incubation time (min)			
		1	2	5	10
C. intybus var. *sativum* 'VL52'	Binary fusion[x]	8.4 ± 1.3a[y]	5.2 ± 0.7ab	2.4 ± 0.7bc	0.6 ± 0.4c
+ *C.endivia* var. *crispum* 'Wallone Despa'	Total fusion	16.6 ± 1.2a	11.6 ± 0.9b	5.2 ± 0.4c	1.0 ± 0.3d
C. intybus var. *sativum* 'VL52'	Binary fusion	3.8 ± 0.4a	2.8± 0.6a	1.0 ± 0.3b	0.2 ± 0.2b
+ *C. intybus* 'Pi531291'	Total fusion	7.6 ± 0.5a	6.0± 0.3a	2.0 ± 0.4b	0.6 ± 0.4b

Data are means ± SE (n = 9, collected from three experiments)

[x] Binary fusions included binary heterofusion events. Total fusion included all binary (hetero and homokaryon) and multifusion events.

[y] a, b, c, d significant differences based on Tukey's Post Hoc test, $p \leq 0.05$. Results were compared for binary heterofusion and total fusion rate within each fusion event for the four incubation times.

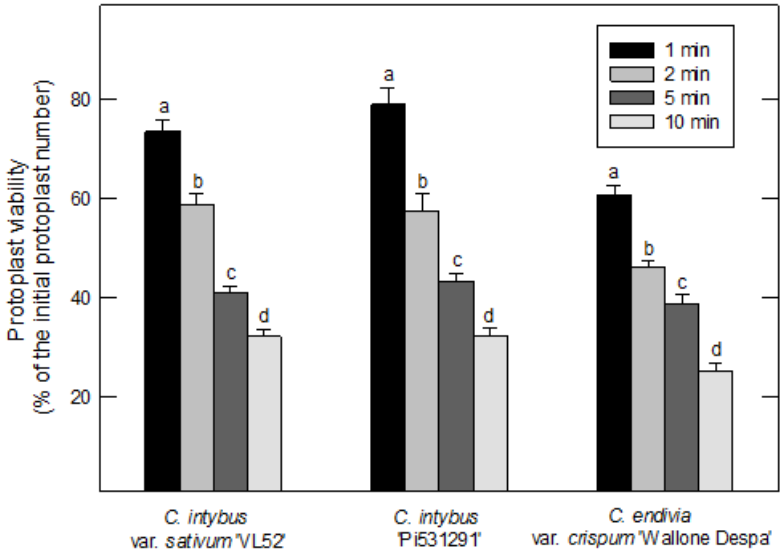


Fig. 3-1 Effect of different incubation times (1, 2, 5 and 10 min) in PEG MW 3350 solution on the viability of *C. intybus* var. *sativum* 'VL52', *C. intybus* 'Pi531291' and *C. endivia* var. *crispum* 'Wallone Despa' protoplasts (% of the initial protoplast number). Data are means ± SE (n = 9, collected from three experiments). a, b, c, d significant differences based on Tukey's Post Hoc test, $p \leq 0.05$. Results were compared within each *Cichorium* type for the four incubation times.

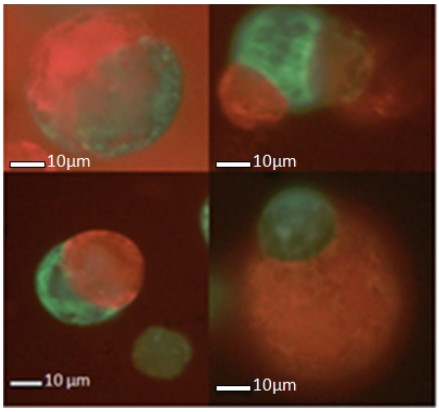


Fig. 3-2 Formation of heterokaryons through binary and multi fusions using PEG MW 3350. Red protoplasts: *C. endivia* var. *crispum* 'Wallone Despa' stained with Rho. Green protoplasts: *C. intybus* var. *sativum* 'VL52' stained with FDA.

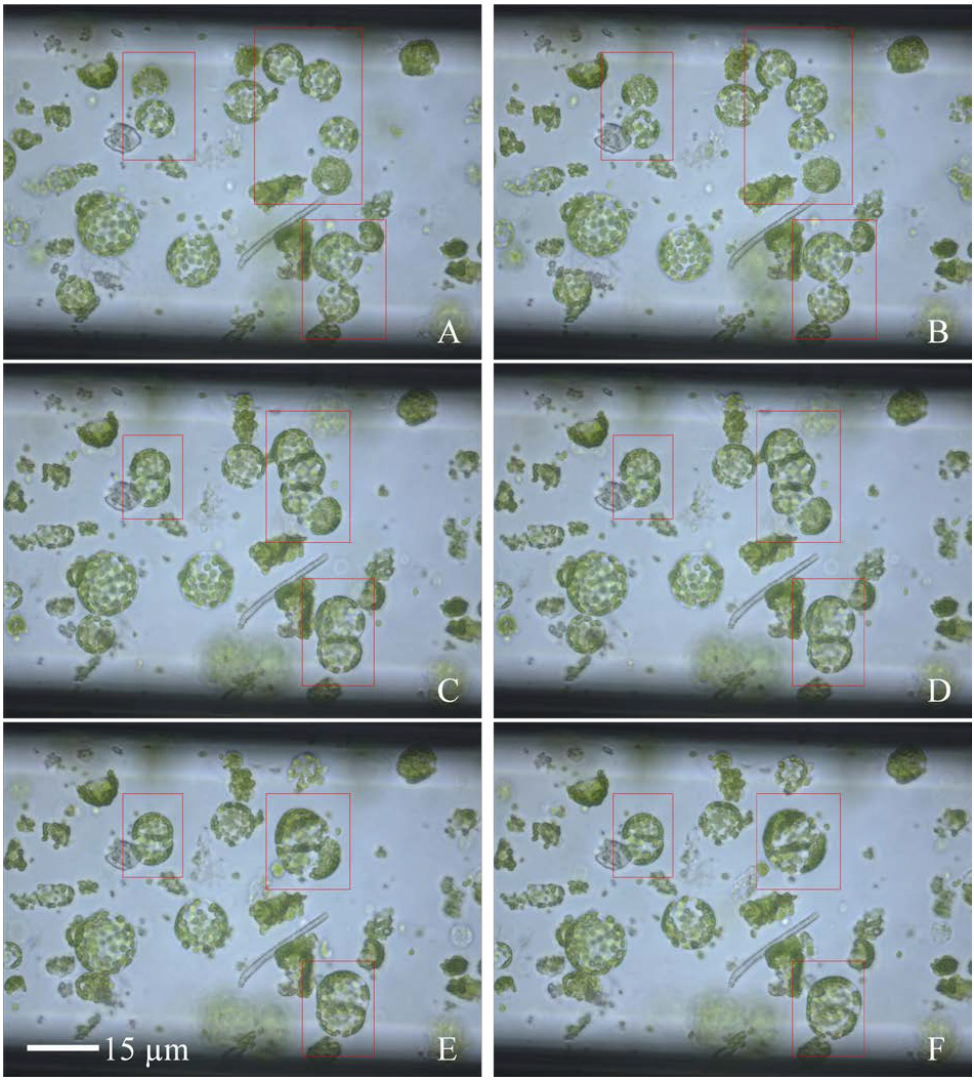


Fig. 3-3 Different steps during electrofusion of *C. intybus* var. *sativum* 'VL52' and *C.endiva* var. *crispum* 'Wallone Despa' protoplasts using an AC of 1.5 V for 40 s for alignment and post-alignment and 2 DC pulses of 40 V for 25 µs for fusion. Boxes indicate (A and B) neighbouring protoplasts before adding DC pulses, (C and D) merging of protoplast membranes due to DC pulses, (E and F) post-alignment.

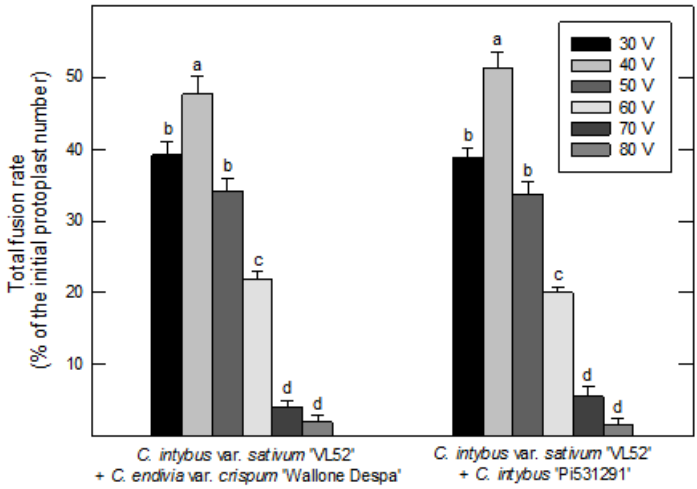


Fig. 3-4 Effect of DC pulse voltage on the total fusion rate of *C. intybus* var. *sativum* 'VL52' + *C. endivia* var. *crispum* 'Wallone Despa' and of *C. intybus* var. *sativum* 'VL52' + *C. intybus* 'Pi531291' protoplasts (% of the initial protoplast number). Data are means ± SE (n = 9, collected from three experiments). a, b, c, d significant differences based on Tukey's Post Hoc test, $p \leq 0.05$. Results were compared within each fusion event for the six DC pulse voltages

Plant regeneration

All fusion products were cultured in low melting point agarose beads. The protoplasts obtained after chemical fusion showed the first cell divisions after 3 days and four-cell stages after 5 days. Microcolonies formed after two weeks. Within two months, calli developed and were screened. All fusion combinations regenerated plantlets. Protoplasts of parental, non-fused *C. intybus* var. *sativum* 'VL52' and *C. intybus* 'Pi531291' showed full plantlet regeneration; protoplasts of *C. endivia* var. *crispum* 'Wallone Despa' did not regenerate in the applied light conditions.

Protoplasts after electrical fusion showed an average of first division rates of 15% of the initial number of cultivated protoplasts. However, they were not able to sustain cell division and subsequently died.

Hybrid screening

Because chicory and endive nuclei contain a different amount of DNA, flow cytometric analysis could easily distinguish *C. intybus* var. *sativum* 'VL52' and *C. endivia* var. *crispum* 'Wallone Despa'. Consequently, the peak values of hybrid fusion products obtained after

symmetric fusion between chicory and endive is the sum of the peak values of both parents (Fig. 3-5). Flow cytometric analysis of regenerating calli produced 17.8 ± 3.2% somatic hybrids containing the full genomes of both parents. In total, 23.9 ± 1.4% and 58.3 ± 1.4% of the regenerants were tetraploid and diploid *C. intybus* var. *sativum* 'VL52', respectively. Moreover, two calli showed hexaploid peak values. No regeneration of *C. endivia* var. *crispum* 'Wallone Despa' occurred. Microsatellite analysis confirmed the data obtained by flow cytometry (an example of the microsatellite outcome is shown in Table 3-2).

Due to the comparable DNA content of the fusion partners, flow cytometry could not distinguish putative fusion products of *C. intybus* var. *sativum* 'VL52' and *C. intybus* 'Pi531291'. Microsatellite markers demonstrated the presence of 4.2 ± 1.0% hybrids among the *C. intybus* var. *sativum* 'VL52' + *C. intybus* 'Pi531291' regenerants (an example of the microsatellite outcome is shown in Table 3-2). Regeneration rates for *C. intybus* var. *sativum* 'VL52' were 8.5 ± 0.8% tetraploid and 40.2 ± 1.3% diploid. In contrast, 5.2 ± 1.1% tetraploid and 41.9 ± 1.5% diploid *C. intybus* 'Pi531291' were obtained.

Table 3-2 Part of the microsatellite analysis of the parents and 11 putative hybrid regenerants after fusion of *C. intybus* var. *sativum* 'VL52' and *C. endivia* var. *crispum* 'Wallone Despa' and of *C. intybus* var. *sativum* 'VL52' and *C. intybus* 'Pi531291' (This table serves as an example and doesn't contain all the microsatellite analysis performed)

Microsatellite markers

Parents	GA118				GA187				GA014				GA019				GA304				GA386				GA495				GA519(2)				GA571			
C. endivia var. *crispum* 'Wallone Despa'	109y				154				138				338				220				140				201				187				201			
C. intybus var. *sativum* 'VL52'	178	180			161	165			130				352				244				151	161			235				191				213			
C. intybus 'Pi531291'	170	172			1	1			120	122			314	330			224	232			142	147			237	238			173	183			223	225		
Putative hybrids																																				
ILVO01z	109	178			154	161			130	138			338	352			220	244			140	151			201	235			187	191			201	213		
ILVO02	109	178			154	161			130	138			338	352			220	244			140	151			201	235			187	191			201	213		
ILVO03	109	178			154	161			130	138			338	352			220	244			140	151			201	235			187	191			201	213		
ILVO04	109	178			154	165			130	138			338	352			220	244			140	151	161		201	235			187	191			201	213		
ILVO05	109	178			154	165			130	138			338	352			220	244			140	151	161		201	235			187	191			201	213		
ILVO06	109	178			154	161	165		130	138			338	352			220	244			140	151	161		201	235			187	191			201	213		
ILVO07	109	178			154	161	165		130	138			338	352			220	244			140	151	161		201	235			187	191			201	213		
ILVO08	178				161	165			130				352				244				151	161			235				191				213			
ILVO09	178				161	165			130				352				244				151	161			235				191				213			
ILVO10	109	180			154	161			130	138			338	352			220	244			140	151			201	235			187	191			201	213		
ILVO11x	1w				1				120	122	130		315	332	352		225	232	244		142	147	151	161	234	235			173	183	191		213	223	225	

Microsatellite markers

Parents	GA395				TGA001				GA007				GA036				GT082				GT082-X				GA355				GA075				GA357			
C. endivia var. *crispum* 'Wallone Despa'	193				1				1				1				1				1				1				1				1			
C. intybus var. *sativum* 'VL52'	207	228			329	332			218				179				190				120				214				219				180			
C. intybus 'Pi531291'	207	244			302	329			218				165	179			190	200			1				202	208			205	215			200	214		
Putative hybrids																																				
ILVO01	193	207			1	332			1	218			1	179			1	190			1	120			1	214			1	219			1	180		
ILVO02	193	207			1	332			1	218			1	179			1	190			1	120			1	214			1	219			1	180		
ILVO03	193	207	228		1	332			1	218			1	179			1	190			1	120			1	214			1	219			1	180		
ILVO04	193	207	228		1	332			1	218			1	179			1	1			1	1			1	214			1	219			1	180		
ILVO05	193	207			1	332			1	218			1	179			1	1			1	1			1	214			1	219			1	180		
ILVO06	193	207	228		1	332			1	218			1	179			1	190			1	120			1	214			1	219			1	180		
ILVO07	193	207	228		1	332			1	218			1	179			1	190			1	120			1	214			1	219			1	180		
ILVO08	207	228			332				218				179				190				120				214				219				180			
ILVO09	207	228			332				218				179				190				120				214				219				180			
ILVO10	193	207			1	332			1	218			1	179			1	190			1	120			1	214			1	219			1	180		
ILVO11	207	207	228	244	302	329	332		218	218			1				1				1				202	208	214		203	215	219		1			

w The absence of a signal is symbolized 1

x ILVO011 is a putative hybrids from PEG-mediated symmetric fusion between *C. intybus* var. *sativum* 'VL52' and *C. intybus* 'Pi531291'

y A genotype is characterized as the base pair length for a certain microsatellite marker. One number represents homozygosity, two numbers heterozygosity for this marker. Each genotype is characterized by 18 microsatellite markers.

z ILVO001-ILVO010 are putative hybrids from PEG-mediated symmetric fusion between *C. intybus* var. *sativum* 'VL52' and *C. endivia* var. *crispum* 'Wallone Despa'

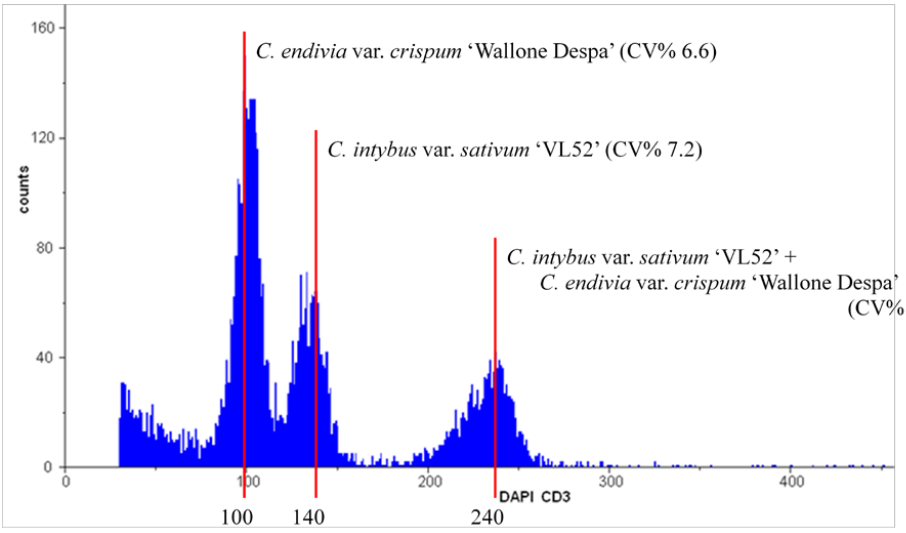


Fig. 3-5 Flow cytometric analysis of the two fusion partners *C. intybus* var. *sativum* 'VL52', *C.endiva* var. *crispum* 'Wallone Despa' and their somatic hybrid (*C. intybus* var. *sativum* 'VL52' + *C.endiva* var. *crispum* 'Wallone Despa').

3.4 Discussion

In this study, intra- and interspecific somatic hybrid *Cichorium* plantlets could be obtained. We compared the two main somatic hybridization tools, electrical and chemical protoplast fusion, for the fusion of *Cichorium* species: industrial chicory *C. intybus* var. *sativum* 'VL52' protoplasts fused with endive *C. endivia* var. *crispum* 'Wallone Despa' protoplasts and with wild type chicory *C. intybus* 'Pi531291' protoplasts. Protoplasts were successfully fused using both the PEG-mediated chemical fusion and electrical fusion. Optimal electrical fusion conditions resulted in more total fusion events than could be induced through chemical fusion. These findings support the review of Jansky (2006). However, they are in contrast with former research describing higher fusion frequencies after chemical fusion (Hidaka and Omura, 1992; Assani et al., 2005). Due to the absence of fluorescent markers during electrical fusion, no clear comparison of the binary heterofusion rate could be made between chemical and electrical fusion. However, a larger proportion of undesired hybrids with higher ploidy levels is expected by electrical fusion (Jansky, 2006). The highest binary heterofusion rate obtained in this study was 8.4 ± 1.3%. Guan et al. (2010) found binary heterofusion rates up

to 4.2 ± 0.9% with PEG MW 6000 in ginger protoplasts. Rezazadeh et al. (2011) obtained up to 18% of binary fusions when using PEG MW 3000-3700 (Sigma-Aldrich) mediated protoplast fusion for intraspecific somatic hybridization in mango. Assani et al. (2005) observed an average binary fusion rate of 10% after electrical fusion and 17% after PEG-mediated fusion. However, these percentages included heterokaryon as well as homokaryon binary fusions. We noted a decrease in (total and binary) fusion frequencies with an increasing incubation time in PEG MW 3350 (Table 3-1). This is probably due to the decreasing viability of the protoplasts when longer incubation times were applied (Fig. 3-1). Guan et al. (2010), however, described a higher binary fusion rate as the fusion time was prolonged in both 15% PEG MW 4000 and PEG MW 6000. A decrease was only observed at a prolonged fusion time when 30% PEG MW 4000/6000 was used. By contrast, the multifusion rate increased with prolonged fusion time, however, serious protoplast damage occurred when applying an incubation time of more than 30 min. The differences between their observations and our results might indicate that protoplast sensitivity towards the toxicity of PEG is species dependent.

Chemical fusion enabled sustained division of protoplasts, while electrical fusion inhibited protoplast development. This is in accordance with findings of protoplast fusion in ginger, where the protoplast viability 24 h after electrical fusion was lower than after PEG-mediated fusion (Guan et al., 2010). Nonetheless, electrical fusion has already been reported as a more efficient method in terms of plant regeneration due to the cytotoxicity of PEG (Assani et al., 2005; Olivares-Fuster et al., 2005; Rezazadeh et al., 2011). Moreover, Assani et al. (2005) stated that an electric field pulse technique stimulated the somatic embryogenesis and mitotic activities. The negative effect of electrical fusion found in this study is possibly due to toxic effects of the iso-osmotic buffer used for electrical fusion. The sensitivity of cells towards electric pulses is highly variable. In this study, electrical fusion parameters were optimized in the micro-fusion chamber, then applied in the helix fusion chamber. The optimal conditions for protoplast viability might therefore have changed. Another disadvantage of electrical fusion compared to chemical fusion is the inability to analyze binary heterofusion formations between the same cell types.

It may be interesting to note that a novel protoplast fusion method, electrochemical fusion, which combines the advantages of both methods, can overcome the abovementioned problems. This alternative procedure is based on chemically induced protoplast aggregation and DC pulse-promoted membrane fusion. Classical chemical fusion protocols required two to three washes to remove the cytotoxic PEG. Removal of PEG in the novel technique is

simpler because of its low concentration. Together with the DC pulse-promoted membrane fusion, high yields in symmetric somatic hybrids and cybrids have already been obtained in *Citrus* (Olivares-Fuster et al., 2005).

Putative hybrids of the *C. intybus* var. *sativum* 'VL52' and *C. endivia* var. *crispum* 'Wallone Despa' fusion were screened using flow cytometry. Flow cytometry has been used in previous studies to analyze ploidy levels of somatic hybrids after both electrical and chemical fusion (Sun et al., 2004; Guan et al., 2010). The flow cytometric analysis is fast, easy and does not require full plantlet regeneration. However, integration of one or two chromosomes or of small chromosomal fragments, which lead to asymmetric hybrids, are not always visible through flow cytometry. Therefore, flow cytometry can only be used for screening alleged symmetric hybrids between parents with a distinguishable DNA content. *In situ* hybridizations, karyotype analysis and molecular markers are convenient tools to obtain more detailed information about the hybrid genomic constitution.

The chemical fusion between *C. intybus* var. *sativum* 'VL52' and *C. endivia* var. *crispum* 'Wallone Despa' produced up to 18% somatic hybrids. Cappelle et al. (2007) fused protoplasts of two *C. endivia* varieties with *C. intybus* var. Cassel protoplasts according to a modified method of Kao (1982) based on PEG-mediated fusion. That study yielded 288 regenerants, including 192 (77.42%) hybrid types with intermediate leaf shapes. However, not all of these hybrid types were tetraploid, suggesting the presence of asymmetric hybrids. Likewise, in our study, no plant was regenerated from the two *C. endivia* parents. In addition to somatic hybrids, we observed more than 20% tetraploid *C. intybus* var. *sativum* 'VL52' among the regenerants. Rambaud et al. (1992) noted similar results when inducing tetraploidy by chemical protoplast fusion in *C. intybus* var. Magdebourg. The fusion of *C. intybus* var. *sativum* 'VL52' with *C. intybus* 'Pi531291' produced up to 4% somatic hybrids. This lower percentage can be contributed to the higher regeneration capacities of the parental wild type *C. intybus* 'Pi531291' compared to the endive in the abovementioned fusion, leading to a lower percentage of somatic hybrids among the regenerants.

In this study for the first time protoplasts of genotypes of industrial chicory (*C. intybus* var. *sativum* 'VL52'), wild chicory (*C. intybus* 'Pi531291') and endive (*C. endivia* var. *crispum* 'Wallone Despa') were successfully fused using electrical fusion. These findings can contribute to the further development of electrical somatic hybridization within *Cichorium* species. However, further research is needed to develop a protoplast regeneration protocol after electrical fusion. In this study, PEG-mediated chemical fusion has confirmed its value as

a protoplast-based breeding tool. The development of the somatic hybridization technique offers an alternative for interspecific *Cichorium* breeding.

3.5 Conclusion

Somatic hybridization may provide an alternative for interspecific sexual *Cichorium* breeding. To develop an efficient breeding tool, the protoplast fusion conditions must be optimized. We have evaluated the effect of chemical and electrical protoplast fusion on symmetric somatic hybrid production in *Cichorium* species.

Protoplasts of industrial chicory (*C. intybus* var. *sativum* 'VL52') were successfully fused electrically as well as chemically with wild chicory (*C. intybus* 'Pi531291') and endive (*C. endivia* var. *crispum* 'Wallone Despa') protoplasts. Binary heterofusion could only be evaluated after chemical fusion; rates up to 8% were achieved. The highest total fusion rates (up to 51.2 ± 2.3%) were obtained after electrical fusion, whereas chemical fusion induced a fusion rate of 16.6 ± 1.2%. However, regeneration was only established after chemical fusion. Out of the regenerants after industrial chicory and endive fusion, 18% were somatic hybrids. Fusion of industrial chicory with wild chicory produced on average 4% hybrid regenerants.

Our findings can contribute to the research needed to develop a protoplast regeneration protocol after electrical fusion. The methods for symmetric protoplast fusion described in this study can contribute to advanced asymmetric protoplast fusion experiments in *Cichorium* species.

Chapter 4 - Chromosome fragmentation and cytoplasm inactivation in *Cichorium* species

4.1 Introduction

Symmetric protoplast fusion combines desirable as well as undesirable traits of different parents, often leading to a disturbed regeneration capacity or fertility of the somatic hybrid. By reducing the amount of transmitted nuclear and/or cytoplasmic information through asymmetric protoplast fusion, these problems can possibly be overcome. To create cybrids, only the cytoplasm from a donor after complete fragmentation of its nucleus is transmitted to a cytoplasmic inactivated recipient. Several techniques can be used for donor nuclear genome fragmentation, but UV treatment is currently the most widely applied because of its easy application and high reproducibility (Hall et al., 1992). Within a single species susceptibility towards UV radiation can strongly differ (Wang et al., 2012). A general problem is the difficult quantification of DNA damage after an irradiation treatment. Denaturing (alkaline) gel electrophoresis and pulse field gel electrophoresis (PFGE) were used by Hall et al. (1992). Abas et al. (2007) used the Comet assay Single Cell Gel Electrophoresis (SCGE) to observe DNA strand breaks in protoplasts of *Nicotiana plumbaginifolia*. However, the DNA loading densities seemed critical for reproducible results with these techniques. Most of the DNA analysis is performed after partial or full plantlet regeneration using molecular techniques. Next to DNA analysis, the negative effect of UVC irradiation on several cell processes including cell wall regeneration, protoplast viability and cell division are observed (Navratilova et al., 2008).

Metabolic inhibitors, as iodoacetamide (IOA) inhibit protoplast division by inactivating the cytoplasm and are suitable for the selection of somatic hybrids in plant cells (Iriawati et al., 1996). Fusion of IOA-treated recipient parental protoplasts with irradiated donor protoplasts could produce 100% asymmetric fusion products.

In *Cichorium*, successful asymmetric protoplast fusion has already been performed; for the creation of cybrids, Varotto et al. (2001) fused ^{135}Cs γ-rays-irradiated sunflower (*H. annuus*) protoplasts (2n = 34) and iodoacetate-treated red chicory protoplasts (2n = 18). Out of 33 regenerants, 6 had a chromosome number ranging from 30 to 36. Among the remainder, all containing the chromosome number of chicory, two regenerants showed mtDNA of both parents, and two others showed the hybridization pattern of sunflower mtDNA or recombination of the mt genomes of *C. intybus* and *H.annuus*, respectively. Cybrids of *C. intybus* and sunflower have also been obtained through symmetric protoplast fusion by Rambaud et al. (1993). Some regenerants showed the incorporation of sunflower mtDNA into

chicory mtDNA. No sunflower chromosomes were found in these regenerants, suggesting the elimination of the sunflower chromosomes during mitosis.

As described in previous studies, a large variability exists among plant species and cultivars in their sensitivity to UV radiation (Teramura and Sullivan, 1994). The main goal of this study was to analyze the UVC sensitivity of *Cichorium* species that can be used as donor plants in further asymmetric protoplast research to obtain CMS in *Cichorium*. We therefore evaluated *Cichorium* protoplast viability, cell wall resynthesis and protoplast regeneration after UVC irradiation. The quantification of DNA damage after an irradiation treatment was measured using SCGE (Comet assay) and standard gel electrophoresis. To analyze the IOA sensitivity of recipient industrial chicory cultivars, we evaluated *Cichorium* protoplast viability and regeneration after IOA treatment.

4.2 UV irradiation

4.2.1 Materials and methods

Plant material and protoplast isolation

Seeds of one wild type chicory (*C. intybus* 'Pi531291') from Hungary and one endive (*C. endivia* var. *crispum* 'Wallone Despa') were used in the experiments. *In vitro* plant production, protoplast isolation and culture were performed as described in §Chapter 2.

UVC irradiation

After protoplast isolation, 2 ml of protoplast solution (5 x 10^5 protoplasts ml^{-1} MC_2 [Table 2-1]) were dispersed in 90 mm petri dishes and irradiated by a UVC germicidal lamp (G30T8/OF of Sylvania, UVC output of 13.4 W at 254 nm) at a dose rate of approximately 170 µW cm^{-2} during 0, 1, 2, 4, 6, 10, 15, 20 and 30 min. Protoplasts were then washed with MC_2 (Table 2-1), centrifuged (100 g, 10 min) and resuspended in either MC_1 or MC_2 (Table 2-1); to analyze protoplast viability and cell wall resynthesis, 3 ml of protoplast solution (5 x 10^4 protoplasts ml^{-1} MC_1) was cultured in 50 mm petri dishes under complete dark conditions. To analyze protoplast regeneration, protoplasts resuspended in MC_2 were cultured in low melting point agarose (LMPA) beads following §Chapter 2, under complete dark conditions.

Microscopic analysis of protoplast viability and cell wall resynthesis

Microscopic detection was carried out using an inverted fluorescence microscope (Leica DMIRB) equipped with a Leica Camera System (Leica DFC320). For protoplast viability staining, 100 µl protoplast solution of the liquid cultures was mixed with 1 µl of a 0.5 % (w/v) fluorescein diacetate (FDA) stock solution (5 mg FDA dissolved in 1 ml acetone), incubated for 10 min at room temperature, rinsed and observed through excitation by 485 nm light. Observations were made directly after UV irradiation and after 5 days of culture in liquid medium. Cell wall regeneration was studied by using Calcofluor White M2R (CFW) (Sigma-Aldrich). 100 µl protoplast solution of the liquid cultures were mixed with a stock solution of CFW resulting in a final dye concentration of 0.01% (w/v), rinsed and observed through excitation by 360 nm. Cell wall resynthesis was determined 5 days after protoplast culture in liquid medium. For both protoplast viability and cell wall regeneration, experiments were repeated 3 to 4 times. Data were collected from 2 to 3 petri dishes per experiment and per treatment.

Analysis of protoplast regeneration after UVC irradiation

Protoplast regeneration was evaluated in the LMPA beads. After 7 days, the formation of four-cell stages was analyzed. After 14 days, the microcolony development was determined. Experiments were repeated 3 to 5 times. Data were collected from 2 to 3 petri dishes per experiment and per treatment.

Evaluation of UVC damage using Single Cell Gel Electrophoresis (SCGE) and standard gel electrophoresis

Ssbrs and dsbs were evaluated using SCGE (Comet assay). SCGE measures DNA damage from individual protoplasts based on the migration of denatured DNA through an electrophorectic field. SCGE was performed following manufacturer's instructions (USB products, Affymetrix, Single Cell Gel Electrophoresis Comet Assay, www.usbweb.com). One side of microscope slides were coated with 1.5% molten agar and dried. Control and UVC irradiated protoplasts were diluted in 1 ml 1x phosphate buffered saline (PBS) solution, centrifuged at 100 g for 5 min at 4°C and resuspended in 1x PBS at a concentration of approximately 25,000 cells ml^{-1}. The protoplast suspension (5 µl) was mixed with 35 µl liquified LMPA 0.8% (w/v) solution. The LMPA-embedded protoplast mixture was placed on a coated microscope slide and covered with a cover slip to allow gel formation. After removal of the cover slips, the slides were treated for 1 h at 4°C with lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris-HCl, pH 10) to expose DNA. The slides were washed 3 times with MQ water, before treated with denaturation solution (300 mM NaOH, 1 mM EDTA, pH > 13) for 20 min at 4°C. The slides were washed 3 times with MQ water and electrophoresed for 3, 4, 5, 10 and 15 min at 25 V and visualized by ethidium bromide (EtBr) under a fluorescence microscope.

For standard gel electrophoresis, DNA from control and UVC irradiated protoplasts was isolated using the Qiagen DNeasy Plant Tissue Mini kit, according to the manufacturer's instructions. The DNA concentration and quality was analyzed by an Eppendorf Nanodrop ND-1000 spectrophotometer. Samples were diluted to a 250 and 500 ng DNA $µl^{-1}$. Gel electrophoresis was performed at 125 V in 1x tris-acetate-EDTA (TAE) buffer during no longer than 3 h due to heating of the agarose gels (0.5, 1, 1.5% agarose in 1x TAE (w/v)). A mixture of 10 µl DNA and 2 µl loading dye (Thermo Scientific Fermentas 6x DNA Loading Dye) were loaded on the gel for each sample. Because of the absence of alkalic conditions, this technique only reveals the presence of dsbs in DNA molecules.

Statistical analysis

One-way analysis of variance (ANOVA) and Tukey's Post-Hoc test were used to analyze the effect of different incubation times with UVC irradiation on the protoplast viability and cell wall resynthesis in liquid culture and four-cell stage and microcolony formation in the LMPA beads for both *C. intybus* 'Pi531291' and *C. endivia* var. *crispum* 'Wallone Despa'. All calculations were obtained using the statistical software package Statistica v.11.

4.2.2 Results

Microscopic analysis of protoplast viability and cell wall resynthesis

Protoplast viability observed directly after UVC irradiation, decreased stepwise with longer incubation times. After 4 min of UVC irradiation, the viability of both *C. intybus* 'Pi531291' and *C. endivia* var. *crispum* 'Wallone Despa' protoplasts decreased significantly. However, still more than 80% of the protoplasts were viable. After 30 min irradiation, the protoplast viability for *C. intybus* 'Pi531291' and *C. endivia* var. *crispum* 'Wallone Despa' dropped below 70% and 60%, respectively. After 5 days incubation in liquid medium, the protoplast viability dropped to on average 50% for both *Cichorium* types in the control group. *C. endivia* var. *crispum* 'Wallone Despa' protoplast viability decreased significantly when they had been incubated for 2 min; *C. intybus* 'Pi531291' protoplast viability declined significantly after 6 min incubation. However, a major decrease in protoplast viability for both *Cichorium* types was only observed when 15 min of UVC irradiation was applied (Fig. 4.2-1).

Cell wall resynthesis in the control group of both *Cichorium* types was observed during 24-48 h after protoplast isolation. Cell wall formation of *C. intybus* 'Pi531291' protoplasts decreased more than 20 % after 5 days of liquid culture when 1 min irradiation had been applied. *C. endivia* var. *crispum* 'Wallone Despa' protoplasts formed 7 % less cell walls after 1 min and 13 % less cell walls after 2 min irradiation (Fig. 4.2-2).

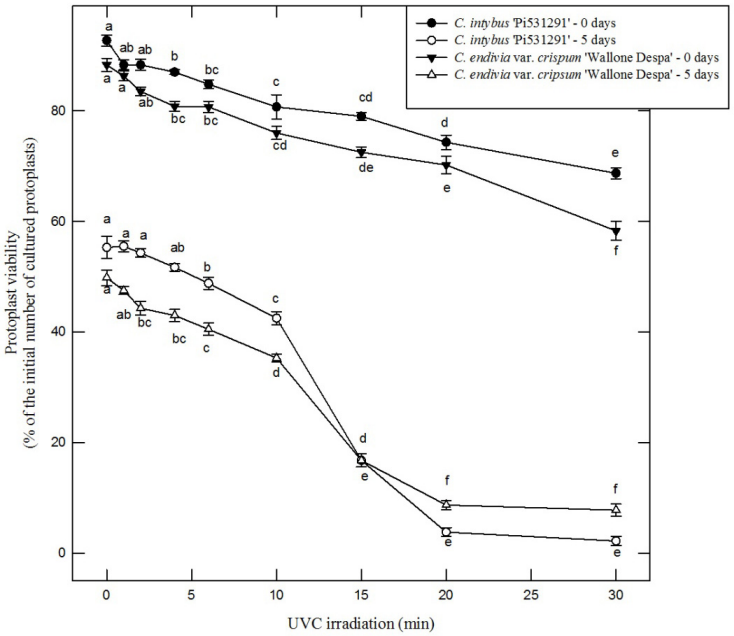


Fig 4.2-1 Protoplast viability (% of the initial number of cultured protoplasts) of two *Cichorium* species (*C. intybus* 'Pi531291' and *C. endivia* var. *crispum* 'Wallone Despa') after different UVC irradiation incubation times (0, 1, 2, 4, 6, 10, 15, 20 and 30 min), measured directly after irradiation (0 days) and after 5 days of protoplast culture. Data are means ± SE (n = 10, collected from three to four experiments). a, b, c, d, e, f are significant differences based on Tukey's Post Hoc test, $p \leq 0.05$. Results compared within each *Cichorium* type after 0 and 5 days of protoplast culture.

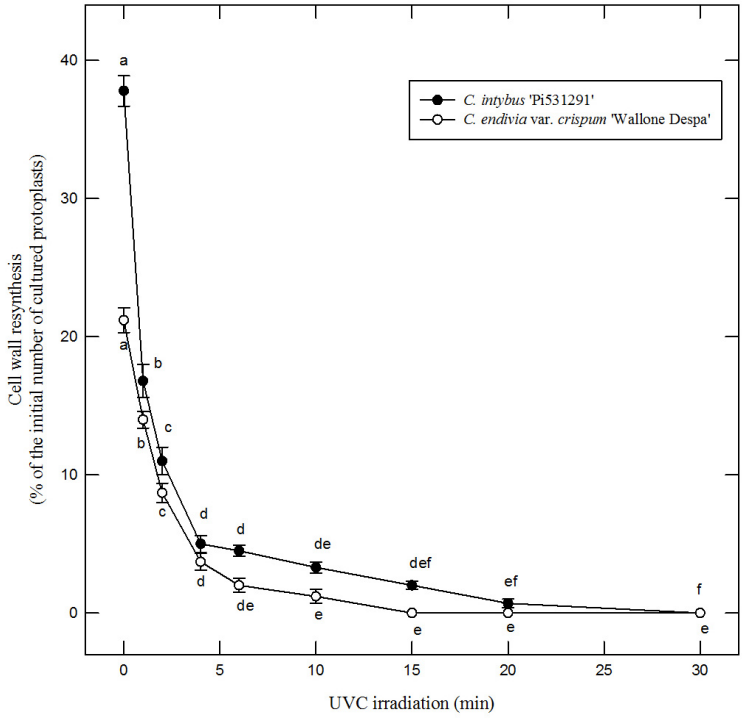


Fig. 4.2-2 Cell wall resynthesis (% of the initial number of cultured protoplasts) of two *Cichorium* species (*C. intybus* 'Pi531291' and *C. endivia* var. *crispum* 'Wallone Despa') after different UVC irradiation incubation times (0, 1, 2, 4, 6, 10, 15, 20 and 30 min), measured after 5 days of protoplast culture. Data are means ± SE (n = 10, collected from three to four experiments). a, b, c, d, e, f are significant differences based on Tukey's Post Hoc test, $p \leq 0.05$. Results compared within each *Cichorium* type.

Analysis of protoplast regeneration after UVC irradiation

Four-cell stage formation after 7 days culturing in LMPA beads significantly dropped after 2 min of irradiation for *C. intybus* 'Pi531291' protoplasts, and 6 min of irradiation resulted in less than 5% of four-cell stage formation. Microcolony formation after 14 days significantly diminished after 2 min of irradiation. After 6 min of irradiation only 14% formed microcolonies, compared to 55% in the control group (Fig. 4.2-3).

Four-cell stage formation after 7 days of *C. endivia* var. *crispum* 'Wallone Despa' protoplasts significantly decreased after 4 min irradiation. After 6 min irradiation, the protoplast regeneration rate dropped under 1%. Microcolony formation after 14 days was significantly reduced when the endive protoplasts had been UVC irradiated for 4 min (Fig. 4.2-4).

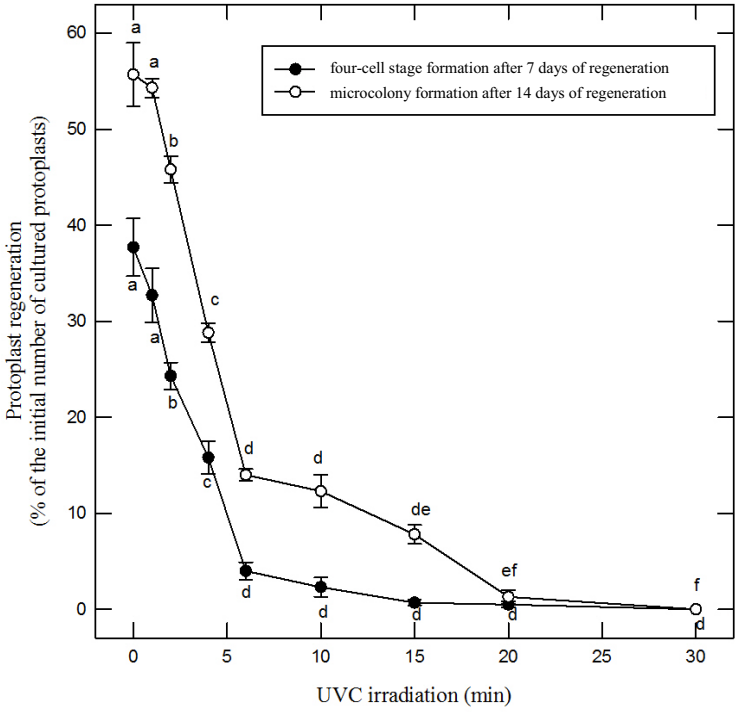


Fig. 4.2-3 Four-cell stage and microcolony formation (% of the initial number of cultured protoplasts) of *C. intybus* 'Pi531291' protoplasts after different UVC irradiation incubation times (0, 1, 2, 4, 6, 10, 15, 20 and 30 min). Data are means ± SE (n = 10, collected from three to five experiments). a, b, c, d, e, f are significant differences based on Tukey's Post Hoc test, $p \leq 0.05$. Results compared within four-cell stage formation and microcolony formation phase.

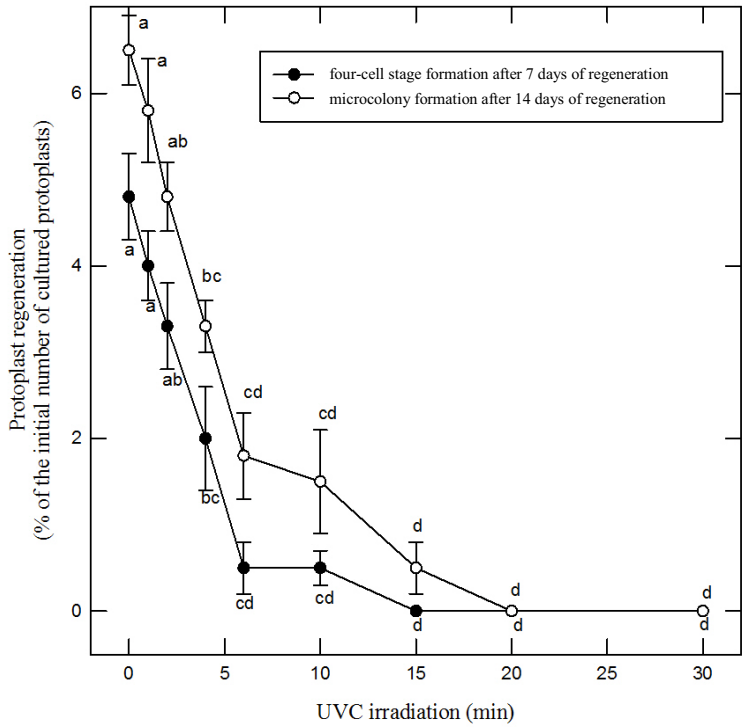


Fig. 4.2-4 Four-cell stage and microcolony formation (% of the initial number of cultured protoplasts) of *C. endivia* var. *crispum* 'Wallone Despa' protoplasts after different UVC irradiation incubation times (0, 1, 2, 4, 6, 10, 15, 20 and 30 min). Data are means ± SE (n = 10, collected from three to five experiments). a, b, c, d are significant differences based on Tukey's Post Hoc test, $p \leq 0.05$. Results compared within four-cell stage formation and microcolony formation phase.

Evaluation of UVC damage using SCGE and standard gel electrophoresis

Using SCGE, damaged DNA containing strand breaks will migrate further in the gel than intact DNA, creating an image similar to a comet (core and tail). Dependent on the amount of migrated DNA, the tail length will vary. The absence of a comet core would indicate the absence of large DNA parts, and therefore, would prove the positive effect of UVC on chromosome fragmentation. However, SCGE analysis of our control and UVC irradiated protoplasts delivered no clear results. No comet signals were obtained from UVC irradiated protoplasts. Nuclei of control protoplast samples were only slightly visible using this technique.

The standard gel electrophoresis showed no clear distinction in DNA damage between control and UVC irradiated protoplasts. Only UVC irradiation of 60 min revealed a decreased

intensity of the EtBr-stained DNA in comparison to the control and 10, 20 and 30 min irradiated protoplasts (Fig. 4.2-5). The loading density (250 versus 500 ng DNA μl^{-1}) and the agarose concentration in the gel did not influence the results.

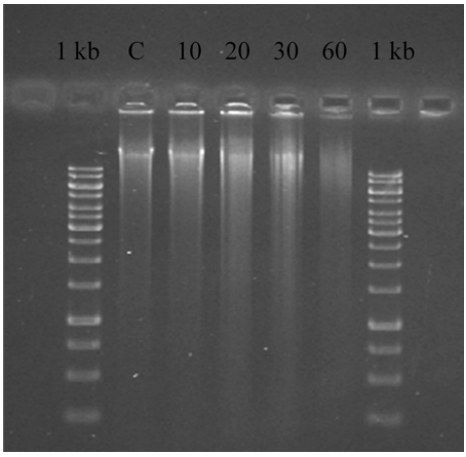


Fig. 4.2-5 The effect of UV dose on the mobility of *C. intybus* 'Pi531291' protoplasts DNA using gel electrophoresis (0.5% agarose) and EtBr staining. C = control. 10, 20, 30 and 60 refer to the min of UVC irradiation. 1 kb ladders were used as reference.

4.2.3 Discussion

Preliminary studies were performed to analyze UVC irradiation dosage effects on *Cichorium* protoplasts to be used in prospective asymmetric fusion experiments. *C. intybus* and *C. endivia* protoplasts were submitted to 170 $\mu W\ cm^{-2}$ UVC irradiation during 0, 1, 2, 4, 6, 10, 15, 20 and 30 min. The protoplast viability measured directly after irradiation was not severely hampered. Even after longer irradiation times (15, 20 and 30 min) more than 55% of the endive protoplasts and more than 65% of the chicory wild type protoplasts showed active cytoplasmic activity as determined by FDA staining. The higher decrease of protoplast viability after long irradiation times could be mainly due to the evaporation of water from the liquid MC_2 medium, leading to lethal dehydration. After 5 days liquid medium culture, protoplast viability clearly decreased, even in the control group. The viability of *C. intybus* 'Pi531291' protoplasts decreased from 92% directly after irradiation to 55% after 5 days. For *C. endivia* var. *crispum* 'Wallone Despa' protoplasts, a decline was observed from 88% directly after irradiation to 50% after 5 days of incubation. This can probably be ascribed to the unfavorable protoplast regeneration conditions for *Cichorium* protoplasts (Deryckere et

al., 2012); Liquid culture systems showed to be less appropriate for protoplast development than the LMPA bead culture system. Therefore, an overestimation of the UVC effect on protoplast viability (and cell wall formation) can be expected. For both *Cichorium* types, the protoplast viability of the control group and the irradiated protoplasts after 5 days culture showed a minor gradual decrease when the protoplasts had been irradiated up to 10 min. Irradiation during 15 min or more caused a drop in protoplast viability. The high protoplast viability rate after UVC irradiation is in accordance with previous studies on sugar beet protoplasts (Hall et al., 1992). Sugar beet protoplasts irradiated at the highest UV dose (4200 J m^{-2}) showed comparable viability rates as the untreated control cells after 6 days culture. The treated protoplasts, however, died after 14 days of culturing. High viability rates of *Cucumis sativus* protoplasts after UVC irradiation with a germicidal lamp during 10 min were also observed. These irradiated *Cucumis* protoplasts survived for 2 weeks and could be used in asymmetric somatic hybridizations (Navratilova et al., 2008).

Cell wall formation was heavily inhibited by UVC irradiation in both *Cichorium* types. 1 min of irradiation was sufficient to cause a significantly high decrease of cell wall resynthesis; 6 min of irradiation reduced the cell wall formation rate to less than 5%. These results are comparable with observations made by Hall et al. (1992). Cell wall resynthesis of sugar beet protoplasts showed a dose-dependent inhibition. Moreover, applying a short period of illumination during protoplast regeneration restored cell wall formation in cultures irradiated at the lowest doses (700 J m^{-2}). At higher UV doses, no restoration was observed. Navratilova et al. (2008) suggested that enzymes participating in cell wall regeneration are encoded by nuclear DNA. As a result of UVC damage, the expression of these enzymes is heavily disturbed.

Regeneration of *C. endivia* var. *crispum* 'Wallone Despa' protoplasts was already described in Deryckere et al. (2012). Compared to *C. intybus* protoplasts, *C. endivia* protoplasts are more recalcitrant, leading to a lower protoplast regeneration rate. This made it difficult to analyze the influence of UVC irradiation on endive protoplast regeneration. However, UVC irradiation caused a dose-dependent significant decrease in four-cell stage and microcolony formation. A comparable result was obtained for *C. intybus* 'Pi531291' protoplasts. Because of the higher regeneration capacity of *C. intybus* protoplasts, a more profound distinction between the control and irradiated culture was obtained. The increased percentage of microcolonies after 14 days in comparison to the percentage of cells in the four-cell stage after 7 days can be attributed to the refreshment of medium as suggested in Deryckere et al. (2012). Another suggestion is the reversion towards cell division through the repair of

damaged DNA. Light dependent and independent repair processes can undo the effects of UV-induced DNA damage (Stapleton, 1992). The aforementioned beneficial effect of a short illumination period, inducing repair mechanisms, during protoplast regeneration that restored cell wall formation in UV-irradiated cultures, did not restore cell division (Hall et al., 1992).

The visualization of the DNA damage performed in this study did not yield valuable results. The use of SCGE was useful in the UV damage evaluation in *N. plumbaginifolia* protoplasts (Abas et al., 2007). However, in our study, no clear comets could be obtained. When comets were obtained in UVC-treated protoplasts, no clear quantification could be made based on tail moment, tail length and percentage of migrated DNA, because of the poor visualization quality. A possible explanation for the failure of the technique in our study is that the comet assay is not able to detect small DNA fragments (smaller than 50 kb) since they are mostly washed during the lysis and electrophoresis steps (Olive, 1999). The results obtained by the standard gel electrophoresis are also not valuable for UVC-induced DNA damage quantification. Although a slight difference was observed when performing 60 min of UVC irradiation, no clear quantification could be made. This is partly due to the relative short electrophoresis time of 3 h compared to the 6 days used in Hall et al. (1992). Possibly, fragments could be visualized when performing longer electrophoresis times, however, due to agarose gel melting, this was not possible in our laboratory.

The problem of DNA damage visualization after UVC-irradiation during 10, 20 and 30 min is in contrast of the results obtained with the protoplast regeneration experiments, through which became clear that UVC irradiation had already profound effects after short incubation of the protoplasts on the cell wall formation, which was significantly hindered after 1 min UVC irradiation. UVC irradiation has profound effects on the cell's mechanism already. Further research should focus on the effective rearrangements in the cell, not only on DNA levels, but also on RNA and protein levels.

In this study, the effect of UVC irradiation on the regeneration capacities of *Cichorium* protoplasts was tested. UVC irradiation prevented cell wall resynthesis and cell division, probably due to induced DNA damage. No severe toxic effects of UVC irradiation on the cytoplasm of the protoplasts were observed, as protoplast viability was not heavily influenced. Furthermore, the use of UV is cheap, safe and easily applicable in comparison to ionizing radiation. UVC irradiation showed promising properties to obtain asymmetric somatic hybrids in *Cichorium* species. Further research on dose-dependent damage to the DNA structure by UVC irradiation in *Cichorium* protoplasts is needed to quantify the DNA damage. Optimal UVC irradiation parameters to obtain different levels of donor DNA damage are required

when we want to create different asymmetric products: for instance, the formation of chromosome addition lines needs the introgression of only one or few chromosomes from wild or cultivated relatives, whereas in the formation of complete asymmetric cybrids the introgression of nuclear donor DNA is undesired, however, no effect on the donor cytoplasm must be observed. Next to this dose-dependent damage, more research on UVC irradiation-activated cell defence mechanisms and repair systems are needed. Moreover, the use of UVC irradiation in the production of asymmetric fusion products in *Cichorium* species should also be investigated.

4.3 IOA-induced cytoplasmic inactivation

4.3.1 Materials and methods

Plant material

One industrial chicory inbred line, *C. intybus* var. *sativum* 'VL52' (seeds) and one industrial chicory genotype *C. intybus* var. *sativum* 'K1093' (shoots)were used. *In vitro* plant production, protoplast isolation and culture were performed as described in §Chapter 2.

IOA treatment

After isolation, protoplasts (1.5 x 10^5 protoplasts ml^{-1} MC_2) were treated with different IOA concentrations (1, 1.5, 1.625, 1.75, 2 mM IOA dissolved in MC_2) for 20 min at room temperature in dark conditions. After treatment, protoplasts were washed twice with MC_2, centrifuged (100 g, 10 min) and resuspended in either MC_1 or MC_2 (Table 2-1); to analyze protoplast viability, 3 ml of protoplast solution (5 x 10^4 protoplasts ml^{-1} MC_1) was cultured in 50 mm petri dishes under complete dark conditions. To analyze protoplast regeneration, protoplasts resuspended in MC_2 were cultured in low melting point agarose (LMPA) beads at a final density of 5 x 10^4 protoplasts ml^{-1} following §Chapter 2, under complete light conditions.

Analysis of protoplast viability and regeneration after IOA treatment

Microscopic detection of protoplast viability was carried out using FDA, as described in §4.2.1. Observations were made directly after IOA treatment and after 5 days of culture in liquid medium. Protoplast regeneration was evaluated in the LMPA beads. After 4 weeks, the microcalli development was determined. Experiments were repeated 3 to 5 times. Data were collected from 2 to 3 petri dishes per experiment and per treatment.

Statistical analysis

One-way analysis of variance (ANOVA) and Tukey's Post-Hoc test were used to analyze the effect of different IOA concentrations on the protoplast viability in liquid culture and on the microcalli development in the LMPA beads for both industrial chicories. All calculations were obtained using the statistical software package Statistica v.11.

4.3.2 Results

Analysis of protoplast viability and regeneration after IOA treatment

The protoplast viability directly after IOA treatment of is shown in Fig. 4.3-1. No severe viability reduction was seen directly after treatment. For *C. intybus* var. *sativum* 'VL52' protoplasts, only an IOA concentration of 1.75 and 2 mM yielded significantly less viable protoplasts. Parallel observations were made for *C. intybus* var. *sativum* 'K1093' protoplasts: an IOA concentration of 2 mM yielded significantly less viable protoplasts. However, still more than 80 % showed FDA fluorescence at these concentrations for both industrial chicories.

After 5 days liquid medium culture, protoplast viability for both clearly dropped from 90 % to 60 % in the control group. This is probably due to the unfavorable protoplast regeneration conditions for *Cichorium* protoplasts (Deryckere et al., 2012). More than 30 % protoplast viability was observed at 1 mM IOA concentration. IOA treatment at 1.625 mM or higher concentrations yielded less than 10 % of viable protoplasts after 5 days culture (Fig. 4.3-2).

Four weeks after IOA treatment and protoplast culture in LMPA beads, microcalli formation reached 4.4 and 5.3 % for *C. intybus* var. *sativum* 'VL52' and *C. intybus* var. *sativum* 'K1093' control protoplasts, respectively. This is in comparison to results obtained for the same genotypes in Deryckere et al. (2012). IOA treatment with a concentration of 1 and 1.5 mM was not sufficient to prevent cell division, although a significant lower proportion of microcalli were formed. IOA treatment with concentrations of 1.625 and 1.75 yielded almost no divisions. 2 mM IOA concentration totally inhibited cell division (Fig 4.3-3).

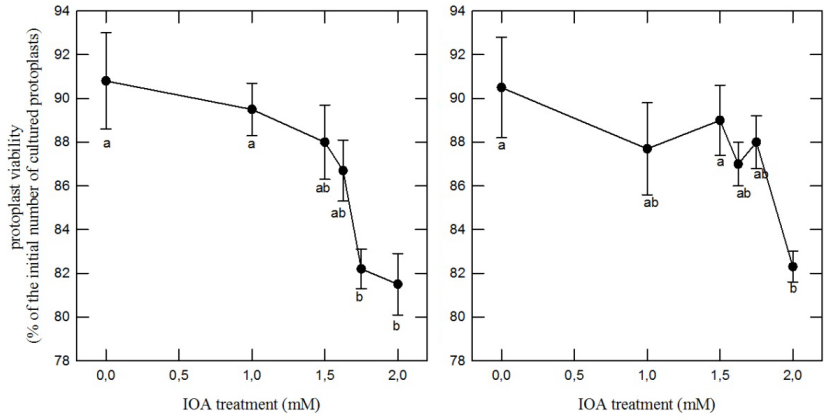


Fig. 4.3-1 Protoplast viability (% of the initial number of cultured protoplasts) Left; *C. intybus* var. *sativum* 'VL52' and right; *C. intybus* var. *sativum* 'K1093' after different IOA treatments (0, 1, 1.5, 1.625, 1.75 and 2 mM), measured directly after treatment. Data are means ± SE (n = 10, collected from three to five experiments). a, b are significant differences within each *Cichorium* type based on Tukey's Post Hoc test, $p \leq 0.05$

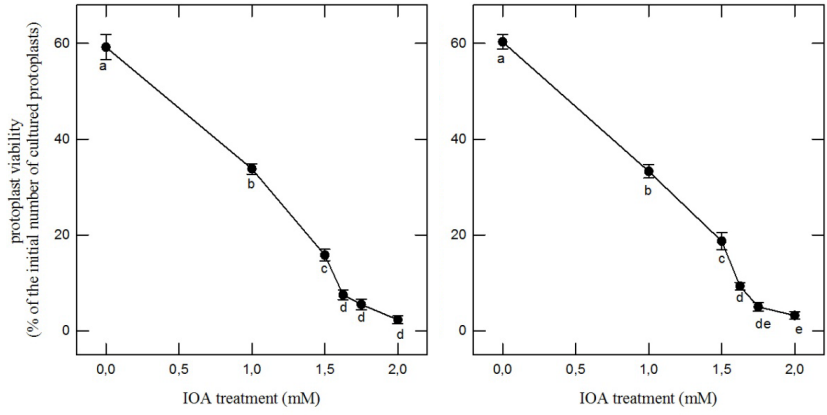


Fig. 4.3-2 Protoplast viability (% of the initial number of cultured protoplasts) Left; *C. intybus* var. *sativum* 'VL52' and right; *C. intybus* var. *sativum* 'K1093' after different IOA treatments (0, 1, 1.5, 1.625, 1.75 and 2 mM), measured after 5 days culture. Data are means ± SE (n = 10, collected from three to five experiments). a, b, c, d, e are significant differences within each *Cichorium* type based on Tukey's Post Hoc test, $p \leq 0.05$

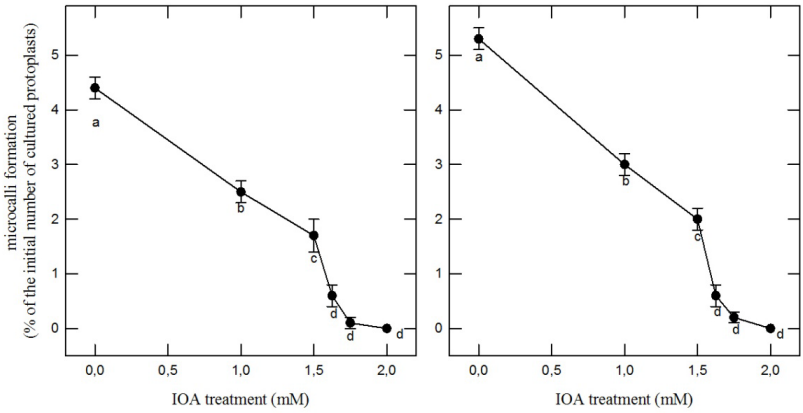


Fig. 4.3-3 Microcalli formation (% of the initial number of cultured protoplasts) Left; *C. intybus* var. *sativum* 'VL52' and right; *C. intybus* var. *sativum* 'K1093' after different IOA treatments (0, 1, 1.5, 1.625, 1.75 and 2 mM), observed after 4 weeks culture. Data are means ± SE (n = 10, collected from three to five experiments). a, b, c, d are significant differences within each *Cichorium* type based on Tukey's Post Hoc test, $p \leq 0.05$

4.3.3 Discussion

We observed that protoplast viability after 5 days culture and protoplast regeneration was heavily affected after IOA treatment of 1.625 mM and higher. However, an overestimation of the IOA effect on protoplast viability is most likely due to the used liquid culture systems which showed to be less appropriate for protoplast development than the LMPA bead culture system (Deryckere et al., 2012). Varotto et al. (2001) obtained totally inhibited cell division of red chicory mesophyll protoplasts when using 2 and 4 mM iodoacetate. In our study, iodoacetamide (IOA) was used instead of iodoacetate. IOA has been stated to penetrate the cells more easily than iodoacetate. The lower IOA concentrations we needed to totally inhibit cell division can partly be ascribed to this better cell penetration. A genotype effect is a possible, alternative explanation. Differences in sensitivity to iodoacetate and IOA were described by Creemers-Molenaar et al. (1992). Perennial ryegrass protoplasts were extremely sensitive to iodoacetate, whereby cell division was absent, even after heterokaryon formation. IOA treatments prevented cell division but allowed proliferation of heterokaryons after fusion with irradiated protoplasts.

The optimal IOA concentration obtained in this study is 1.625 mM. Higher concentrations did not inhibit microcalli formation significantly more. The optimal IOA concentration obtained is in accordance to observations made in other IOA treatments used for asymmetric fusions:

A. thaliana (Yamagishi et al., 2002) and *Musa* (Xiao et al., 2009) protoplasts were treated for 15 min at room temperature with 2 mM of IOA before cell fusion. Lower IOA concentrations (0.5 mM) stopped growth of *Gossypium hirsutum* protoplasts, whereas 3mM and 7.5 mM IOA were needed to stop cell proliferation in *Citrus* (de Bona et al., 2009a) and *B. scorzonerifolium* (Minqin et al., 2005), respectively.

Wright (1978) noted that successful IOA treatment is very cell density-dependent. Optimal IOA concentrations (i.e. a dose that causes 100 % inactivation but does not prevent rescue) differ between experiments due to miscounting of cell densities or different amounts of cell aggregation. Xiao et al. (2009) used 1 x 10^6 protoplasts ml^{-1} treated with 0.07 - 2.5 mM IOA for 15 min at room temperature. After 1.5 mM IOA treatment, less than 5 % of the protoplasts formed cell colonies. Treatment with 2.5 mM IOA completely inhibited cell division, although no statistically significant difference was observed between 1.5 and 2.5 mM. Therefore, 1.5 mM was used as the optimal IOA concentration. In our study, we used 1.5 x 10^5 protoplasts ml^{-1} for IOA treatment. Due to severe stickyness of the protoplasts at higher protoplast densities in the presence of IOA, this seemed to be the optimal protoplast density. After 1.625 mM IOA treatment, less than 10 % of the protoplasts of both *Cichorium* types showed protoplast viability. Microcalli formation dropped below 1 % after 1.625 mM IOA treatment. Using 1.75 and 2 mM IOA further inhibited cell division, although no significant difference was observed between 1.625, 1.75 and 2 mM. At high IOA concentrations, the solution became more viscous, causing protoplast agglutination. Therefore, 1.625 mM IOA was used as the optimal concentration for cytoplasm inactivation in our recipient chicories.

4.4 Conclusion

In this chapter, we evaluated the UVC irradiation dosage effects on putative *Cichorium* donor protoplasts and the IOA sensitivity of putative recipient industrial chicory cultivars to be used in prospective asymmetric fusion experiments. *C. intybus* and *C. endivia* protoplasts were submitted to 170 $\mu W\ cm^{-2}$ UVC irradiation during 0, 1, 2, 4, 6, 10, 15, 20 and 30 min. Irradiation times of 6 to 10 min were appropriate for further asymmetric fusions because of their ability to significantly reduce protoplast regeneration without heavily disturbing protoplast viability. UVC-induced DNA damage quantification was not obtained using SCGE and the standard gel electrophoresis. Further research is required.

The optimal IOA concentration for inhibiting recipient protoplast regeneration was 1.625 mM for the two industrial chicory types tested.

The fragmentation techniques as described in this chapter were used for asymmetric *Cichorium* fusion experiments to obtain cybrids (§Chapter 7).

Chapter 5 - *Cichorium* karyotype analysis and fluorescence *in situ* hybridization (FISH)

5.1 Introduction

The transfer of nuclear and/or cytoplasmic features from wild to cultivated species is of significant importance in crop improvement. Symmetric or asymmetric protoplast fusion overcomes sexual incompatibility and eliminates extensive backcrossing. However, the introduction of alien DNA in intra- or interspecific fusions may cause chromosome rearrangements. To determine these, identification of individual chromosomes in cytological preparations is essential. Among cytogenetic studies, chromosome identification and karyotype constructions are included. Karyotypes show the chromosomes ordered in sequence of decreasing length, characterized by arm length, centromere position and secondary constrictions, such as the location of nucleolar organizers (Van Laere et al., 2008). An additive cytogenetic tool for individual plant chromosome characterization is fluorescence *in situ* hybridization (FISH).

For karyotyping, the ribosomal genes rDNA segments 5S and 45S and multicopy gene families can deliver unique FISH patterns, by which chromosomes within a species can be distinguished. A critical requirement for successful chromosome identification is an efficient chromosome preparation procedure. The most common targets for FISH are mitotic metaphase chromosomes from root tips. The chance of obtaining a satisfactory FISH mapping depends also on the position of the target DNA inside the chromosome and the chromosome condensation state (Jiang and Gill, 2006).

The Asteraceae, containing over 20,000 species, are well known for their broad karyotypic variation between and within species (Fregonezi et al., 2004). So far, *Cichorium* has been poorly studied cytogenetically and a better cytogenetic understanding of the genus can help future breeding programs. Within the *Cichorium* genus, several studies have already provided valuable insights into the molecular background: nuclear (Bellamy et al., 1996; Kiers et al., 2000; Lucchin et al., 2008) and plastid molecular markers (Bellamy et al., 1995; Varotto et al., 2001; Cappelle et al., 2007; Gonthier et al., 2010) have been developed to study the genetic relationship among *Cichorium* species and a genetic map of the nine chromosomes of the *C. intybus* genome was established (Cadalen et al., 2010). To our knowledge, only one study was performed on chromosome visualization in *Cichorium* species. Rambaud et al. (1992) estimated the ploidy level of regenerated rooted plantlets after protoplast fusion by counting young root-tip chromosomes after Feulgen staining. However, no karyotype characterization has been reported yet.

To this end, the genome size was estimated and detailed karyotypes of two *Cichorium* cultivars based on DAPI staining, arm lengths and centromeric index were established. Moreover, the distribution and organization of two ribosomal gene families (5S and 45S) were examined using fluorescence in situ hybridization (FISH).

5.2 Materials and methods

Plant material

One industrial chicory inbred line *C. intybus* var. *sativum* 'VL52' (2n = 2x = 18) and one endive cultivar *C. endivia* var. *crispum* 'Wallone Despa' (2n = 2x = 18) were used for karyotype analysis. Seeds of *in vivo* plants were initiated *in vitro*. After rinsing for 1 min in 70% ethanol, seeds were surface sterilized for 20 min in 6.5% NaOCl and germinated in 60 mm petri dishes on solid Murashige and Skoog (1962) medium containing 150 mg l^{-1} casein hydrolysate and 30 g l^{-1} sucrose at pH 5.8. After germination, the plantlets were placed on solid half strength Murashige and Skoog medium containing 20 g l^{-1} sucrose and grown in Meli jars (Meli NV Veurne, Belgium) at 23 ± 2°C under a 16 h/8 h (light/dark) photoperiod at 40 µmol m^{-2} s^{-1} photosynthetic active radiation.

Genome size

The genome size was determined using flow cytometry analysis on 10 samples of each genotype. A PAS III flow cytometer (Partec, Münster, Germany) equipped with a 20 mW solid state laser (Sapphire 488-20) emitting at a fixed wavelength of 488 nm was used. Nuclei suspensions were prepared according to a modified protocol described by Galbraith et al. (1983). The extraction and staining buffers and the PI and RNase stock solutions were available in the CyStain PI Absolute P kit (Partec GmbH, Münster, Germany). Young leaves (0.5 cm^2) were put in a 55-mm-wide petri dish together with a known internal standard. Four hundred microliter of extraction buffer was added before chopping the leaves for 1 min with a razor blade. The total suspension was filtered through a 50 µm filter and 1600 µl staining buffer including 10 µl propidium iodide (PI) stock solution and 5µl RNAse was added. The nuclei were incubated for 2 h in darkness. The DNA content was calculated assuming a linear relationship between the fluorescence signals from the PI-stained nuclei of *Cichorium* and the known internal standard. The internal standard used for the genome size determination of *C. intybus* var. *sativum* 'VL52' was *Lycopersicon esculentum* L. cv. Stupicke (2C = 1.96 pg)

(Dolezel et al., 1992). For *C. endivia* var. *crispum* 'Wallone Despa', *Glycine max* L. cv. Polanka (2C = 2.5 pg) was used as internal standard (Dolezel et al., 1994).

Chromosome preparation

Mitotic metaphase chromosome spreads were obtained from actively growing root meristems by squashing according to Van Laere et al. (2008) with adaptations. Therefore, root tips were pretreated with 0.001% (v/v) α-bromonaphthalene (Merck) solution for 2 h at 4°C, then fixed in Carnoy solution (ethanol - acetic acid 3 : 1) for 2 h at room temperature and stored at -20°C until use. The root tips were rinsed in tap water before incubation in an enzyme mixture of 0.5 % pectolyase (Sigma-Aldrich) and 0.5 % cellulase 'Onozuka RS' (Duchefa Biochemie BV) dissolved in a 10 mM citrate buffer (10 mM tri sodium citrate, 10 mM citric acid, pH 4.6) for 45 min at 37°C. The partially digested root tips were squashed on slides in a small drop of 10 mM citrate buffer. Thereafter, a few drops of 60% acetic acid were added to dissolve the cytoplasm. The slides were placed on a heating plate (42°C) and the nuclei solution was spread for 2 min over the slide. A few drops of Carnoy solution were added, after which the slides were dried after rinsing in 98% ethanol. The presence of well-spread metaphase chromosomes was evaluated with a phase contrast microscope (Leica DMIRB) using 100x and 400x magnification. Slides could be stored at 4°C until further use.

Karyotype analysis

Before DAPI staining, slides containing well-spread metaphase chromosome sets were washed twice with 2x saline sodium citrate (SSC, containing 0.03 M trisodium citrate, 0.3 M sodium chloride, pH 7). Thereafter, the slides were dehydrated by 3 min incubation in 70%, 90% and 98% ethanol, successively. Slides were stained with 20 µl Vectashield (Vector Laboratories) containing 1 µg ml^{-1} DAPI. Chromosome measurements and karyotype construction were determined by analyzing 10 well-spread metaphase chromosomes sets of each genotype using a freeware computer application, MicroMeasure v.3.3 (Reeves, 2001). Centromere positions (centromeric index Ci% = [short arm/long arm] x 100), chromosome arm length and chromosome type were determined following the nomenclature of Levan et al. (1964) (Figs. 5-1 and 5-2). Also the asymmetry of the karyotype [(∑ length short arms)/(mean chromosome complement)*100] and the condensation index [(genome size 1C)/(mean chromosome complement)] (Mbp/µm).

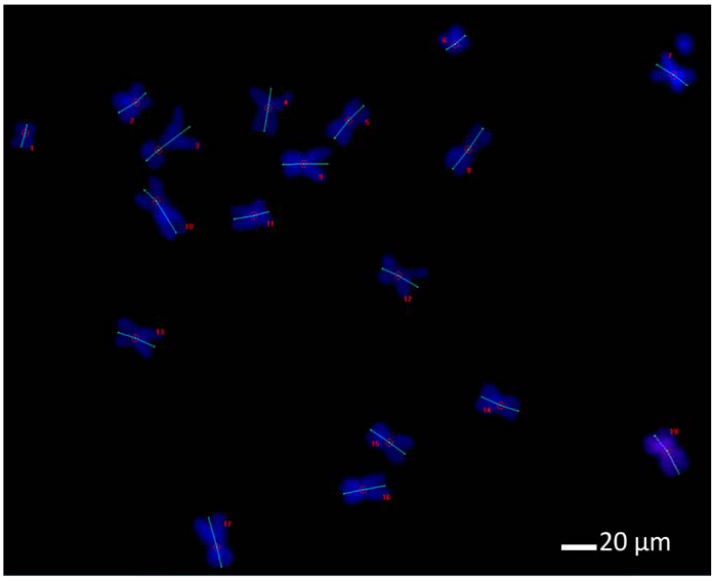


Fig. 5-1 Spread of metaphase chromosomes of *C. endivia* var. *crispum* 'Wallone Despa', stained with DAPI; Centromere position and chromosome arm length were measured with MicroMeasure v.3.3

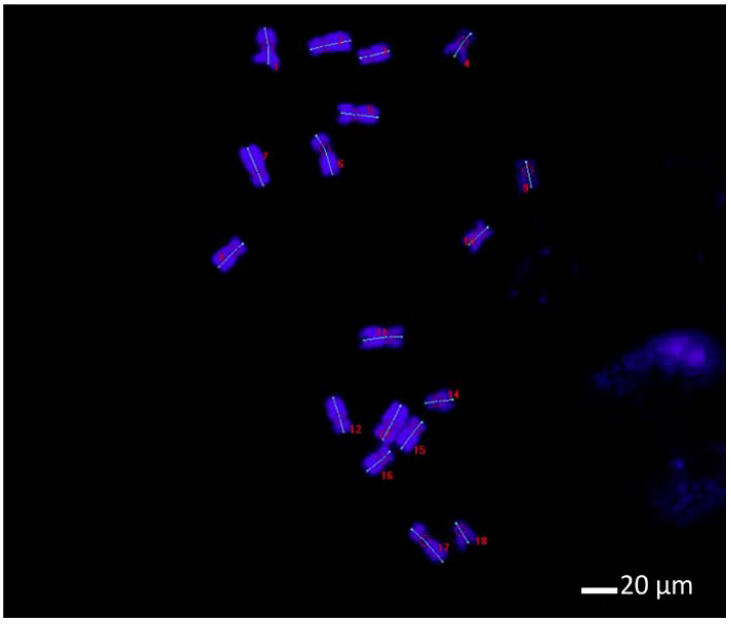


Fig. 5-2 Spread of metaphase chromosomes of *C. intybus* var. *sativum* 'VL52', stained with DAPI; Centromere position and chromosome arm length were measured with MicroMeasure v.3.3

Fluorescence in situ hybridization

Mitotic metaphases were hybridized *in situ* with the probes pTa71 (provided by University of Wageningen, The Netherlands) and pTa794 (provided by Université Paris Sud, France). The clone pTa71 (Gerlach and Bedbrook, 1979), containing a 9 kb EcoRI fragment of *Triticum aestivum* L. consisting of the 45S (18S - 5.8S - 25S) rDNA and the transcribed and nontranscribed intergenic spacer regions, and the clone pTa794 (Gerlach and Dyer, 1980), including a 410 bp 5S rDNA and the intergenic spacer isolated from *Triticum aestivum* L., were labeled with biotin-16-dUTP or digoxigenin (DIG)-11-dUTP (Roche Applied Science) (Fig. 5-3) by nick translation (according to the manufacturer's instructions). 1 µg DNA was dissolved in 16 µl MilliQ water, mixed with 4 µl biotin/digoxigenin-nick translation mix, centrifuged and incubated for 90 min at 15°C. The reaction was stopped by adding 1 µl 0.5 M EDTA (pH 8) and heating for 10 min at 65°C. The labeling was confirmed using dot blot: 1 µl of each DNA probe was put on a Hybond N+ membrane (Amersham pharmacia biotech, Buckinghamshire, England) and UV-irradiated for 1 min to dry. Alkaline phosphatase (AP)-labeled anti-DIG antibody and AP-labeled streptavidin were added for hybridization to a DIG-labeled or biotin-labeled probe, respectively. After incubation with nitro-blue-tetrazolium (NBT) and 5-bromo-4-chloro-3'-indolyphosphate (BCIP) (Roche Applied Science), phosphatase activity was detected by a color reaction in the labeled probes.

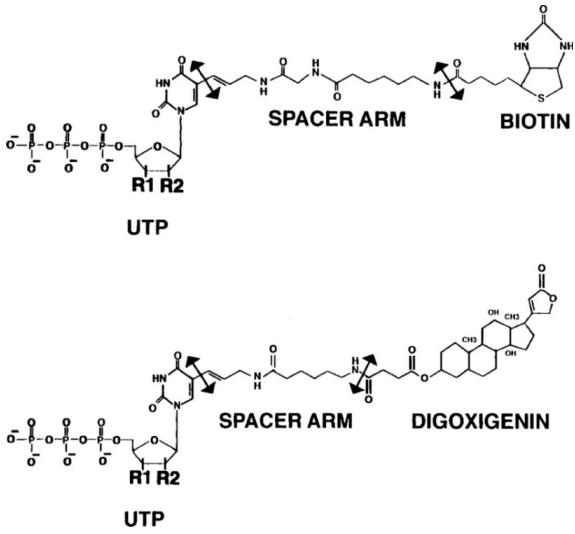


Fig. 5-3 Biotin-14- and digoxigenin-11-uridine triphosphate. Biotin and digoxigenin are linked through a spacer arm whose length can vary from 7 to 20 C/N atoms, to uridine (R1 = OH; R2 = OH), deoxyuridine (R1 = OH; R2 = H) or dedeoxyuridine (R1 = H; R2 = H) (after Chevalier et al., 1997)

Pretreatment and hybridization were performed according to Van Laere et al. (2008). Before hybridization, the selected chromosome slides were dried overnight and pretreated with 1 µg ml^{-1} RNase A for 1 h at 37°C, washed with 2x SSC and 0.01 M HCl, incubated with 0.05 ng ml^{-1} pepsin for 15 min at 37°C, washed with 2x SSC and finally incubated in 4% paraformaldehyde and washed with 2x SSC before air-drying (Leitch and Heslop-Harrison, 1994). The hybridization mixture included 50% (v/v) formamide, 10% (w/v) dextran sulphate, 2x SSC, 0.25% (w/v) sodium dodecyl sulphate (SDS) and 1.25 ng μl^{-1} probe DNA. The hybridization mixture was denaturated for 10 min at 70°C, placed on ice for 5 min and added to the pretreated chromosome slides. After a 5-min denaturation at 80°C, hybridization was performed overnight in a humid chamber at 37°C. Thereafter, the slides were washed with 2x SSC for 15 min at room temperature (RT), 0.1x SSC for 30 min at 48°C and 2x SSC for 15 min at RT. Biotin-labeled DNA was detected with CY3-conjugated streptavidin (Jackson Immuno Research Laboratories, West Grove, Pa., USA) and amplified with biotinylated goat-antistreptavidin (Vector Laboratories, Burlingame, Calif., USA) and again CY3-conjugated streptavidin. Digoxigenin-labeled DNA was detected with fluorescein isothiocyanate (FITC)-conjugated antidigoxigenin from sheep (Roche Apllied Science) and amplified with FITC-conjugated antisheep (Roche Applied Science). Slides were counterstained with 20 µl Vectashield (Vector Laboratories) containing 1 µg ml^{-1} DAPI. 45S rDNA FISH was carried out on both *C. intybus* var. *sativum* 'VL52' and *C. endivia* var. *crispum* 'Wallone Despa'. The 45S rDNA probes were labeled both with digoxigenin and biotin. Bicolor FISH with 5S and 45S rDNA was carried out on *C. intybus* var. *sativum* 'VL52'. The 5S rDNA and 45S rDNA fragments were labeled with digoxigenin and biotin, respectively.

5.3 Results

Genome size

The diploid genome size (2C) of *C. intybus* var. *sativum* 'VL52' was 2.43 ±0.08 pg, based on the internal standard *Solanum lycopersicum* L. (2C = 1.96 pg). For *C. endivia* var. *crispum* 'Wallone Despa', 2C was 1.92 ±0.01 pg, based on the internal standard *Glycine max* L. (2C = 2.5 pg).

Karyotype analysis

The mean total length of the haploid metaphase complement was 152.8 and 132.3 µm for *C. intybus* var. *sativum* 'VL52' and *C. endivia* var. *crispum* 'Wallone Despa' respectively. The karyotype of *C. intybus* var. *sativum* 'VL52' consisted of 5 metacentric (M) and 4 submetacentric (SM) chromosomes. The karyotype of *C. endivia* var. *crispum* 'Wallone Despa' included 8 M and 1 SM chromosomes. The longest and shortest chromosome of *C. intybus* var. *sativum* 'VL52' was on average 22.3 and 12.8 µm, respectively. For *C. endivia* var. *crispum* 'Wallone Despa' the longest chromosome was on average 19.2 µm, the shortest 9.9 µm (Table 5-1, Figs. 5-4 and 5-5). The asymmetry index of both species was comparable; 38.8% for *C. intybus* var. *sativum* 'VL52' and 41.6% *C. endivia* var. *crispum* 'Wallone Despa'. Also the condensation index showed a minor difference between both; 7.8 for *C. intybus* var. *sativum* 'VL52' and 7.1 for *C. endivia* var. *crispum* 'Wallone Despa' (Table 5-1).

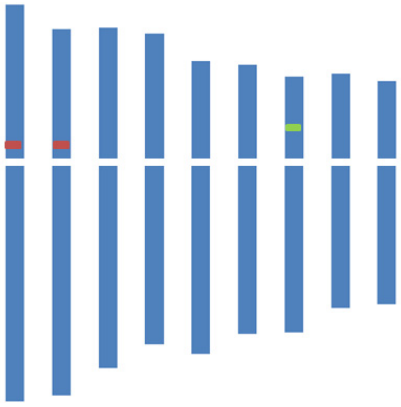
Chromosome localization of 45S and 5S rDNA

Digoxigeninated 45S rDNA probes bound more specifically to the target DNA than the biotinylated probes. Biotinylated probes delivered an unclear, unspecific spot. Digoxigenin-labeled probes, on the contrary, specifically elucidated the 45S rDNA position on the chromosomes. FISH with 45S rDNA on *C. intybus* var. *sativum* 'VL52' revealed hybridization signals in the pericentromeric regions of chromosomes 1 and 2 (Fig. 5-6). Moreover, the bicolor FISH revealed a 5S rDNA hybridization signal on the short arm of chromosome 7 (Fig. 5-7). Hybridization signals of 45S rDNA were observed on chromosome 1, 2 and 6 of *C. endivia* var. *crispum* 'Wallone Despa'. The spots on chromosomes 1 and 2 were located in the pericentromeric region, comparable to the observations of *C. intybus* var. *sativum* 'VL52'. The hybridization signal on chromosome 6, however, was situated more distally from the centromeric region (Fig. 5-8). The idiograms of the two genotypes, with indication of chromosome length, Ci and hybridization signals are presented in Figs. 5-4 and 5-5.

Table 5-1 Karyotypic data for *C. intybus* var. *sativum* 'VL52' and *C. endivia* var. *crispum* 'Wallone Despa'

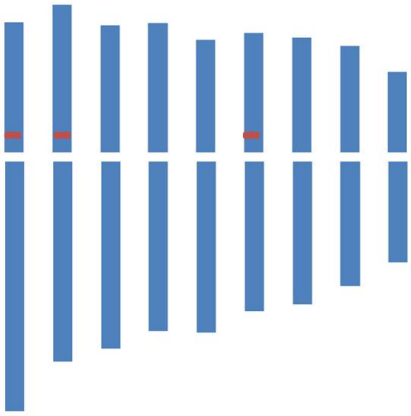
	C. intybus var. *sativum* 'VL52'	*C. endivia* var. *crispum* 'Wallone Despa'
Chromosome number	2n = 2x = 18	2n = 2x = 18
Total chromosome complement (µm)[1]	152.8 ± 6.1	132.3 ± 2.8
Length of longest chromosome (µm)	22.3 ± 0.7	19.2 ± 0.4
Length of shortest chromosome (µm)	12.8 ± 0.6	9.9 ± 0.5
Asymmetry index (%)	38.8	41.6
Condensation index (Mbp/µm)[2]	7.8	7.1
Chromosome formula	5 M + 4 SM	8 M + 1 SM

Data are means ± SE (n = 10)
[1]total chromosome complement on haploid level
[2] 1pg = 978 Mbp (Dolezel et al., 2003)



Chromosome	Total length (µm)	Centromere index (Ci)	Chromosome type
1	22.3 ± 0.7	39.5 ± 1.1	M
2	20.8 ± 1.1	37.7 ± 1.2	M
3	19.2 ± 1.1	40.1 ± 1.0	M
4	17.7 ± 1.3	42.3 ± 1.0	M
5	16.7 ± 1.0	34.8 ± 1.0	SM
6	15.3 ± 1.0	36.5 ±1.2	SM
7	14.6 ± 1.0	35.0 ± 1.6	SM
8	13.5 ± 1.0	39.3 ± 1.1	M
9	12.8 ± 0.6	36.3 ± 1.2	SM

Fig. 5-4 Left: Idiogram of *C. intybus* var. *sativum* 'VL52'; Red spots indicate 45S rDNA fragments, the green spot indicates the 5S rDNA fragment. Right: Total length, centromere index en chromosome type of the 9 chromosomes of the haploid complement (Data are means ± SE, n = 10).



Chromosome	Total length (µm)	Centromere index (Ci)	Chromosome type
1	19.2 ± 0.4	34.8 ± 1.0	SM
2	17.2 ± 0.2	42.6 ± 1.0	M
3	16.0 ± 0.3	40.7 ± 1.3	M
4	15.3 ± 0.3	43.3 ± 0.6	M
5	14.6 ± 0.3	40.0 ± 1.0	M
6	13.9 ± 0.3	44.3 ± 1.0	M
7	13.3 ± 0.3	44.7 ± 0.9	M
8	12.5 ± 0.3	44.2 ± 0.6	M
9	9.9 ± 0.5	42.9 ± 0.7	M

Fig. 5-5 Left: Idiogram of *C. endivia* var. *crispum* 'Wallone Despa'; Red spots indicate 45S rDNA fragments. Right: Total length, centromere index en chromosome type of the 9 chromosomes of the haploid complement (Data are means ± SE, n = 10).

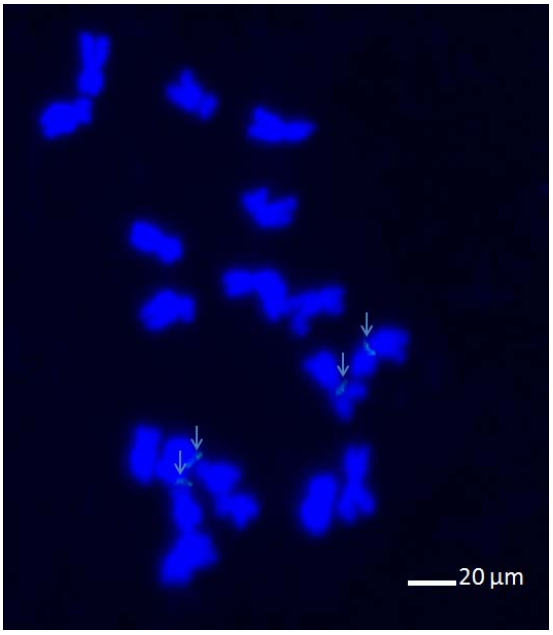


Fig. 5-6 FISH of digoxigenin-labeled 45S rDNA on a metaphase spread of *C. intybus* var. *sativum* 'VL52' (arrows).

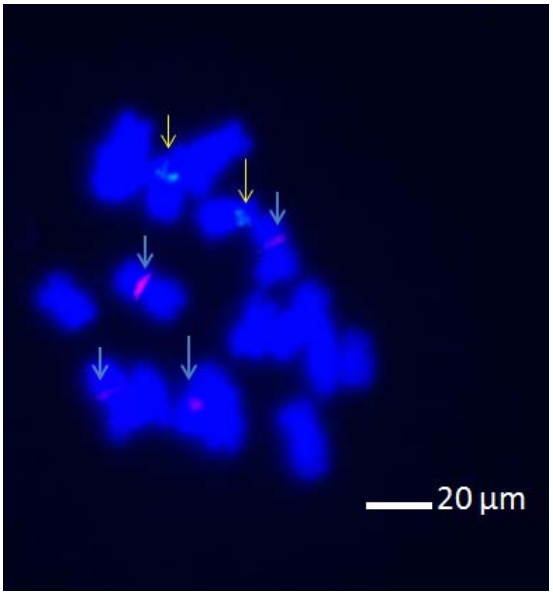


Fig. 5-7 FISH of digoxigenin-labeled 5S rDNA (yellow arrows) and biotin-labeled 45S rDNA (blue arrows) on a metaphase spread of *C. intybus* var. *sativum* 'VL52'.

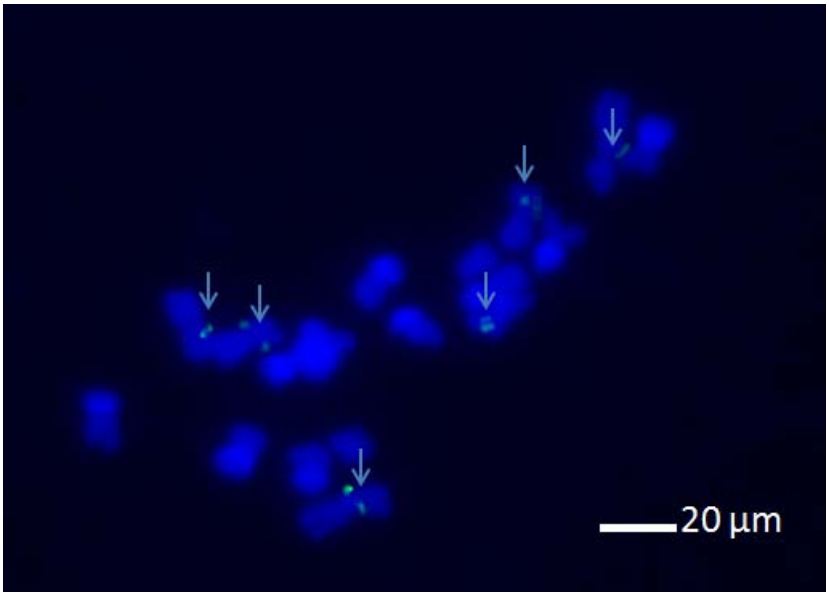


Fig. 5-8 FISH of digoxigenin-labeled 45S rDNA on a metaphase spread of *C. endivia* var. *crispum* 'Wallone Despa' (arrows).

5.4 Discussion

Clear DNA content differences were observed between the two *Cichorium* species. For industrial chicory, 2C = 2.43 ±0.08 pg. For the endive, 2C was 1.92 ±0.01 pg. These findings are in accordance with the differences we observed in their total (haploid) chromosome complement, which was bigger for industrial chicory than for endive. Although, different internal standards were used to calculate the DNA content and nonlinearity can arise in flow cytometry due to undersaturated staining of nuclei or machine errors (Mortreau et al., 2009), the genome sizes of the two species are clearly different. To our knowledge, no information of the genome size in *Cichorium* species was available from earlier reports.

In our study, *C. intybus* and *C. endivia* were also karyotyped for the first time. Their karyotypes were different in the number of metacentric (M) and submetacentric (SM) chromosomes and chromosome length. Despite this difference, the two karyotypes also showed similarities: The SM chromosomes 6 and 9 of *C. intybus* are closely situated near a Ci of 37.5, distinguishing SM from M chromosomes. In comparison, chromosomes 6 and 9 of *C. endivia* are metacentric. This demonstrates the close cytological relationship and can explain the possibility of sexual crossing between these two *Cichorium* species. The chromosome size of our *Cichorium* genotypes varied from 10 to 22 µm. These sizes are big in comparison to other plant species from the Asteraceae family; *Crepis japonica* (~ 1.9 - 2.2 µm), *Galinsoga parviflora* (~ 1.4 - 1.9 µm) and *Chaptalia nutans* chromosomes (~ 1.3 - 2.5 µm) are tenfold smaller (Fregonezi et al., 2004). Big chromosomes were also found in other families such as the Liliaceae and Alliaceae: *Lilium longiflorum* (2C = 77.1 pg) and *L. rubellum* (2C = 73.6 pg) contain chromosomes varying from 18 up to 34 µm (Lim et al., 2001). Chromosomes of *Allium cepa* have a mean total length of 69.7 µm (Khrustaleva and Kik, 2001).

Chromosome localization of 5S and 45S rDNA on *C. intybus* var. *sativum* 'VL52' chromosomes revealed one and two hybridization signals, respectively. The 5S rDNA fragment was located on the short arm of chromosome 7. The two 45S rDNA hybridization signals were situated in the pericentromeric region of chromosomes 1 and 2. Three 45S rDNA hybridization signals were found in the pericentromeric region on *C. endivia* var. *crispum* 'Wallone Despa' chromosomes 1, 2 and 6. This variability of the number of 45S rDNA loci among plant species of the same genus has already frequently been reported (Pedrosa-Harand et al., 2006). The rDNA clusters are believed to be highly mobile due to the existence of transposons observed in plant rDNA clusters (Britton-Davidian et al., 2012). In plants and animals, 45S rDNA is mainly associated with a NOR in active state. Moreover, a NOR region

is often associated with a secondary constriction, like satellites, in the chromosome. Our findings, however, showed 45S rDNA signals in the pericentric region on chromosomes not containing secondary constrictions. These secondary constrictions can be lost during chromosome preparations, or totally absent in *Cichorium*. The detection of 45S rDNA signal without a nucleolar organizing region (NOR) has also been observed in *Allium cepa* (Ricroch et al., 1992) and *Hydrangea* species (Van Laere et al., 2008).

Our best FISH results were obtained using digoxigenin-labeled probes. The use of biotin-labeled probes yielded diffuse spots, through which accurate localization of our target DNA was hindered. However, size and charge differences between biotinylated and digoxigeninated probes are absent (Fig. 5-3). The nonspecific binding of biotin has previously also been reported in Chevalier et al. (1997), attributing this problem to the stickiness of the protein caused by its attached carbohydrates. Endogenous biotin interferes with specific signals, causing false-positive results, observed when using biotin-labeled probes (Chevalier et al., 1997). Generally, hybridization of labeled probes is also influenced by the accessibility of the target. The position of the target at the chromosomes and the chromosome condensation state are key factors who determine the accessibility of the target. Yet, another reason for the better results obtained using digoxigenin-labeled probes can be included: in our study, biotin-streptavidin-labeled target DNA was detected with two amplifying layers containing biotinylated goat-antistreptavidin and CY3-conjugated streptavidin. This layered construction, enhanced the signal detection, but could mask the overall biotin residues which could not be detected. The digoxigenin-labeled probes, however, could be detected using only one amplification layer.

The successful application of FISH on *Cichorium* species opened perspectives for the application of genomic *in situ* hybridization (GISH). GISH is able to differentiate the parental chromosomes or different chromosomal fragments in interspecific hybrids. As GISH is mostly used in plant species with large-sized chromosomes (Van Laere et al., 2010), this technique was promising for further research in our *Cichorium* genotypes. Preliminary GISH experiments, however, revealed a high degree of homology between *C. intybus* and *C. endivia* genotypes, impeding the differentiation in allotetraploid hybrids. Also, the detectable GISH signals were often restricted to pericentromeric regions, where repetitive DNA clusters are present, and disabling the clear difference between parental genomes in hybrids. Further research on the optimization of GISH in *Cichorium* can be made by improving more stringent hybridization conditions and changing the probe/block DNA ratio. However, the high homology between the chicory and endive genome can thwart the GISH technique. For this

reason, performing GISH on intraspecific somatic hybrids of industrial and wild type chicory is probably not recommended. Possible differences of the 5S probe would provide a solution to distinguish these hybrids. But therefore, first 5S localization through FISH on wild type chicory and endive should be observed.

Our results demonstrated that FISH and bicolor FISH in combination with DAPI counterstaining can be a powerful tool to provide chromosomal landmarks for developing karyotypes in *Cichorium* species. More cytological markers can be added to develop chromosome portraits which can be used to more profoundly characterize progeny in plant breeding purposes.

5.5 Conclusion

To determine alien DNA in intra- or interspecific fusions between *Cichorium* species, karyotype analysis and FISH of two ribosomal gene families (5S and 45S) were performed on parental industrial chicory *C. intybus* var. *sativum* 'VL52' and endive *C. endivia* var. *crispum* 'Wallone Despa'. The karyotype of *C. intybus* var. *sativum* 'VL52' consisted of 5 metacentric (M) and 4 submetacentric (SM) chromosomes. The karyotype of *C. endivia* var. *crispum* 'Wallone Despa' included 8 M and 1 SM chromosomes. FISH of 45S rDNA with *C. intybus* var. *sativum* 'VL52' revealed two hybridization signals on chromosomes 1 and 2. A single 5S rDNA hybridization signal was observed on chromosome 7. Three 45S rDNA hybridization signals were observed on chromosome 1, 2 and 6 of *C. endivia* var. *crispum* 'Wallone Despa'. The karyotype information of industrial chicory and endive can be used for further plant breeding purposes or for the characterization of somatic hybrids after protoplast fusion.

Chapter 6 - Plasmotype characterization

Based on the published article: Deryckere D, De Keyser, E, Eeckhaut T, Van Huylenbroeck J, Van Bockstaele E. (2012) High resolution melting analysis as a rapid and highly sensitive method for Cichorium plasmotype characterization. Plant Molecular Biology Reporter doi: 10.1007/s11105-012-0547-y

6.1 Introduction

Somatic hybridization has been used in many species to circumvent sexual incompatibility and to enable the direct transfer of nuclear and cytoplasmic genome features into plant cells. In *Cichorium* species, somatic hybridization through protoplast fusion and regeneration has already been established (Varotto et al., 2001; Cappelle et al., 2007; Deryckere et al., 2012). This has created a great demand for the development of organelle markers to study somatic hybrids/cybrids. Molecular markers have been used to study the genetic relationship among *Cichorium* species and cultivar groups (Bellamy et al., 1996; Kiers et al., 2000; Lucchin et al., 2008). Cadalen et al. (2010) calculated a genetic map based on 472 simple sequence repeats (SSR) and single nucleotide polymorphisms (SNP) markers covering the nine chromosomes of the *C. intybus* genome. Mitochondrial (mt) and chloroplast (cp) DNA sequence polymorphisms in *Cichorium* species have been investigated in the 1990s (Bellamy et al., 1995). Cappelle et al. (2007) described specific mtDNA coding regions (*atp6*, *atpA*, *coxII*, *coxIII*, *nad3*, *nad4* and *orfB*) and the cp DNA *trnL-trnF* intergenic spacer region as probes in Southern blot experiments. This revealed which parental mtDNA or cpDNA was present in interspecific symmetric somatic hybrids between *C. intybus* and *C. endivia*. Varotto et al. (2001) reported the use of the mt genes *coxI*, *coxII* and *cob* from the maize mt genome and the *orf522* and *atpA* genes from the sunflower mt genome to characterize asymmetric somatic hybrid plants between *C. intybus* and *Helianthus annuus* L. Gonthier et al. (2010) used the mt genes *atpA*, *atp9*, *cob* and *coxII* and the cp DNA sequences *matK*, *ndhF*, *rbcL* and *trnL-trnF* to evaluate the level of contamination by organelle DNA in the construction of two BAC libraries representing deep coverage of the nuclear genome of *C. intybus*. In all of these studies, the visualization of the organelle DNA fragments was done using the cumbersome Southern blot analysis.

High-resolution melting (HRM) analysis is a much faster and more sensitive detection technique for cytoplasms of various plant species. HRM was introduced in the 1970s with the genotyping of yeast mitochondrial DNA (Michel et al., 1974; Wu et al., 2008). Improvements to the saturating intercalating dyes have made HRM analysis a highly sensitive method for genotyping, discovering mutations and tracking SNPs (Han et al., 2012). Originally, the technique was used extensively in medical diagnostic applications to locate mutations in human genetics (Wittwer et al., 2003; Krypuy et al., 2006; Sinthuwiwat et al., 2008). HRM was soon inserted in the SNP genotyping of many plant species including barley (Lehmensiek et al., 2008), grapevine and olive (Mackay et al., 2008), perennial ryegrass (Studer et al.,

2009), potato (De Koeyer et al., 2010), wheat (Botticella et al., 2011), rice (Li et al., 2011), *Brassica rapa* (Lochlainn et al., 2011), almond (Costa et al., 2012), alfalfa (Han et al., 2012), *Capsicum* (Jeong et al., 2012) and bean (Ganopoulos et al., 2012). HRM combines a polymerase chain reaction (PCR) using a double stranded DNA (dsDNA) binding dye with the melting behavior of the PCR amplicons. When dsDNA dissociates through melting in single stranded DNA (ssDNA), changes in fluorescence can be monitored. Shifts in melting temperature curves can be attributed to variations in amplicon sequences caused by SNPs, insertions/deletions (INDELS) or SSR variants and amplicon length (Lehmensiek et al., 2008; Wu et al., 2010). In contrast with other techniques such as Southern blotting, digestion with restriction enzymes and others, HRM is a fast and low cost technique due to its 384-well format. Moreover, HRM is non-destructive; the DNA can be recovered after melting and used for sequencing or cloning (Hofinger et al., 2009). No information is currently available on HRM analysis in *Cichorium* species.

The aim of the present study was to evaluate HRM analysis for the detection of specific mitochondrial and chloroplast markers to distinguish the industrial chicory plasmotypes *C.intybus* var. *sativum* from the wild type chicory *C. intybus* and the endive *C. endivia* plasmotypes. The technique was used to characterize the cytoplasms of symmetric protoplast fusion products between *C. intybus* var. *sativum* 'VL52' and *C. endivia* var. *crispum* 'Wallone Despa'.

6.2 Materials and methods

Plant material and DNA extraction

One industrial chicory inbred line, *C. intybus* var. *sativum* 'VL52', one industrial chicory clone *C. intybus* var. *sativum* 'K1093', five chicory wild types of *C. intybus* ('Ames23224', 'Ames26033', 'Ames22531', 'Ames22532' and 'Pi531291') and two endive cultivars *C. endivia* (*C. endivia* var. *crispum* 'Wallone Despa' and *C. endivia* var. *latifolium* 'nr. 5') were used in the experiments. The plants were grown at the Institute for Agricultural and Fisheries Research (ILVO) and provided by the COSUCRA- Groupe Warcoing S.A., Chicoline division. Seeds of *in vivo* plants were rinsed for 1 min in 70% ethanol, surface sterilized for 20 min in 6.5% NaOCl and germinated in 60 x 15 mm petri dishes on solid Murashige and Skoog (1962) medium containing 150 mg l^{-1} casein hydrolysate and 30 g l^{-1} sucrose at pH 5.8. After germination, the plantlets were grown on solid Murashige and Skoog medium

containing 20 g l^{-1} sucrose and grown in Meli jars (Meli NV Veurne, Belgium) at 23 ± 2°C under a 16 h/8 h (light/dark) photoperiod at 40 µmol m^{-2} s^{-1} photosynthetic active radiation supplied by cool white fluorescent tube lamps (Sylvania Standaard F40W/33-640/RS Cool White). The HRM technique was tested on 50 somatic hybrids. The somatic hybrids resulted from symmetric protoplast fusions between *C. intybus* var. *sativum* 'VL52' and *C. endivia* var. *crispum* 'Wallone Despa' and were regenerated following the protocol of Deryckere et al. (2012). Their hybridity was demonstrated by genomic microsatellite markers provided by the COSUCRA-Groupe Warcoing S.A., Chicoline division (data not shown).

Plant DNA was extracted from 100 mg fresh *in vitro* leaf material using the Qiagen DNeasy Plant Tissue Mini kit, according to the manufacturer's instructions. The DNA concentration and quality was analyzed by an Eppendorf Nanodrop ND-1000 spectrophotometer. Samples were diluted to a 15 ng DNA μl^{-1} working concentration.

Identification of cytoplasmic SNPs

To obtain information on the presence of putative cytoplasmic SNPs in the nine *Cichorium* species, we selected five mtDNA (*atpA*, *coxII*, *orfB*, *nad3* and *cob*) and two cpDNA (*trnL-trnF* and *ndhF*) regions from literature (Cappelle et al., 2007; Gonthier et al., 2010) and sequenced them after PCR amplification. The 25-µl PCR reaction volume consisted of 75 ng total plant DNA template, 1.5 U AmpliTaq® DNA polymerase (Applied Biosystems), 1x PCR buffer (Applied Biosystems), 0.025 mM dNTP each, 0.2 µM each primer and deionized water up to 25 µl. For PCR amplification, a preheating period at 94°C for 4 min preceeded 30 cycles of 93°C for 45 s, 55°C for 45 s and 72°C for 2 min; a postamplification incubation at 72°C for 10 min completed the reaction. PCR amplification was performed on the GeneAmp® PCR System 9700 (Applied Biosystems). The PCR fragments obtained were sequenced using the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions, using the gene-specific primers. The sequencing reactions were analyzed on a 3130*xl*® Genetic Analyzer (Applied Biosystems) using the software program Sequence Analysis v5.2 Patch2 (Applied Biosystems).

HRM primer design

Primer pairs targeting SNP and INDEL sites were evaluated for HRM analysis. By using the online primer design tool Primer 3 Plus, the primer pairs were designed to be 20 ± 2 bp long and to have an annealing temperature of 60 ± 2°C. The amplicon length was chosen between

100 and 200 bp spanning one (several) SNP(s) and/or INDELs. The primer pairs tested for the HRM analysis are summarized in Table 6-1.

PCR amplification and HRM analysis

All PCR reactions were performed in 384-well plates using a LightCycler® 480 Real-Time PCR system (Roche Applied Science) in a total volume of 10 µl per well. The reaction mixture contained 30 ng total DNA, 2.5 mM $MgCl_2$, 0.2 µM forward and reverse primers and 1 x High-Resolution Melting Master Mix (Roche Applied Science). Before performing HRM, an initial denaturation at 95°C for 10 min was followed by 50 cycles of amplification (denaturation at 95°C for 10 s, annealing at 60°C for 15 s and extension at 72°C for 25 s). The amplification was followed by the high-resolution melting: denaturation at 95°C for 1 min, cooling to 40°C for 1 min, a one-step temperature increase to 65°C and a continuous further increase to 95°C at 0.02°C s^{-1} increments. During the incremental melting step, fluorescence data were acquired continuously. After samples were amplified using PCR and melted using HRM, the gene scanning software (LightCycler® 480 Gene Scanning Software) analyzed the T_m of the individual curves, calculated the relative fluorescence signal differences between the plasmotypes, and autogrouped those with similar melting curves (no melting standards were required).

Testing the HRM sensitivity for heterozygous plasmotypes

To determine whether our HRM markers could distinguish between homozygous and heterozygous samples, we tested the detection efficiency of the *coxII-2, cob-1*, *cob-2, trnL-trnF*, *trnL-trnF-2* and *ndhF-1* fragments in pooled samples. For each amplicon type, fragments of *C. intybus* var. *sativum* 'VL52' were mixed with amplicons of *C. endivia* var. *crispum* 'Wallone Despa' at ratios of 0:100, 5:95, 25:75, 50:50, 75:25, 95:5 and 100:0. HRM analysis was performed on these mixed samples.

HRM on protoplast fusion products

The putative HRM markers *coxII-2*, *cob-1*, *cob-2, trnL-trnF*, *trnL-trnF-2*, and *ndhF-1* obtained in the previous experiments were used to characterize the cytoplasm of the 50 somatic hybrids. The parent plasmotypes *C. intybus* var. *sativum* 'VL52' and *C. endivia* var. *crispum* 'Wallone Despa' were included as references. Furthermore, to confirm the sequence variations detected by HRM, sequence analysis was performed on 8 clones of PCR products from the somatic hybrids and the original fusion partners.

Before cloning, a PCR reaction was performed using Gotaq® Hot Start Polymerase (Promega) that consisted of preheating at 94°C for 2 min then 35 cycles of 94° for 30 s, 56°C for 30 s and 72°C for 1 min, finalized at 72°C for 5 min. PCR amplification was performed on the GeneAmp® PCR System 9700 (Applied Biosystems). The PCR products were cloned into a pCR™2.1-TOPO® vector (Invitrogen) using the TOPO® TA Cloning® Kit (Invitrogen) according to the manufacturer's protocol. After direct colony PCR using the universal M13 primer, the sequences of the 8 clones for each plant and each cytoplasmic fragment were determined on a 3130*xl*® Genetic Analyzer (Applied Biosystems) and analyzed using the software program Sequence Analysis v5.2 Patch2 (Applied Biosystems).

Table 6-1 Mitochondrial and chloroplast primer pair sequences used for HRM analysis of the *Cichorium* species

Fragment	Size	Primer sequences (5' → 3')	Amplicon variations	Industrial chicory	Wild chicory	Endive	HRM detection
			mitochondrial fragments				
coxII-1	207 bp	F: GCCCCTGACAGGATAGATGA R: CTGGAACCGAAAAAGACCTG	SNP (T/G)	T	G	G	No
coxII-2	102 bp	F: CTTGCCCCTGACAGGATAGA R: GAGAAATGCGCAGTGGAAAG	SNP (T/G)	T	G	G	Yes
cob-1	129 bp	F: TTGGGGTCAGATGAGCTTTT R: CGATTTAAGGTGGCATTGTC	SNP (G/T)	G	T	T	Yes
cob-2	150 bp	F: CCGGAATGGTATTTCCTACC R: CGGGCGAAAACTTGAACTAC	SNP (A/C)	A	C	C	Yes
cob-3	100 bp	F: GGGAGGTGTAGCCGCAATAG R: GTGAATCGGGCGAAAACTT	SNP (A/C)	A	C	C	No
			chloroplast fragments				
trnL-trnF	395 bp	F: GGTTCAAGTCCCTCTATCCCCA R: CTACCAGCTGAGCTATCCCG	SNP (T/C) INDEL (11 bp) INDEL (1 bp) INDEL (3 bp)	T absent present absent	C absent absent present	C present absent present	Yes
trnL-trnF-1	145 bp	F: TTAGCGGCTCAAAACTCCTT R: TCATTCAAATGGGGATTCCT	SNP (T/C)	T	C	C	No
trnL-trnF-2	104 bp	F: AAATTATAGGCCCGGGATGA R: CCCGACTATTCTTCATGTCTCA	INDEL (3 bp)	absent	present	present	Yes
trnL-trnF-3	102 bp	F: TCCCTCTATCCCCAAAAAGA R: AAGGAGTTTTGAGCCGCTAAC	INDEL (11 bp) INDEL (1 bp)	absent present	absent absent	present absent	No
trnL-trnF-4	135 bp	F: TTCGTTAGCGGCTCAAAACT R: TTCCTTGCTCAAAGATGTTCA	SNP (T/C)	T	C	C	No
trnL-trnF-5	150 bp	F: TTCGTTAGCGGCTCAAAACT R: TCATTCAAATGGGGATTCCT	SNP (T/C)	T	C	C	No
ndhF-1	94 bp	F: TGCATTTGTTAAAATGGGTCCT R: GGCATCTATGTAACCCCGATT	SNP (C/A)	C	A	A	Yes
ndhF-2	107 bp	F: TGGATTTCCTTAATGCATTTGT R: GGCATCTATGTAACCCCGATT	SNP (C/A)	C	A	A	No
ndhF-3	187 bp	F: TGCATTTGTTAAAATGGGTCCT R: CTCCATTTGGAATTGCATCA	SNP (C/A) SNP (G/A)	C G	A G	A A	No

6.3 Results

Identification of cytoplasmic SNPs

Sequence analysis of the seven cytoplasmic fragments revealed the presence of several SNPs and INDELs. In the mitochondrial fragments *atpA* (1500 bp) and *orfB* (450 bp), no variation was observed between the sequences of different *Cichorium* species. The fragment *nad3* (335 bp) showed one non-species-specific transversion (C/A) at 216 bp which discriminated the wild type *C. intybus* 'Ames22532' from all other plasmotypes. However, only SNPs or INDELs discriminating the industrial chicory plasmotypes from the endive and the wild type plasmotypes were useful for future HRM experiments. The sequences of the mitochondrial *coxII* and *cob* fragments yielded one and two valuable SNPs, respectively. One transversion (T/G) at 46 bp was observed in the *coxII* amplicon sequence (1700 bp) (Supplemental Fig. 6-1). The *cob* fragment (698 bp) contained two transversions (G/T and A/C) at 193 bp and 620 bp, respectively (Supplemental Fig. 6-2).

For the chloroplast fragment *ndhF* (579 bp), three SNPs were found. One SNP (C/A) at 278 bp discriminated between the industrial chicory plasmotypes and the others (Supplemental Fig. 6-3). The sequence of the chloroplast fragment *trnL-trnF* (395 bp) revealed three INDELs and one SNP. The endive plasmotypes showed an 11 bp insertion at 50 bp. A 1 bp insertion was observed at 68 bp in the industrial chicory plasmotypes and a 3-bp-long insertion at 298 bp was observed in the wild type chicories and the endives. The SNP (T/C) at 153 bp distinguished the industrial chicory plasmotypes from the endive and wild type plasmotypes (Supplemental Fig. 6-4).

HRM analysis

No valuable HRM distinctions were obtained when using primer pairs *coxII-1*, *cob-3*, *trnL-trnF-1*, *trnL-trnF-3*, *trnL-trnF-4*, *trnL-trnF-5*, *ndhF-2* and *ndhF-3*. Plasmotypes were distinguishable on the melting curves of the fragments *coxII-2*, *cob-1*, *cob-2, trnL-trnF*, *trnL-trnF-2* and *ndhF-1*. A clear difference in fluorescence was observed between the industrial chicory plasmotypes versus the wild type chicory and endive plasmotypes when using the mitochondrial fragments *coxII-2*, *cob-1* and *cob-2* (Fig. 6-1). HRM analysis of the chloroplast fragments *trnL-trnF-2* and *ndhF-1* separated the industrial chicory plasmotypes from the wild type chicory and endive plasmotypes (Fig. 6-2). HRM analysis of the *trnL-trnF* fragment yielded three groups that represented the industrial chicory, the wild type chicory and the endive plasmotypes (Fig. 6-2). Six of the 14 primer pairs resulted in clearly distinguishable

HRM curves. Among them were three mitochondrial primer pairs (*coxII-2*, *cob-1* and *cob-2*) and three chloroplast primer pairs (*trnL-trnF*, *trnL-trnF-2*, and *ndhF-1*) (Table 6-1).

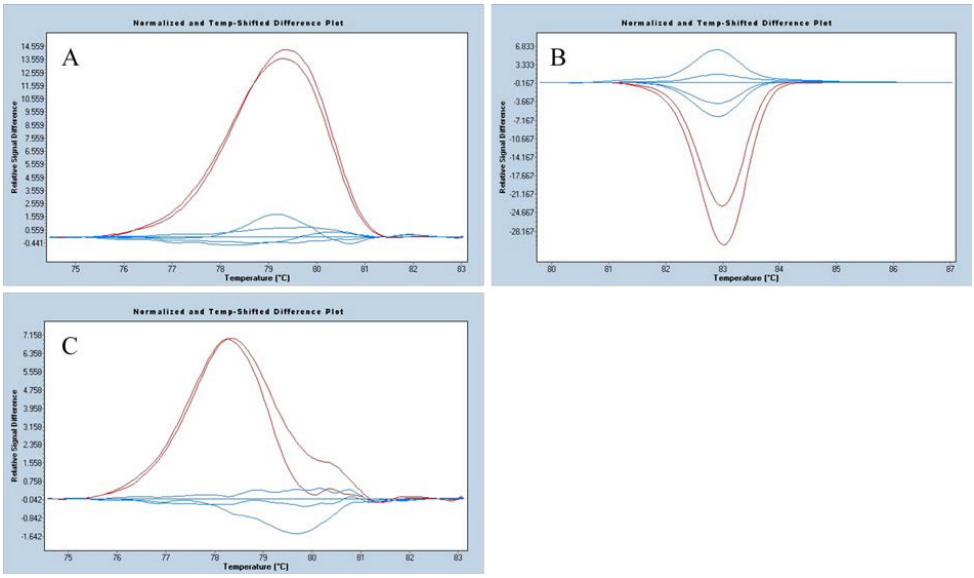


Fig. 6-1 HRM analysis of the mitochondrial fragments (A) *coxII-2*, (B) *cob-1* and (C) *cob-2*. The red curves represent the industrial chicory plasmotypes *C. intybus* var. *sativum* 'VL52' and 'K1093'. The blue curves represent the wild type chicory and endive plasmotypes

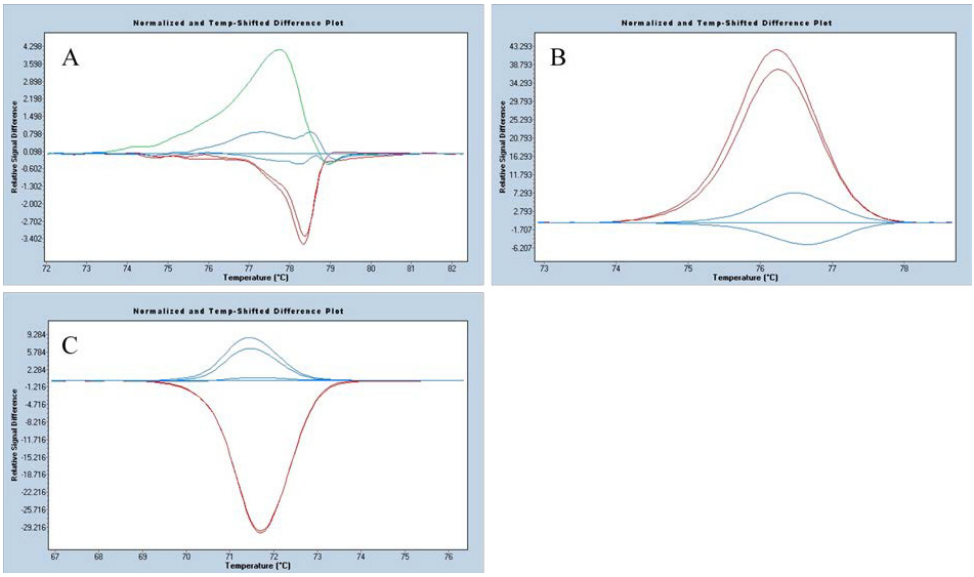


Fig. 6-2 HRM analysis of the chloroplast fragments (A) *trnL-trnF*, (B) *trnL-trnF-2* and (C) *ndhF-1*. The red curves represent the industrial chicory plasmotypes *C. intybus* var. *sativum* 'VL52' and 'K1093'. The blue curves obtained for the fragments *trnL-trnF-2*, and *ndhF-1* represent the wild type chicory and endive plasmotypes. For the fragment *trnL-trnF*, the blue curves represent the wild type chicory, while the green curve represent an endive plasmotype.

Testing HRM sensitivity for heterozygous plasmotypes

A mixture of the two homozygous plasmotypes (*C. intybus* var. *sativum* 'VL52' and *C. endivia* var. *crispum* 'Wallone Despa') should produce a melting curve corresponding to that of a heterozygous plasmotype. The HRM analysis of the mixed plasmotypes for the *coxII-2* fragment revealed HRM curves for the 5:95 and 95:5 ratios parallel to the curves obtained for the homozygous parents *C. endivia* var. *crispum* 'Wallone Despa' and *C. intybus* var. *sativum* 'VL52', respectively (Fig. 6-3). The melting curves of the 25:75, 50:50 and 75:25 ratios could be clearly distinguished from the parental plasmotypes and each other. The mixed plasmotypes for the *trnL-trnF* fragment (Fig. 6-4) yielded higher melting temperatures (T_m) than the original plasmotypes *C. endivia* var. *crispum* 'Wallone Despa' and *C. intybus* var. *sativum* 'VL52'. The melting curves of all the ratios (50:50, 75:25, 25:75, 95:5 and 5:95) could clearly be distinguished from the parental plasmotypes. No discrimination between the parents *C. endivia* var. *crispum* 'Wallone Despa' and *C. intybus* var. *sativum* 'VL52' was observed. The HRM analysis of the other fragments (*cob-1*, *cob-2, trnL-trnF-2*, and *ndhF-1*) yielded no clear distinction between homozygotes and heterozygotes.

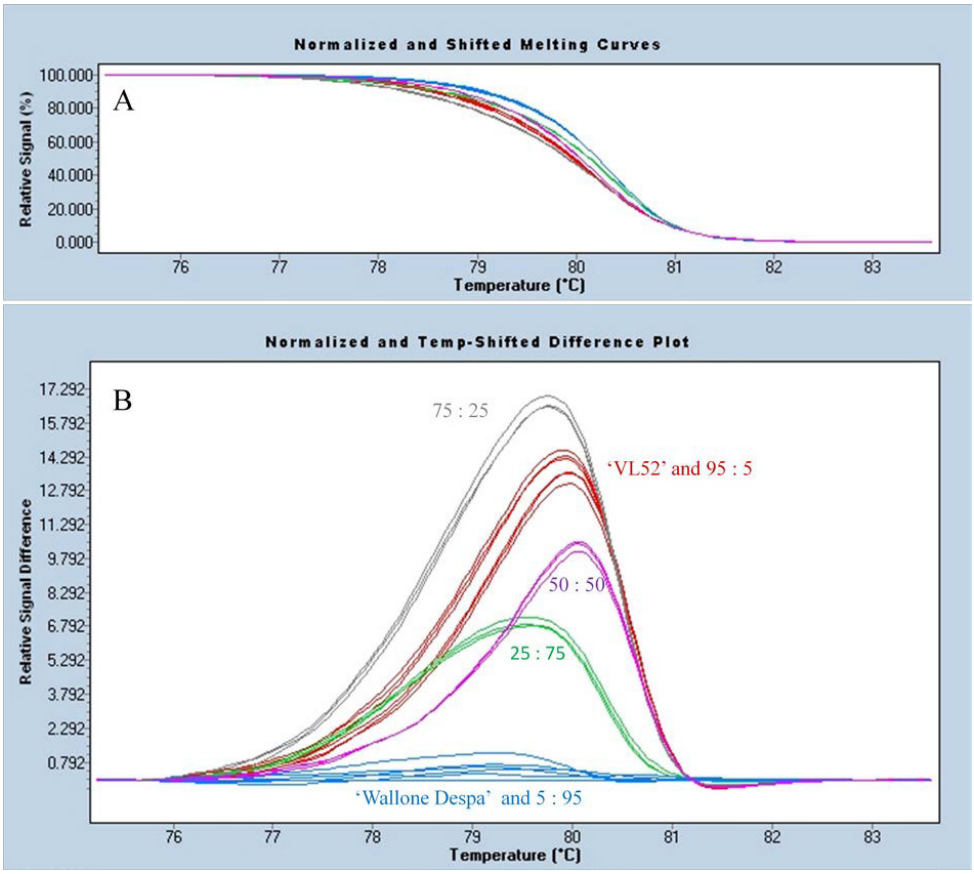


Fig. 6-3 HRM analysis of mixed plasmotypes for the *coxII-2* fragment: (A) melting curve and (B) difference plot of the parental plasmotypes *C. intybus* var. *sativum* 'VL52' (red) and *C. endivia* var. *crispum* 'Wallone Despa' (blue) and the ratios 5:95 (blue), 25:75 (green), 50:50 (pink), 75:25 (grey) and 95:5 (red)

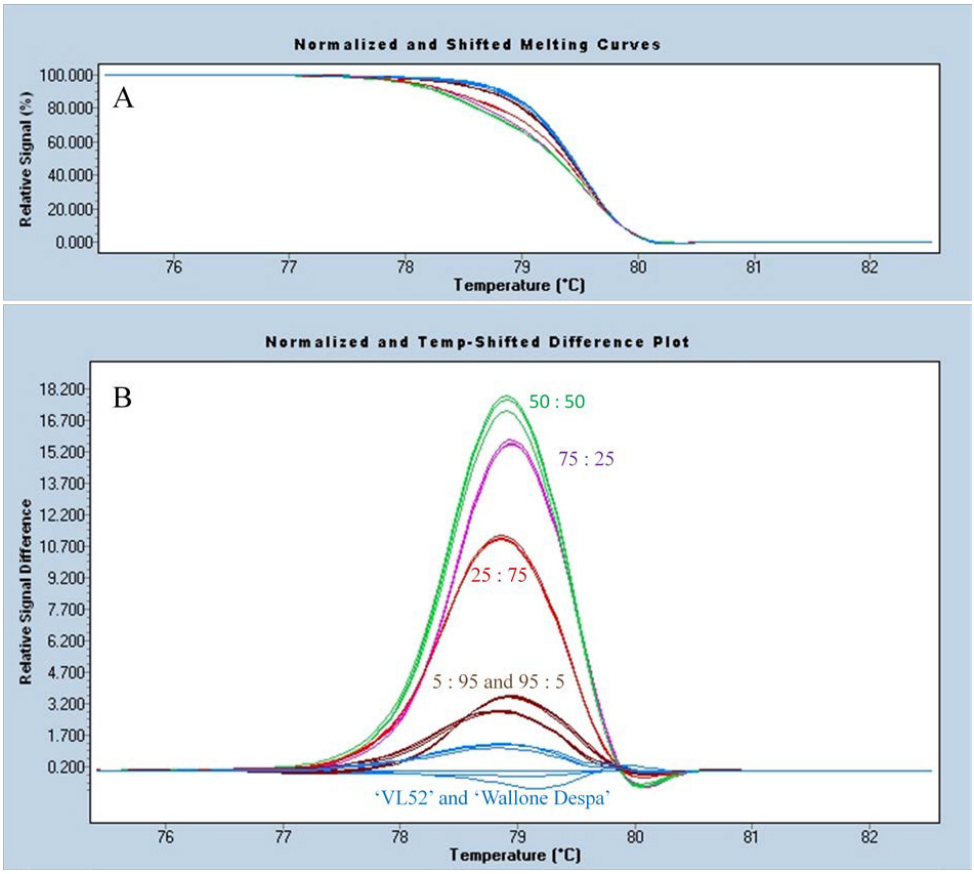


Fig. 6-4 HRM analysis of mixed plasmotypes for the *trnL-trnF* fragment: (A) melting curve and (B) difference plot of the parental plasmotypes *C. intybus* var. *sativum* 'VL52' (blue) and *C. endivia* var. *crispum* 'Wallone Despa' (blue) and the ratios 5:95 and 95:5 (brown), 25:75 (red), 50:50 (green) and 75:25 (pink)

HRM analysis of protoplast fusion products

We tested the ability of the HRM markers *coxII-2*, *cob-1*, *cob-2, trnL-trnF*, *trnL-trnF-2* and *ndhF-1* to characterize the cytoplasm of 50 *Cichorium* somatic hybrids. Figure 6-5 shows the outcome of the HRM analysis for the *coxII-2* and *cob-2* fragments. The melting curves of the somatic hybrids corresponded to either the parent *C. intybus* var. *sativum* 'VL52' or to the parent *C. endivia* var. *crispum* 'Wallone Despa'. Out of 50 somatic hybrids, 26 contained the mitochondrial *coxII-2, cob-1* and *cob-2* fragments of the industrial chicory, whereas 23 contained the mitochondrial fragments of the endive plasmotype. In one somatic hybrid, the *coxII-2* and *cob-1* endive fragments and the *cob-2* industrial chicory fragment were present.

No intermediate melting curves were observed, suggesting the abscence of heterozygosity within each fragment. All the chloroplast fragments (*trnL-trnF*, *trnL-trnF-2*, and *ndhF-1*) corresponded to the industrial chicory plasmotypes. Sequence analysis after cloning of the PCR-fragments was consistent with the results obtained by HRM.

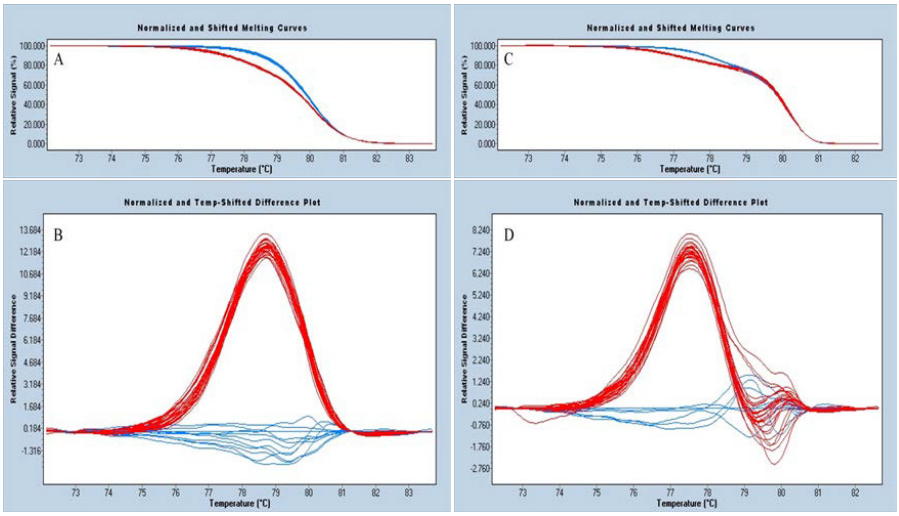


Fig 6-5 HRM analysis of the 50 somatic hybrids (A) melting curve and (B) difference plot for the *coxII-2* fragment and (C) melting curve and (D) difference plot for the *cob-2* fragment. The red and blue curves represent the *C. intybus* var. *sativum* 'VL52' and *C. endivia* var. *crispum* 'Wallone Despa' plasmotypes, respectively

6.4 Discussion

We have developed three mt (*coxII-2*, *cob-1* and *cob*-2) and three cp markers (*trnL-trnF*, *trnL-trnF-2*, and *ndhF*-1) for HRM analysis to discriminate two industrial chicories (*Cichorium intybus* var. *sativum* 'VL52' and 'K1093') from five wild types (*C. intybus* 'Ames23224', 'Ames26033', 'Ames22531', 'Ames22532' and 'Pi531291') and two endive cultivars *C. endivia* (*C. endivia* var. *crispum* 'Wallone Despa' and *C. endivia* var. *latifolium* 'nr.5') (Figs. 6-1, 6-2).

The sequence analysis of mt and cp fragments previously used in *Cichorium* species (Cappelle et al., 2007; Gonthier et al., 2010) revealed several SNPs and INDELs in our plasmotypes. The resulting SNP and INDEL data were used to construct primer pairs suitable for HRM analysis. Six (*coxII-2*, *cob-1*, *cob-2, trnL-trnF*, *trnL-trnF-2*, and *ndhF-1)* out of 14 primer pairs produced different HRM profiles for industrial chicory plasmotypes and endive

and wild type chicory plasmotypes. SNPs as well as INDELs were responsible for the different melting behavior of the amplicons. Amplicon size ranged from 94 to 207 bp (except for the cp fragment *trnL-trnF*) and the primer pairs spanned at least one SNP or INDEL (Table 6-1). This is in accordance with De Koeyer et al. (2010) who obtained similarly sized fragments for SNP detection using HRM in potato. Shorter amplicons are assumed to result in more distinctive HRM curves (Reed and Wittwer, 2004; Seipp et al., 2007; Hofinger et al., 2009; Han et al., 2012). Despite its 395-bp size, the cp fragment *trnL-trnF* resulted in clearly distinctive melting graphs for our plasmotypes. This is probably due to the presence of both a SNP and three INDELs.

The lack of a good HRM analysis after implementation of the eight other primer pairs (*coxII-1*, *cob-3*, *trnL-trnF-1*, *trnL-trnF-3*, *trnL-trnF-4*, *trnL-trnF-5*, *ndhF-2* and *ndhF*-3) may be due to an inefficient PCR amplification. PCR conditions must be carefully chosen in order to amplify only one fragment. The presence of non-specific bands, primer dimers or secondary structures can interfere with HRM performance (White and Potts, 2006; Lehmensiek et al., 2008). Another reason for the failure of putative HRM primer pairs can be the inability to identify SNPs located closely to the amplicon's primers (<20 bp) as described by Hofinger et al. (2009) and Botticella et al. (2011). However, similar to the observations of Reed and Wittwer (2004), our HRM analysis did not suffer from the distance between primers and SNP position. The primers *coxII-2F*, *cob-1R*, *cob-2R*, *ndhF-1F* and *trnL-trnF-2F* were all located less than 15 bp from the SNP or INDEL. Also, the effect of amplicon concentration on the melting curve shape was negligible because any initial product concentration difference is equalized during the PCR plateau phase (Liew et al., 2004).

In addition to the abovementioned possible problems during PCR amplification, the lack of different melting curves can be another reason for HRM analysis failure. The ability to discriminate homozygous plasmotypes depends on the melting temperature (T_m) difference between the plasmotypes and the potential of the LightCycler® 480 Real-Time PCR system to observe these T_m differences (Herrmann et al., 2006). Liew et al. (2004) has mentioned that differences in T_m depend on the class of SNPs. The following SNP classes were described: class 1, C/T or G/A; class 2, C/A or G/T; class 3, C/G and class 4, T/A. Homozygous genotypes containing class 1 or 2 SNPs were easily distinguished through different T_m. T_m differences between homozygous genotypes containing class-3 or class-4 SNPs were smaller and not always dinstiguisable (Liew et al., 2004; Herrmann et al., 2006). The different alleles of the homozygous *Cichorium* plasmotypes could be discriminated using HRM markers *coxII-2*, *cob-1*, *cob-2* and *ndhF-1* which included class-2 SNPs. The HRM markers *trnL-trnF*

and *trnL-trnF-2* were embedding INDELs that caused a different melting pattern of the different alleles based on differences in amplicon size.

The HRM markers developed were tested on regenerated fused protoplasts to determine the origin of the cytoplasmic organelles of 50 SSR confirmed somatic hybrids. The markers could easily distinguish between the industrial chicory and endive plasmotypes (Fig. 6-5). The results obtained were confirmed by sequencing eight clones of each somatic hybrid for each fragment. The absence of heterozygosity within the mitochondrial fragments in 49 hybrids suggests the preferential establishment of a single plasmotype within each somatic hybrid. No preference for either the *C. intybus* or the *C. endivia*-like mitochondria was observed. On the contrary, all the chloroplast fragments (*trnL-trnF*, *trnL-trnF-2*, and *ndhF-1*) corresponded to the industrial chicory plasmotypes. Cappelle et al. (2007) described a recombinant mitochondrial genome in one somatic hybrid analyzed with the restriction/probe combination *Hin*dIII/*coxII*. However, when tested with other mitochondrial probes (*nad4ex1*, *atp6*, *nad3* and *orfB*), these hybrids showed either the industrial chicory or the endive plasmotype. Varotto et al. (2001) produced 33 asymmetric somatic hybrid plants between *C. intybus* and *Helianthus annuus*. Southern blot analysis of total DNA with the *coxII* probe after *Hin*dIII restriction revealed three regenerants presenting both the sunflower and the chicory plasmotype. One plant showed a recombinant mitochondrial genome.

Cappelle et al. (2007) also used the *trnL-trnF* marker for chloroplast DNA analysis and performed a long migration in 2.5% agarose gels to detect the 13 bp size polymorphism in somatic hybrids of *C. intybus* and *C. endivia*. In their hybrid plants, either the *C. intybus* fragment or the *C. endivia* fragment was observed. According to our findings they did not detect any heterozygosity or recombination in the chloroplast fragments of the somatic hybrids.

Several ways to track SNPs have been developed recently. All of these techniques require a separation step including restriction fragment length polymorphsims (RFLP) analysis, single nucleotide extension (SNE) and sequencing (Liew et al., 2004). Although sequencing is considered a reference technique because of its ability to specifically reproduce the DNA sequence, it is time consuming and expensive to use for tracking SNPs. HRM, in contrast, enables rapid (less than 2 h) and efficient high-throughput scanning of multiple fragments due to the 384-well plates. Moreover, HRM is cost-efficient because of its small reaction volume (10 µl). Because of its low toxicity to PCR, the ResoLight high-resolution melting dye can be used at high concentrations to saturate the dsDNA PCR product and therefore contributes to higher resolution melting sensitivity. The non-destructive character of the technique also

allows a postmelting recovery of DNA fragments for sequencing or cloning (Hofinger et al., 2009).

Two HRM markers (*coxII-2* and *trnL-trnF*) were also able to discriminate heterozygous plasmotypes containing at least 25% of one parental plasmotype (Figs. 6-3, 6-4). However, the heterozygous plasmotypes detected here are mixtures of both parental plasmotypes and not recombinations. When recombinations would occur, new HRM profiles would be detected, not representing the parent plasmotypes nor the mixture of both. These new HRM profiles, however, indicate the presence of irregularities requiring additional analysis. Another disadvantage of HRM is that not all SNPs elicit changes in the melting profile (Liew et al., 2004; Herrmann et al., 2006); parallel melting curves therefore do not implicate the absence of SNPs.

This is the first report on the use of HRM analysis on *Cichorium* species. Our technique provides a fast and simple approach for plasmotyping a pool of DNA from different *Cichorium* plants. Applying this technique when searching for cybrids among protoplast regenerants reduces the labor and expense associated with sequencing.

6.5 Conclusion

Somatic hybridization in *Cichorium* species has already been extensively investigated. Hybrid or cybrid characterization requires an effective plasmotype screening method. We evaluated high-resolution melting (HRM) analysis for the detection of specific mitochondrial and chloroplast markers to distinguish two industrial chicory (*C.intybus* var. *sativum*) plasmotypes from five wild type chicory (*C. intybus*) and two endive (*C. endivia*) plasmotypes. Three mitochondrial (*coxII-2*, *cob-1* and *cob*-2) and three chloroplast HRM markers (*trnL-trnF*, *trnL-trnF-2*, and *ndhF*-1) were successfully developed. Two markers (*coxII-2* and *trnL-trnF*) were additionally able to discriminate heterozygous plasmotypes containing at least 25% of one parental plasmotype. Moreover, the technique was successfully used to characterize the cytoplasms of 50 SSR confirmed somatic hybrids of *C. intybus* var. *sativum* 'VL52' and *C. endivia* var. *crispum* 'Wallone Despa'. HRM enables a rapid (less than 2 h) and efficient high-throughput scanning of multiple fragments due to the 384-well plates, and is cost-efficient because of its small reaction volume of 10 µl. This is the first report on the use of HRM analysis on *Cichorium* species. The technique is a fast and simple alternative for laborious and costly sequencing in plasmotyping regenerants obtained after somatic fusion.

Chapter 7 - Asymmetric protoplast fusion

7.1 Introduction

CMS in chicory has already been introduced by Varotto et al. (2001) through asymmetric protoplast fusion between chicory and CMS-sunflower. These asymmetric hybrids are fusion products between two non-crossable species and thus controversial because of the GMO legislation in Europe. Cappelle et al. (2007) introduced CMS in chicory by symmetric fusion between chicory and endive. This tetraploid hybrid is composed of two sexually crossable (fertile) species. However, these tetraploid plants contained undesired genes from endive, making time-consuming backcrosses needed. Although Cappelle showed the possibility to produce an alloplasmic CMS plant through protoplast fusion of two fertile lines, the real challenge is to create a diploid CMS chicory plant. Therefore, we searched for the introduction of CMS and the broadening of the genetic variation in the industrial chicory by means of asymmetric protoplast fusion between protoplasts of different *Cichorium* species, without producing GMOs, while maintaining the diploid status.

We implemented the knowledge obtained by the aforementioned experiments in §Chapters 2-6; based on the fragmentation techniques of §Chapter 4 and the protoplast fusion conditions obtained in §Chapter 3, we should be able to fuse recipient (industrial chicory) nuclei with donor (wild type chicory and endive) cytoplasms. The protoplast regeneration protocol of §Chapter 2 enables full plantlet development of putative cybrids. §Chapters 5 and 6 describe screening techniques to characterize these regenerants.

7.2 Materials and methods

Plant material

The plants used as acceptor and donor in the experiments were chosen based on their genetic distance calculated through microsatellite analysis, provided by COSUCRA-Groupe Warcoing S.A., Chicoline division. A longer genetic distance between two fusion partners enhances the occurrence of alloplasmic CMS in the regenerants. As acceptors, an inbred line *C. intybus* var. *sativum* 'VL52' and a clone of a heterozygous genotype *C. intybus* var. *sativum* 'K1093' were selected. As donors, the endive *C. endivia* var. *crispum* 'Wallone Despa' and the wild type chicories *C. intybus* 'Ames22531' and 'Pi531291' were used.

Fragmentation, fusion and regeneration

Asymmetric fusion was performed using the PEG-induced chemical protoplast fusion, optimized in §Chapter 3. The acceptor *C. intybus* var. *sativum* 'VL52', treated with 1.625 mM IOA (see §Chapter 4), was fused with the donors *C. endivia* var. *crispum* 'Wallone Despa' and *C. intybus* 'Pi531291'. These donors were UV-irradiated for 1, 2, 6, 10 and 15 min. As a control, asymmetric fusions were performed using untreated acceptor or donor plants; in addition, symmetric fusions between untreated *C. intybus* var. *sativum* 'VL52' and untreated *C. endivia* var. *crispum* 'Wallone Despa' or *C. intybus* 'Pi531291' were performed.

The acceptor *C. intybus* var. *sativum* 'K1093', treated with 1.625 mM IOA, was fused with the donor *C. intybus* 'Ames22531', UV-irradiated for 1, 2, 6, 10 and 15 min. Similar to the former fusion, both asymmetric and symmetric fusions were performed between untreated acceptor and/or donor plants, as a control.

After fragmentation and fusion, protoplasts were regenerated according to §Chapter 2, but the first week of culture was performed under dark conditions, followed by one week under partial light. From week 3 onward, full light conditions were applied (16 h light / 8 h dark). After 4 weeks, the microcalli formation in the beads was analyzed to estimate the regeneration efficiency after fragmentation and fusion. As a control for regeneration efficiency, untreated and unfused acceptor plants were also regenerated.

Regenerant screening

Screening of the asymmetric fusion regenerants was performed using flow cytometry for ploidy determination (see §Chapter 3), microsatellite marker analysis provided by the COSUCRA-Groupe Warcoing S.A., Chicoline division for nuclear genome confirmation and HRM for cytoplasmic genome confirmation (see §Chapter 6). Symmetric fusion products were only screened for their nuclear genome constitution.

7.3 Results

Regeneration efficiency

After 4 weeks of regeneration in the LMPA beads, microcalli formation was analyzed (Table 7-1). For the fusions of untreated *C. intybus* var. *sativum* 'VL52' with untreated and treated *C. endivia* var. *crispum* 'Wallone Despa', microcalli formation was not significantly different from the unfused control acceptor when UV irradiation up to 6 min was used. Longer

irradiation diminished the regeneration capacity in the beads. Fusion of 1.625 mM IOA treated *C. intybus* var. *sativum* 'VL52' protoplasts with irradiated (up to 6 min) *C. endivia* var. *crispum* 'Wallone Despa' protoplasts, yielded higher microcalli formation rates than the unfused control acceptor. After 10 and 15 min irradiation, regeneration efficiencies decreased and were not significantly different from the percentage of microcalli formation from the unfused control acceptor. Similar effects were observed after fusion between *C. intybus* var. *sativum* 'VL52' and *C. intybus* 'Pi531291' and between *C. intybus* var. *sativum* 'K1093' and *C. intybus* 'Ames22531' (Table 7-1).

Regenerant screening

Table 7-2 shows the regenerants obtained after the fusion events. After fusion between *C. intybus* var. *sativum* 'VL52' and *C. endivia* var. *crispum* 'Wallone, 176 asymmetric and 55 symmetric fusion regenerants were obtained. Out of the 55 symmetric fusion regenerants, 7 were hybrid when analyzed with flow cytometry. HRM analysis of the 176 asymmetric fusion regenerants showed 1 heterozygous plasmotype containing a 75 : 25 ratio mixture of the mt *coxII-2* fragment of *C. intybus* var. *sativum* 'VL52' and *C. endivia* var. *crispum* 'Wallone Despa', respectively (Fig. 7-1). This plasmotype was a fusion product between 1.625 mM IOA-treated *C. intybus* var. *sativum* 'VL52' and 2 min UV-irradiated *C. endivia* var. *crispum* 'Wallone Despa' protoplasts. HRM analysis on the cp genome of this plasmotype showed the presence of 100 % parental *C. intybus* var. *sativum* 'VL52' *trnL-trnF* fragments. The mt and cp constitution of the 175 other regenerants were all parental *C. intybus* var. *sativum* 'VL52' following HRM analysis. Due to the regeneration inability of *C. endivia* var. *crispum* 'Wallone Despa' protoplasts, only *C. intybus* var. *sativum* 'VL52' regenerants were observed. This was confirmed through flow cytometry and microsatellite marker analyis.

After fusion between *C. intybus* var. *sativum* 'VL52' and *C. intybus* 'Pi531291', 213 asymmetric and 63 symmetric fusion regenerants were obtained. Within the latter one, 3 symmetric hybrids were detected. HRM analysis and microsatellite marker analysis on the 213 asymmetric fusion regenerants yielded no hybrids nor cybrids. Both parents were found among the regenerants.

Fusion of *C. intybus* var. *sativum* 'K1093' with *C. intybus* 'Ames22531', yielded 128 asymmetric and 45 symmetric fusion regenerants, respectively. Among the regenerants of both the asymmetric and symmetric fusions, no hybrids were found.

Table 7-1 Microcalli formation observed in the LMPA beads (% of the initial number of cultures protoplasts) after fusion between *C. intybus* var. *sativum* 'VL52' and *C. endivia* var. *crispum* 'Wallone Despa' or *C. intybus* 'Pi531291' protoplasts, and between *C. intybus* var. *sativum* 'K1093' and *C. intybus* 'Ames22531'

Acceptor	IOA treatment(mM)	Acceptor microcalli formation[x]	UV irradiation time (min)					
			0	1	2	6	10	15
			donor: *C.endivia* var. *crispum* 'Wallone Despa'					
C. intybus var. *sativum* 'VL52'	0	4.0± 0.7a	4.1± 0.1a	3.7± 0.1a	3.7± 0.2a	3.7± 0.1a	2.5± 0.2b	2.1± 0.1b
	1.625	0.6± 0.2b	2.1± 0.1a	1.7± 0.1a	2.0± 0.2a	1.9± 0.2a	0.6± 0.1b	0.7± 0.2b
			donor: *C. intybus* 'Pi531291'					
C. intybus var. *sativum* 'VL52'	0	4.4± 0.8a	4.6± 0.1a	3.8± 0.1b	3.8± 0.1b	3.8± 0.1b	2.6± 0.2c	2.5± 0.2c
	1.625	0.7± 0.2c	3.9± 0.1a	2.5± 0.1b	2.5± 0.1b	2.3± 0.2b	0.9± 0.1c	0.6± 0.1c
			donor: *C. intybus* 'Ames 22531'					
C. intybus var. *sativum* 'K1093'	0	5.1 ± 0.9a	4.7 ± 0.7a	3.8 ± 0.1ab	3.8 ± 0.1ab	3.8 ± 0.1ab	3.0 ± 0.1b	3.0 ± 0.1b
	1.625	0.5 ± 0.1b	2.5 ± 0.1a	2.4 ± 0.1a	2.3 ± 0.1a	2.2 ± 0.1a	0.4 ± 0.1b	0.4 ± 0.1b

[x] microcalli formation of the untreated and unfused acceptor

Data are means ± SE (n = 6)

a, b, c are significant differences within each line based on Tukey's Post Hoc test, $p \leq 0.05$. Results were compared between the acceptor microcalli formation and the microcalli formation of the fusion events with this acceptor.

Table 7-2 Number of regenerants after fusion between *C. intybus* var. *sativum* 'VL52' and *C. endivia* var. *crispum* 'Wallone Despa' or *C. intybus* 'Pi531291' protoplasts, and between *C. intybus* var. *sativum* 'K1093' and *C. intybus* 'Ames22531'

Acceptor	IOA treatment (mM)	UV irradiation time (min)					
		0	1	2	6	10	15
		donor: *C.endivia* var. *crispum* 'Wallone Despa'					
C. intybus var. *sativum* 'VL52'	0	55	30	35	33	8	8
	1.625	18	15	14	15	0	0
		donor: *C. intybus* 'Pi531291'					
C. intybus var. *sativum* 'VL52'	0	63	49	38	42	6	7
	1.625	32	16	14	9	0	0
		donor: *C. intybus* 'Ames 22531'					
C. intybus var. *sativum* 'K1093'	0	45	25	24	24	2	3
	1.625	20	10	11	9	0	0

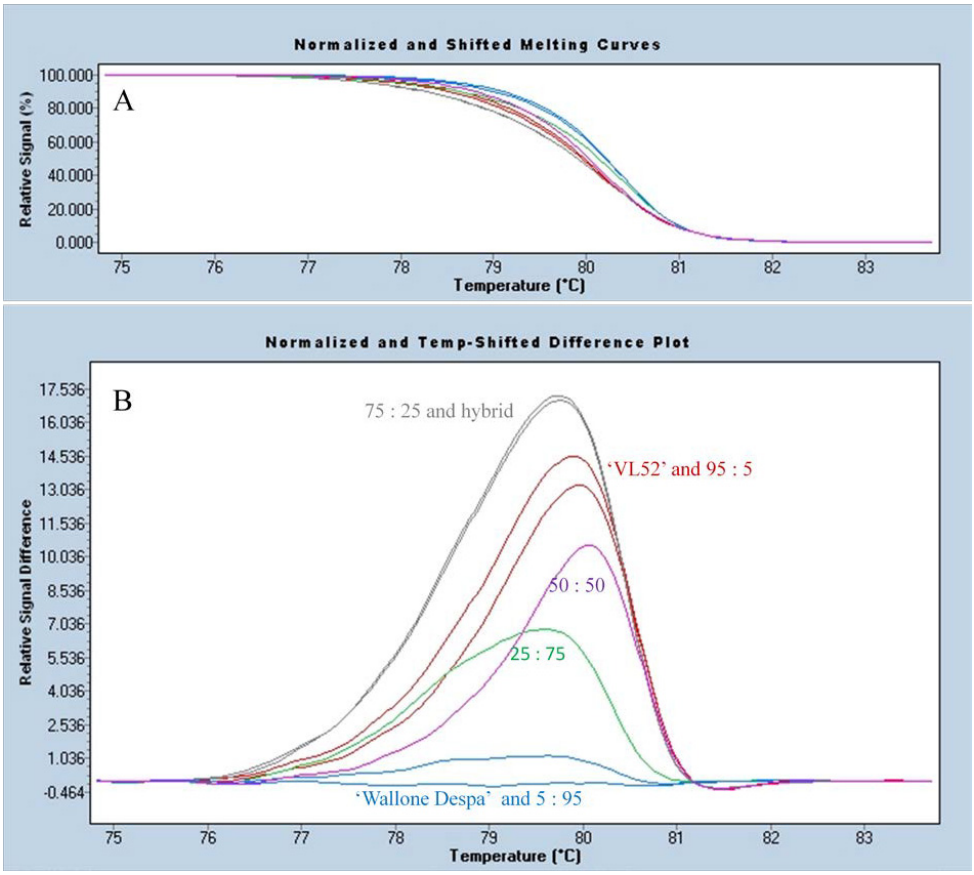


Fig. 7-1 HRM analysis for the *coxII-2* fragment: (A) melting curve and (B) difference plot of a heterozygous plasmotype 'hybrid' of the fusion between 1.625 mM IOA-treated *C. intybus* var. *sativum* 'VL52' and 2 min UV irradiated *C. endivia* var. *crispum* 'Wallone Despa' protoplasts.

7.4 Discussion

Asymmetric protoplast fusions between an industrial chicory and a wild type chicory or endive yielded only one putative cybrid among the 517 tested regenerants. According to HRM analysis, the putative cybrid contained a 75 : 25 ratio mixture of the mt *coxII-2* fragment of *C. intybus* var. *sativum* 'VL52' and *C. endivia* var. *crispum* 'Wallone Despa', respectively. The cybridity still needs to be confirmed by sequencing several clones of its mt *coxII-2* fragment. So far, the sequence information of eight clones revealed only the presence of the mt *coxII-2* fragment of *C. intybus* var. *sativum* 'VL52'. The sequences of more clones are expected soon.

Among the rest of the regenerants, no further hybrids/cybrids were observed. All these regenerants showed the nuclear and corresponding cytoplasmic genome characteristics of one of the parents; *C. intybus* var. *sativum* 'VL52', *C. intybus* 'Pi531291', *C. intybus* var. *sativum* 'K1093' and *C. intybus* 'Ames22531'. This was confirmed through microsatellite marker analysis and HRM analysis for the nuclear and cytoplasmic information, respectively. However, no 100% confirmed absence of asymmetric hybrids among the regenerants can be guaranteed as the 18 microsatellite markers only partially cover 7 of the 9 chromosomes.

Liew et al. (2004) and Herrmann et al. (2006) stated that not all SNPs elicit changes in the HRM melting profiles. Parallel melting curves therefore do not implicate the absence of SNPs. The presence of false-negatives in our results is possible, but minimalized by the HRM markers we developed. We could clearly distinguish different plasmotypes in a pool of putative somatic hybrids after symmetric fusion (§Chapter 6, Fig. 6-5). Two other possible explanations can be suggested; (1) parental escape or (2) cytoplasmic segregation. Although an IOA concentration of 1.625 mM showed severe inhibition of microcalli formation in the control group in these and previous (§Chapter 4.3) experiments, we obtained a large number of IOA-escapes. Parental escape has already been observed in IOA-treated protoplasts used in asymmetric fusions; Terada et al. (1987) has previously described that IOA-treated, inactivated *Brassica* protoplasts were able to regenerate after cell fusion. This was probably due to the 'nurse-effects' of the untreated fusion partner protoplasts. Yamagishi et al. (2002) observed nurse-effects of UV-treated *B. napus* protoplasts by which a higher IOA concentration was needed to inhibit the regeneration of *A. thaliana* protoplast after protoplast fusion. The use of higher IOA concentrations, suggested by Minqin et al. (2005), is no alternative, because of the possibility of protoplast agglutination. In contrast, we did not observe restoration of protoplast regeneration after UV irradiation because of nurse-effects. This is in agreement with observations by Yamagishi et al. (2002) in UV-irradiated *Brassica* protoplasts.

A second hypothesis for the low number of hybrids/cybrids can be cytoplasmic segregation. Chloroplast segregation has already been reported in somatic hybrids of *Brassica* and *Raphanus* species where a bias towards *Brassica* chloroplasts was noticed (Earle et al., 1992). A logically preferential chloroplast segregation was observed from the irradiated fusion partner after double inactivation fusion experiments in *Nicotiana* (Sidorov et al., 1981) and *Brassica* (Kirti et al., 1998) species. However, in contrast, Morgan and Maliga (1987) observed the retention of the chloroplasts of the iodoacetate-treated protoplasts in double inactivation fusions between *Brassica* and *Raphanus* species. These studies indicate that a

biparental transmission of chloroplasts leads to a mixed chloroplast population which is not maintained for a long time before segregation results in cells (plants) with one type of parental chloroplasts (Medgyesy et al., 1980). Butterfass (1989) described the nuclear influence on biased segregation as a control of the number and DNA content of plastids. In somatic hybridizations in *Brassica* (Kirti et al., 1998) and *Nicotiana* (Aviv and Galun, 1988) species a co-transmission of chloroplasts and mitochondria was observed. The nuclear influence and the co-transmission of cytoplasmic organelles may be the reasons for the cytoplasmic segregation towards the chicory plasmotype, because of the absence of a endive nucleus due to UVC-irradiation.

7.5 Conclusion

Through asymmetric protoplast fusion between protoplasts of different *Cichorium* species, we try to induce CMS and broaden the genetic variation in industrial chicory. In this chapter, we analyzed the possibility of producing cybrids containing recipient nuclei and donor cytoplasms. During protoplast regeneration, we observed in all the fusion combinations made, that fusion of 1.625 mM IOA treated acceptor protoplasts with UV-irradiated *C. endivia* var. *crispum* 'Wallone Despa' up to 6 min, yielded higher microcalli formation rates in comparison to the unfused control acceptor. Regenerant screening revealed only one putative cybrid among the 517 tested regenerants of the asymmetric protoplast fusions between industrial chicory and wild type chicory or endive. The putative cybrid contained a 75 : 25 ratio mixture of the mt *coxII-2* fragment of *C. intybus* var. *sativum* 'VL52' and *C. endivia* var. *crispum* 'Wallone Despa', respectively. As possible explanations for the low number of cybrids obtained, parental IOA-escape and cytoplasmic segregation were suggested.

Chapter 8 - Conclusions and future perspectives

Industrial chicory is mainly cultivated for inulin and its hydrolysis products. A high root yield, a high inulin content in the root and high-quality long inulin chains are essential. Both cultural techniques and genotype influence these features (Baert, 1997). Commercial chicory breeding has traditionally been based on intercrossing a number of phenotypically superior parents selected for several commercial traits (Lucchin et al., 2008). The production of chicory hybrids from crosses between distant genotypes showed the expected heterosis effects, indicating that F_1 hybrids can contribute to the future chicory-derived inulin production (Bannerot and Deconinck, 1965; Bannerot and Deconinck, 1970). However, hybrid production requires a good pollination control where selfing of the female line is inhibited (Perez-Prat and van Lookeren Campagne, 2002; Nizampatnam et al., 2009). The development of male sterile lines through biotechnology can open new ways of chicory hybrid breeding. With this PhD work, methods were developed for a protoplast-based somatic hybridization frame by which future (a)symmetric protoplast fusions can be made between *Cichorium* species, delivering an additional tool for chicory breeders. This PhD was performed in close collaboration with the Belgian industrial chicory breeding company COSUCRA-Groupe Warcoing S.A. Chicoline division. The project was based on the background of private knowledge and demands of the COSUCRA-Groupe and on previous studies focusing on somatic hybridization in *Cichorium*:

- COSUCRA-Groupe Warcoing showed through traditional breeding that CMS could be obtained when combining industrial chicory nuclear information with foreign (wild type, endive) cytoplasmic features.
- Reliable protoplast regeneration systems in *Cichorium* were already established, mainly focusing on one particular *Cichorium* species (Crepy et al., 1982; Saksi et al., 1986b; Slabe and Bohanec, 1989; Rambaud et al., 1990; Varotto et al., 1997; Nenz et al., 2000). Somatic hybridization in *Cichorium* species has thoroughly been investigated with symmetric fusions between chicory and endives (Cappelle et al., 2007) and asymmetric fusions between chicory and sunflower (Varotto et al., 2001).

Based on aforementioned knowledge, the aim of this PhD work was to establish a practical protoplast-based somatic hybridization approach that can be used in chicory breeding. For this purpose tools were developed for regeneration, fragmentation, fusion and screening.

First, we successfully regenerated plantlets out of protoplasts of several *Cichorium* types (industrial chicory *C. intybus* var. *sativum*, wild chicory *C. intybus* and endive *C. endivia*) using the LMPA bead technique. For the first time, full plantlet regeneration of *C. endivia*

protoplasts was established. The efficiency of *C. endivia* protoplast regeneration was, however, lower than for *C. intybus* protoplasts. Therefore, efficiency of the regeneration protocol can still be improved by adjusting environmental internal and external conditions. Compared to earlier described protoplast regeneration from *Cichorium* species, our LMPA bead based system is the only one that induces sustained protoplast division and complete regeneration in such a wide *Cichorium* genotype range. Former studies were only applicable on limited chicory genotypes and the culture systems used (liquid, solid, semisolid and Ca-alginate nurse-cultures) showed significant protoplast loss due to clustering and during refreshment steps. We developed a more robust system which by its simplicity also avoids supplementary protoplast loss, enhancing the overall efficiency of the protocol. This was a crucial step for further somatic hybridization in this work and future chicory breeding.
The LMPA bead technique also offers opportunities in other (recalcitrant) crops. The absence of an efficient regeneration protocol for various genotypes is inconceivable when implementing protoplast fusion breeding programmes in any crop. As the LMPA bead technique offers a simple approach through which protoplast loss is reduced, the efficiency of existing protoplast regeneration protocols in other crops should be compared to our technique. Also the search for substantial factors enabling protoplast division should be investigated. For instance, in our case, culture under dark conditions triggered the endive protoplasts to start division.

A second step towards asymmetric protoplast fusion incorporated the fragmentation of the (undesired) nuclear donor genome using UV irradiation. UV irradiation has shown its possibilities in producing asymmetric hybrids in other crops. Moreover, the technique is cheap, safe and easily applicable and preferred over ionizing radiation. Our results highlighted that UVC irradiation prevented cell wall resynthesis and cell division. No cytotoxic effects on *Cichorium* protoplasts were observed. Therefore, UVC irradiation is a promising tool to obtain asymmetric somatic hybrids in *Cichorium* species. The quantification of DNA damage after UVC irradiation treatment by two techniques, standard gel electrophoresis and the Comet assay single cell gel electrophoresis (SCGE) was not successful. As metabolic processes were already heavily hampered at short UVC incubation times, the importance of the long UVC-irradiation effect (10, 20 and 30 min) on DNA is minimalized. Further research should focus not only on DNA levels, but also on RNA and protein levels. Those will probably provide more information on the real dysfunctioning of the nuclear genome. As the distribution of UV-induced DNA damage along the DNA strands depends on the nucleotide

sequence and on the association of DNA with chromosomal proteins (Pfeifer, 1997), it can be valuable to screen the sequence for putative UV-inducible hot spots. These hot spots can be correlated to genes and thus, RNA and proteins. For example, it is known that UV-induced CPDs can occur at DNA sequences containing 5'-TT, 5'-TC, 5'-CT or 5'CC. The 5'-TT is the preferred sequence for CPD formation, whereas 5'-CC is the least preferred sequence (Pfeifer, 1997).

Recipient cytoplasm inactivation was the following step towards asymmetric protoplast fusion. The IOA sensitivity of industrial chicory cultivars was estimated by analyzing protoplast viability and regeneration after IOA treatment. The optimal IOA concentration obtained in this study was 1.625 mM whereby heavily affected protoplast viability and regeneration was observed. At this concentration, still escapes were possible. At higher IOA concentrations no divisions occurred, but the solution became more viscous, causing protoplast agglutination, unsuitable for PEG-induced protoplast fusion. As with UV-induced DNA damage, IOA-induced cytoplasmic damage was not clearly visualized. Although IOA impedes the mitochondrial oxidative phosphorylation and glycolysis and thus reduces the production of ATP, destruction of the cytoplasmic cell organelles was not observed. This indicates that recovery of the cytoplasmic features can be expected after washing and culturing. Moreover, Galtier (2011) noted that this recovery can be enhanced by the nucleus: the copy number and general regulation of the mt genome is under nuclear control, through which efficient DNA repair can be induced. As a consequence, the IOA concentration of 1.625 mM, showing severe inhibition of microcalli formation in §Chapter 4.3, can be too low to be used in asymmetric fusion experiments where 'nurse-effects' of untreated fusion partner protoplasts are likely.

Optimization of the fusion protocol was performed by testing the two common fusion techniques, PEG-induced chemical and electrical fusion. It was expected that electrical fusion was the better option because of its more easily controlled fusion conditions (Bates et al., 1987). However, our results showed that only the PEG-mediated chemical fusion was reliable in *Cichorium* somatic hybridization. The failure of electrical fusion was possibly due to toxic effects of the iso-osmotic buffer, the sensitivity of cells towards electric pulses or the changed optimal conditions in the helix fusion chamber. Both fusion techniques, however, when used in asymmetric fusion, must deal with the IOA viscosity observed in §Chapter 4.3. This IOA viscosity can interfere with the optimal protoplast fusion conditions established in §Chapter 3. One solution is the use of lower IOA and/or PEG concentrations, causing more escapes and/or

less fusion events, respectively. The newly developed electrochemical fusion of Olivares-Fuster et al. (2005), combining a low PEG concentration with a DC pulse-promoted membrane fusion, can be an alternative.

Not much was known about the *Cichorium* species genome and karyotype. Karyological analyses and FISH have already often been used to characterize species (Jiang and Gill, 2006). The karyotypes made in this study for the industrial chicory *C. intybus* var. *sativum* 'VL52' and the endive *C. endivia* var. *crispum* 'Wallone Despa' give a basic view on the different physical chromosome constitution of both species in comparison to molecular techniques. The application of GISH was promising for further research in our *Cichorium* genotypes, as GISH is mostly used in plant species with large-sized chromosomes (Van Laere et al., 2010). Due to a high degree of homology between *C. intybus* and *C. endivia*, a differentiation was not possible, implicating the same problem when performing GISH on intraspecific somatic hybrids of industrial and wild type chicory. Nonetheless, karyotype analysis, FISH and GISH are labor intensive techniques, which allow no high-throughput screening of putative hybrid regenerants. These techniques can be used on hybrid-confirmed regenerants who require further research.

In contradiction to karyotyping and ISH, HRM analysis is a much faster and more sensitive detection technique. Normally, HRM is inserted in the SNP genotyping of plant species. We used HRM as a screening technique, rather than a SNP localization technique. Based on gathered *Cichorium* sequence information, we adjusted HRM to locate specific cytoplasmic organelles in a pool of DNA from different *Cichorium* plants. In our study, two HRM markers (mt fragment *coxII-2* and cp fragment *trnL-trnF*) were able to discriminate heterozygous plasmotypes. However, heterozygous plasmotypes can be mixtures of both parental plasmotypes or recombinations. The HRM markers established will be able to detect the mixture plasmotypes. Recombinations, on the other hand, will produce unique HRM profiles, not representing the parental plasmotypes nor the mixtures and will require additional analysis. Also not all present SNPs will change the HRM melting profiles, causing false-negatives. With our HRM markers, which clearly distinguished different plasmotypes, the presence of false-negatives was minimalized. Applying this technique when searching for cybrids among protoplast regenerants reduces the labor and expense associated with sequencing, although sequencing will be required to characterize the HRM-confirmed hybrids/cybrids. The developed HRM analysis was used on organelle genomes in our study,

but can also be applied on nuclear fragments. Also for other plant species where somatic hybridization research is done, HRM analysis might be a valuable new screening tool. Before application, sequence information of these species is required.

The acquired knowledge of the aforementioned experiments was combined in asymmetric fusion experiments. Only one putative cybrid among the 517 regenerants was found. Among the rest of the regenerants, no further hybrids/cybrids were observed. Although flow cytometric, HRM and microsatellite marker analysis were used, the presence of false-negatives could not be ruled out completely. Other molecular techniques, such as AFLP, RFLP and CAPS can give additional information on the genomic constitution of the regenerants. Through GISH, cytogenetic information of the regenerant chromosomes could be highly valuable to distinguish the (fragments of) donor chromosomes. However, the high homology between the industrial chicory and the wild type chicory/endive genome impedes the use of this technique. Besides the occurrence of false-negatives, two other hypothesis can explain the absence of hybrids/cybrid: parental escape and cytoplasmic segregation. Except for the putative hybrid, the asymmetric fusion regenerants consisted of *C. intybus* var. *sativum* 'VL52' regenerants, containing both the chicory nuclear and cytoplasmic genome. This is in contrast with the results obtained by HRM analysis (§Chapter 5.2) of the symmetric fusion experiments between *C. intybus* var. *sativum* 'VL52' and *C. endivia* var. *crispum* 'Wallone Despa'; out of 50 somatic hybrids, 26 contained the mitochondrial fragments of the industrial chicory, whereas 23 contained the mitochondrial fragments of the endive plasmotype. In one somatic hybrid, the *coxII-2* and *cob-1* endive fragments and the *cob-2* industrial chicory fragment were present. All the chloroplast fragments corresponded to the industrial chicory plasmotypes. The absence of heterozygosity suggests the presence of cytoplasmic segregation towards either one of the parents for mitochondrial fragments and towards the chicory chloroplasts. This also means that no co-transmission between chloroplasts and mitochondria was present, as endive mitochondria coexisted with chicory chloroplasts in 23 of the 50 somatic hybrids. The difference in presence of endive mitochondria in somatic hybrids from symmetric fusions and their absence in asymmetric fusions, can be explained by the presence of the endive nucleus in the tetraploid somatic hybrids. In the asymmetric fusion regenerants, only the chicory nucleus was observed, suggesting the explanation for the chicory plasmotypes in these regenerants. More research, however, is needed to gain a more thorough knowledge on cytoplasmic segregation, starting at early stages of protoplast regeneration. This is thwarted by difficulties that arise when screening single cell plasmotypes, including

the DNA isolation from a single cell/microcolony. If cytoplasmic segregation is indeed hindering the production of desired cybrids, the use of higher IOA concentrations for the inactivation of the recipient parent might be considered, increasing the chance of protoplast agglutionation. An alternative is searching for other *Cichorium* species as donors and recipients by which less or no undesired cytoplasmic segregation is observed.

In this thesis, several tools have been developed for a protoplast-based approach in chicory breeding. So far, one putative cybrid was obtained. Testing larger numbers of regenerants as well as testing different genotype combinations in asymmetric fusions might increase the chance to find cybrids in *Cichorium*. COSUCRA-Groupe Warcoing by intraspecific crossing and Cappelle by interspecific symmetric fusion showed both that CMS can be obtained when combining the industrial chicory nucleus with foreign (wild type, endive) cytoplasmic features. These results clearly demonstrate that also via asymmetric fusion CMS can be introduced in elite breeding material. This will be a necessary step for the creation of hybrids. Therefore, this thesis can be used as a good platform for future protoplast research in chicory breeding, where high-throughput fusions are made to create hybrids and cybrids, introducing CMS and broadening the genetic variation.

Once CMS material is introduced in elite breeding material, further research will focus on hybrid production. For hybrid production, we need inbred lines as pollinators to cross with our CMS line. The hybrid progeny will be analyzed on its root shape, weight, inulin content and chain length and on its male sterility stability. Progeny fertility might be restored after crossing with certain pollinator lines. These lines are withdrawn from the breeding program.

Summary

Since 1990, breeding of industrial root chicory (*C. intybus* var. *sativum*) has known a revival because of the presence of inulin in the root. Inulin chains are fructans with β (2→1) fructosyl-fructose linkages. These β (2→1) bonds are not digested by animal intestinal enzymes and are, therefore, low caloric dietary fibers. Inulin also promotes the absorption of calcium by lowering the pH of the colon. Besides these prebiotic features, oligofructose, inulin's short chain hydrolysis product, has sweetening features. Nowadays, the production of health promoting nutrition based on inulin is marketed. Next to the medicinal and nutritive aspects, inulin is ascribed a promising alternative for raw materials and is found in cosmetics.
Nowadays, industrial chicory breeding is based on the improvement of root yield, inulin content and high-quality long inulin chains. Hybrid breeding trials indicated heterosis effects in F_1 progeny. Hybrid production requires a good pollination control where selfing of the female line is inhibited. Since the observed SI system in chicory is not 100% reliable, an alternative is required. Cytoplasmic male sterility (CMS) can contribute to the creation of 100% true hybrids. However, CMS doesn't naturally occur in chicory. In previous studies, CMS has been introduced by asymmetric protoplast fusion between chicory and sunflower, leading to GMO plants. Symmetric fusion between chicory and endive resulted in a tetraploid CMS chicory plant, containing the undesired genes from endive.

The overall goal of this thesis was to develop several tools for a protoplast-based approach to introduce alloplasmic CMS in chicory. Methods were developed to perform asymmetric protoplast fusions between *Cichorium* species. We established protocols for protoplast regeneration, fragmentation and fusion and protocols for hybrid/cybrid regenerant screening.

We successfully developed a full plantlet regeneration protocol for *Cichorium* protoplasts. Low melting point agarose (LMPA) beads surrounded by liquid medium were used for sustained protoplast division. Several *Cichorium* type protoplasts (industrial chicory *C. intybus* var. *sativum*, wild chicory *C. intybus* and endive *C. endivia*) were regenerated using the LMPA bead technique. For the first time, full plantlet regeneration of *C. endivia* protoplasts was established. We developed a robust system which is characterized by its simplicity, leading to a higher regeneration efficiency.
Fragmentation studies were performed using UV irradiation for nuclear fragmentation and iodoacetamide (IOA) treatment for cytoplasm inactivation. The *Cichorium* sensitivity towards

these two techniques was analyzed. Our results highlighted that UVC irradiation prevented cell wall resynthesis and cell division, but no cytotoxicity of the *Cichorium* protoplasts was observed. The optimal IOA concentration for cytoplasm inactivation was 1.625 mM whereby heavily affected protoplast viability and regeneration was observed. Therefore, UVC irradiation and IOA-induced cytoplasm inactivation are promising tools to obtain asymmetric somatic hybrids in *Cichorium*.

Two protoplast fusion techniques, PEG-induced chemical and electrical fusion, were compared. Our results showed that only the PEG-mediated chemical fusion enabled protoplast regeneration. Symmetric fusion hybrids of *C. intybus* var. *sativum* 'VL52' + *C. endivia* var. *crispum* 'Wallone Despa' and *C. intybus* var. *sativum* 'VL52' + *C. intybus* 'Pi531291'were obtained.

For hybrid screening, two techniques were used: Karyological analyses with fluorescence *in situ* hybridization (FISH) and High resolution melting (HRM) analysis. Karyotypes were made for the industrial chicory *C. intybus* var. *sativum* 'VL52' and the endive *C. endivia* var. *crispum* 'Wallone Despa'. 45S and 5S FISH was also performed on these two species.

We developed three mitochondrial (*coxII-2*, *cob-1* and *cob*-2) and three chloropast markers (*trnL-trnF*, *trnL-trnF-2*, and *ndhF*-1) for HRM analysis to discriminate the cytoplasms of two industrial chicories from five wild type chicories and two endive cultivars. The HRM markers established will be able to effectively detect cybrids after asymmetric protoplast fusion.

Finally, we implemented the knowledge obtained by performing asymmetric protoplast fusions between industrial chicory and endive or wild type chicory. One heterozygous plasmotype containing a 75 : 25 ratio mixture of the mt *coxII-2* fragment of *C. intybus* var. *sativum* 'VL52' and *C. endivia* var. *crispum* 'Wallone Despa', respectively, was found. This plasmotype was a fusion product between 1.625 mM IOA-treated *C. intybus* var. *sativum* 'VL52' and 2 min UV-irradiated *C. endivia* var. *crispum* 'Wallone Despa' protoplasts. HRM analysis on the cp genome of this plasmotype showed the presence of 100 % parental *C. intybus* var. *sativum* 'VL52' *trnL-trnF* fragments.

Samenvatting

Sinds 1990 kent het verbouwen van industriële cichorei (*C. intybus* var. *sativum*) een opmars door de aanwezigheid van inuline in de cichoreiwortel. Inulin is een fructaan bestaande uit β (2→1) fructosyl-fructose ketens. De β (2→1) verbindingen kunnen niet verteerd worden door dierlijke, intestinale enzymes. Daardoor is inuline een voedingsvezel. Inulin promoot ook de absorptie van calcium in de darm door een verlaging van de darm-pH. Naast deze prebiotische kenmerken, wordt oligofructose, een korte-keten hydrolyseproduct van inuline, gebruikt als zoetstof. Vandaag de dag wordt gezondheidspromotend voedsel en drank gebaseerd op inuline, verkocht. Naast deze medicinale en nutritive kenmerken, wordt inuline ook gezien als alternatieve koolstofbron en kan je het vinden in cosmeticaproducten.
De industriële cichoreiteelt is gebaseerd op het verhogen van de wortelopbrengst, de inulineconcentratie en -kwaliteit. Vroegere hybridetesten toonden aan dat er een heterosiseffect waarneembaar was in F_1-nakomelingen. Voor een welonderbouwde hybrid productie is echter een goede bestuivingscontrole vereist, waarbij zelfbestuiving verhinderd wordt. Omdat het zelfincompatibiliteitssysteem in cichorei niet 100% betrouwbaar is, can cytoplasmatische mannelijke steriliteit (CMS) de oplossing bieden. CMS komt niet voor in cichorei. Vroegere studies toonden aan dat CMS kan geïntroduceerd worden door asymmetrische protoplastfusie tussen cichorei en zonnebloem. Maar dit leidde tot GGO planten. Symmetrische fusie tussen cichorei en andijvie resulteerde in tetraploïde CMS cichoreiplanten, maar deze bevatten de ongewenste genen van andijvie.

Het overkoepelende doel van deze thesis was het ontwikkelen van alloplasmische CMS in cichorei gebaseerd op protoplastfusie. Protocols werden ontwikkeld om asymmetrische protoplastfusies uit te voeren tussen *Cichorium*-species onderling. We hebben technieken ontwikkeld voor protoplastregeneratie, - fragmentatie en -fusie en voor hybride en cybride regeneranten screening.

We slaagden erin om een protoplastregeneratieprotocol te ontwikkelen voor verschillende Cichorium-species. Low melting point agarose (LMPA) droplets omgeven door vloeibaar medium induceerde en bevorderde protoplastdeling. Verschillende *Cichorium*-type protoplasten (industriële cichorei *C. intybus* var. *sativum*, wild type cichorei *C. intybus* en andijvie *C. endivia*) konden worden geregenereerd met de LMPA droplet techniek. Voor de eerste keer konden ook planten geregenereerd worden uit *C. endivia* protoplasten. We

ontwikkelden een robuust system dat gekarakteriseerd wordt door zijn eenvoud, waardoor een hogere regeneratie-efficiëntie bereikt wordt.

Fragmentatiestudies met UV-bestraling voor nucleusfragmentatie en met iodoacetamidebehandeling (IOA-) voor cytoplasma-inactivatie werden uitgevoerd. De gevoeligheid van *Cichorium* species tegenover deze twee technieken werd geanalyseerd. De resultaten leerden ons dat UVC-bestraling de celwandsynthese en celdeling van protoplasten verhinderde, zonder daarbij cytotoxisch te zijn. De optimale IOA-concentratie voor cytoplasma-inactivatie was 1,625 mM. Daarbij werd de protoplastleefbaarheid en -regeneratie ernstig verstoord. UVC-bestraling en IOA-geïnduceerde cytoplasma-inactivatie zijn veelbelovende technieken om asymmetrische somatische hybriden te bekomen in *Cichorium*.

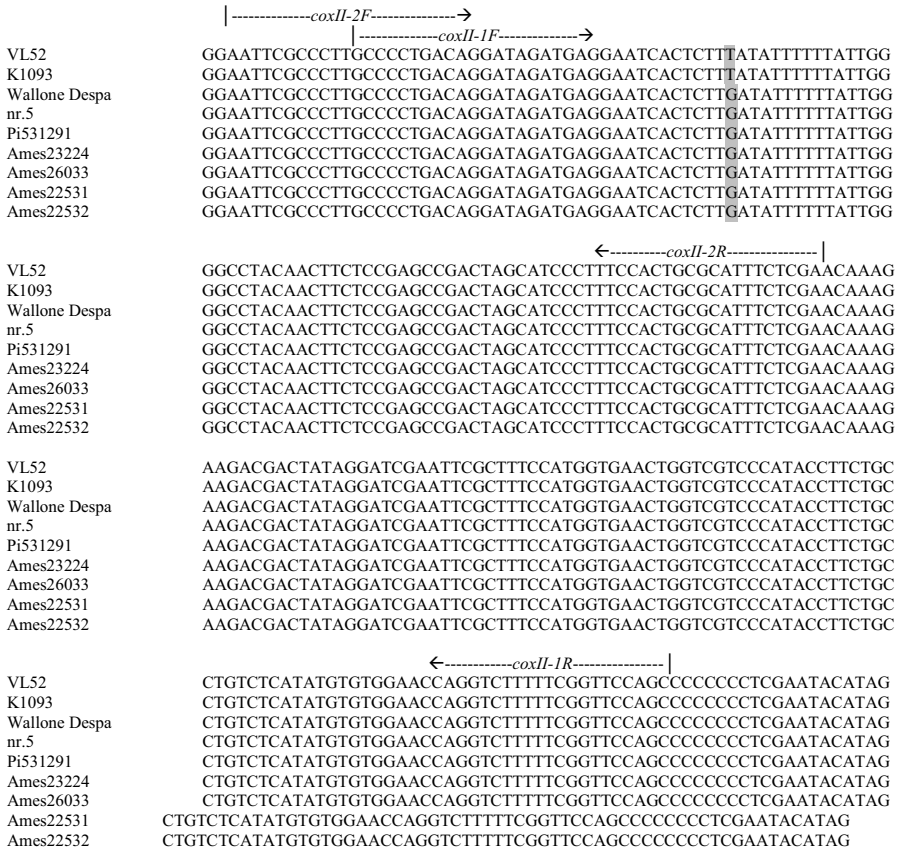
Twee protoplastfusietechnieken werden vergeleken: PEG-geïnduceerde (chemische) en elektrische fusie. We leerden dat enkel de PEG-geïnduceerde fusie protoplastregeneratie toeliet. Symmetrische fusie-hybriden werden bekomen tussen *C. intybus* var. *sativum* 'VL52' + *C. endivia* var. *crispum* 'Wallone Despa' en *C. intybus* var. *sativum* 'VL52' + *C. intybus* 'Pi531291'.

Twee technieken werden toegepast voor hybride screening: Karyologische analyse met fluorescentie *in situ* hybridisatie (FISH) en High resolution melting (HRM) analyse. Karyotypes van industriële cichorei *C. intybus* var. *sativum* 'VL52' en andijvie *C. endivia* var. *crispum* 'Wallone Despa' werden bekomen. Op deze twee species werd ook 45S en 5S FISH uitgevoerd.

We ontwikkelden 3 mitochondriale (*coxII-2*, *cob-1* and *cob*-2) en 3 chloroplastmerkers (*trnL-trnF*, *trnL-trnF-2*, and *ndhF*-1) voor HRM-analyse om de cytoplasma's van twee industriële cichoreien te kunnen onderscheiden van vijf wild types en twee andijvies. Met de HRM-merkers zal het mogelijk zijn om op een efficiënte manier cybriden te detecteren na asymmetrische protoplastfusie.

Uiteindelijk hebben we de opgebouwde kennis gebruikt in asymmetrische protoplastfusies tussen industriële cichorei en wild type cichorei of andijvie. Eén heterozygoot plasmotype werd gevonden waarin een 75 : 25 ratio mengsel van het mt *coxII-2* fragment van *C. intybus* var. *sativum* 'VL52' en *C. endivia* var. *crispum* 'Wallone Despa', respectievelijk, aanwezig was. Dit plasmotype was een fusieproduct va 1.625 mM IOA-behandelde *C. intybus* var. *sativum* 'VL52' en 2 min UVC-bestraalde *C. endivia* var. *crispum* 'Wallone Despa' protoplasten. HRM-analyse van het cp genoom toonde de aanwezigheid van 100% parentale *C. intybus* var. *sativum* 'VL52' *trnL-trnF* fragmenten.

Supplementary Data



Supplemental Fig. 6-1 Part of the alignment of the *coxII* amplicon sequences (1700 bp) of the 9 *Cichorium* species: One SNP (grey) at 46 bp was detected, which discriminated the industrial chicory plasmotypes ('VL52', 'K1093') from the endives ('Wallone Despa', 'nr.5') and wild type plasmotypes ('Pi531291', 'Ames23224', 'Ames26033', 'Ames22531', 'Ames22532'). Two primer pairs for HRM analysis are depicted above the sequences (arrows).

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VL52            GAGTTATAGCAGTCCTAGGGAATTTGTTCGGTGTCTCGGAGTTGTAATCTTCCTATTAATGATTGTGACA
K1093           GAGTTATAGCAGTCCTAGGGAATTTGTTCGGTGTCTCGGAGTTGTAATCTTCCTATTAATGATTGTGACA
Wallone Despa   GAGTTATAGCAGTCCTAGGGAATTTGTTCGGTGTCTCGGAGTTGTAATCTTCCTATTAATGATTGTGACA
nr.5            GAGTTATAGCAGTCCTAGGGAATTTGTTCGGTGTCTCGGAGTTGTAATCTTCCTATTAATGATTGTGACA
Pi531291        GAGTTATAGCAGTCCTAGGGAATTTGTTCGGTGTCTCGGAGTTGTAATCTTCCTATTAATGATTGTGACA
Ames23224       GAGTTATAGCAGTCCTAGGGAATTTGTTCGGTGTCTCGGAGTTGTAATCTTCCTATTAATGATTGTGACA
Ames26033       GAGTTATAGCAGTCCTAGGGAATTTGTTCGGTGTCTCGGAGTTGTAATCTTCCTATTAATGATTGTGACA
Ames22531       GAGTTATAGCAGTCCTAGGGAATTTGTTCGGTGTCTCGGAGTTGTAATCTTCCTATTAATGATTGTGACA
Ames22532       GAGTTATAGCAGTCCTAGGGAATTTGTTCGGTGTCTCGGAGTTGTAATCTTCCTATTAATGATTGTGACA


                                                |----------cob-1F----------→
VL52                  GCTTTTATAGGATACGTACCACCTTGGGGTCAGATGAGCTTTTGGGGGGCTACAGTAATT
K1093                 GCTTTTATAGGATACGTACCACCTTGGGGTCAGATGAGCTTTTGGGGGGCTACAGTAATT
Wallone Despa         GCTTTTATAGGATACGTACCACCTTGGGGTCAGATGAGCTTTTGGGGGGCTACAGTAATT
nr.5                  GCTTTTATAGGATACGTACCACCTTGGGGTCAGATGAGCTTTTGGGGGGCTACAGTAATT
Pi531291              GCTTTTATAGGATACGTACCACCTTGGGGTCAGATGAGCTTTTGGGGGGCTACAGTAATT
Ames23224             GCTTTTATAGGATACGTACCACCTTGGGGTCAGATGAGCTTTTGGGGGGCTACAGTAATT
Ames26033             GCTTTTATAGGATACGTACCACCTTGGGGTCAGATGAGCTTTTGGGGGGCTACAGTAATT
Ames22531       GCTTTTATAGGATACGTACCACCTTGGGGTCAGATGAGCTTTTGGGGGGCTACAGTAATT
Ames22532             GCTTTTATAGGATACGTACCACCTTGGGGTCAGATGAGCTTTTGGGGGGCTACAGTAATT

VL52                  ACAAGCTTAGCTAGCGCCATACCCGTAGTAGGAGATACCATAGTGACTTGGCTTTGGGGT
K1093                 ACAAGCTTAGCTAGCGCCATACCCGTAGTAGGAGATACCATAGTGACTTGGCTTTGGGGT
Wallone Despa         ACAAGCTTAGCTAGCGCCATACCCGTAGTAGGAGATACCATAGTGACTTGGCTTTGGGGT
nr.5                  ACAAGCTTAGCTAGCGCCATACCCGTAGTAGGAGATACCATAGTGACTTGGCTTTGGGGT
Pi531291              ACAAGCTTAGCTAGCGCCATACCCGTAGTAGGAGATACCATAGTGACTTGGCTTTGGGGT
Ames23224             ACAAGCTTAGCTAGCGCCATACCCGTAGTAGGAGATACCATAGTGACTTGGCTTTGGGGT
Ames26033             ACAAGCTTAGCTAGCGCCATACCCGTAGTAGGAGATACCATAGTGACTTGGCTTTGGGGT
Ames22531       ACAAGCTTAGCTAGCGCCATACCCGTAGTAGGAGATACCATAGTGACTTGGCTTTGGGGT
Ames22532             ACAAGCTTAGCTAGCGCCATACCCGTAGTAGGAGATACCATAGTGACTTGGCTTTGGGGT


                                  ←----------cob-1R----------|
VL52                  GGGTTCTCCGTGGACAATGCCACCTTAAATCGTTTTTTTAGTCTTCATCATTTACTCCCC
K1093                 GGGTTCTCCGTGGACAATGCCACCTTAAATCGTTTTTTTAGTCTTCATCATTTACTCCCC
Wallone Despa         GGTTTCTCCGTGGACAATGCCACCTTAAATCGTTTTTTTAGTCTTCATCATTTACTCCCC
nr.5                  GGTTTCTCCGTGGACAATGCCACCTTAAATCGTTTTTTTAGTCTTCATCATTTACTCCCC
Pi531291              GGTTTCTCCGTGGACAATGCCACCTTAAATCGTTTTTTTAGTCTTCATCATTTACTCCCC
Ames23224             GGTTTCTCCGTGGACAATGCCACCTTAAATCGTTTTTTTAGTCTTCATCATTTACTCCCC
Ames26033             GGTTTCTCCGTGGACAATGCCACCTTAAATCGTTTTTTTAGTCTTCATCATTTACTCCCC
Ames22531       GGTTTCTCCGTGGACAATGCCACCTTAAATCGTTTTTTTAGTCTTCATCATTTACTCCCC
Ames22532       GGTTTCTCCGTGGACAATGCCACCTTAAATCGTTTTTTTAGTCTTCATCATTTACTCCCC

VL52                  TTTCTTTTAGTAGGCGCCAGTCTTCTTCATCTGGCCGCATTGCATCAATATGGATCAAAT
K1093                 TTTCTTTTAGTAGGCGCCAGTCTTCTTCATCTGGCCGCATTGCATCAATATGGATCAAAT
Wallone Despa         TTTCTTTTAGTAGGCGCCAGTCTTCTTCATCTGGCCGCATTGCATCAATATGGATCAAAT
nr.5                  TTTCTTTTAGTAGGCGCCAGTCTTCTTCATCTGGCCGCATTGCATCAATATGGATCAAAT
Pi531291              TTTCTTTTAGTAGGCGCCAGTCTTCTTCATCTGGCCGCATTGCATCAATATGGATCAAAT
Ames23224             TTTCTTTTAGTAGGCGCCAGTCTTCTTCATCTGGCCGCATTGCATCAATATGGATCAAAT
Ames26033             TTTCTTTTAGTAGGCGCCAGTCTTCTTCATCTGGCCGCATTGCATCAATATGGATCAAAT
Ames22531       TTTCTTTTAGTAGGCGCCAGTCTTCTTCATCTGGCCGCATTGCATCAATATGGATCAAAT
Ames22532             TTTCTTTTAGTAGGCGCCAGTCTTCTTCATCTGGCCGCATTGCATCAATATGGATCAAAT


VL52                  AATCCATTGGGTGTACATTCAGAGATGGATAAAATTGCTTCTTACCCTTATTTTTATGTA
K1093                 AATCCATTGGGTGTACATTCAGAGATGGATAAAATTGCTTCTTACCCTTATTTTTATGTA
Wallone Despa         AATCCATTGGGTGTACATTCAGAGATGGATAAAATTGCTTCTTACCCTTATTTTTATGTA
nr.5                  AATCCATTGGGTGTACATTCAGAGATGGATAAAATTGCTTCTTACCCTTATTTTTATGTA
Pi531291              AATCCATTGGGTGTACATTCAGAGATGGATAAAATTGCTTCTTACCCTTATTTTTATGTA
Ames23224             AATCCATTGGGTGTACATTCAGAGATGGATAAAATTGCTTCTTACCCTTATTTTTATGTA
Ames26033             AATCCATTGGGTGTACATTCAGAGATGGATAAAATTGCTTCTTACCCTTATTTTTATGTA
Ames22531       AATCCATTGGGTGTACATTCAGAGATGGATAAAATTGCTTCTTACCCTTATTTTTATGTA
Ames22532             AATCCATTGGGTGTACATTCAGAGATGGATAAAATTGCTTCTTACCCTTATTTTTATGTA

VL52                  AAGGATCTAGTAGGTTGGGTAGCTTTTGCTATCTTTTCTTCCATTTTTATTTTTTATGCT
K1093                 AAGGATCTAGTAGGTTGGGTAGCTTTTGCTATCTTTTCTTCCATTTTTATTTTTTATGCT
Wallone Despa         AAGGATCTAGTAGGTTGGGTAGCTTTTGCTATCTTTTCTTCCATTTTTATTTTTTATGCT
nr.5                  AAGGATCTAGTAGGTTGGGTAGCTTTTGCTATCTTTTCTTCCATTTTTATTTTTTATGCT
Pi531291              AAGGATCTAGTAGGTTGGGTAGCTTTTGCTATCTTTTCTTCCATTTTTATTTTTTATGCT
Ames23224             AAGGATCTAGTAGGTTGGGTAGCTTTTGCTATCTTTTCTTCCATTTTTATTTTTTATGCT
Ames26033             AAGGATCTAGTAGGTTGGGTAGCTTTTGCTATCTTTTCTTCCATTTTTATTTTTTATGCT
Ames22531       AAGGATCTAGTAGGTTGGGTAGCTTTTGCTATCTTTTCTTCCATTTTTATTTTTTATGCT
Ames22532             AAGGATCTAGTAGGTTGGGTAGCTTTTGCTATCTTTTCTTCCATTTTTATTTTTTATGCT
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VL52            CCTAATGTTTTGGGGCATCCCGACAATTATATACCTGCTAATCCGATGCCCACCCCGCCT
K1093           CCTAATGTTTTGGGGCATCCCGACAATTATATACCTGCTAATCCGATGCCCACCCCGCCT
Wallone Despa   CCTAATGTTTTGGGGCATCCCGACAATTATATACCTGCTAATCCGATGCCCACCCCGCCT
nr.5            CCTAATGTTTTGGGGCATCCCGACAATTATATACCTGCTAATCCGATGCCCACCCCGCCT
Pi531291        CCTAATGTTTTGGGGCATCCCGACAATTATATACCTGCTAATCCGATGCCCACCCCGCCT
Ames23224       CCTAATGTTTTGGGGCATCCCGACAATTATATACCTGCTAATCCGATGCCCACCCCGCCT
Ames26033       CCTAATGTTTTGGGGCATCCCGACAATTATATACCTGCTAATCCGATGCCCACCCCGCCT
Ames22531   CCTAATGTTTTGGGGCATCCCGACAATTATATACCTGCTAATCCGATGCCCACCCCGCCT
Ames22532       CCTAATGTTTTGGGGCATCCCGACAATTATATACCTGCTAATCCGATGCCCACCCCGCCT

                          |----------cob-2F----------→
VL52            CATATTGTACCGGAATGGTATTTCCTACCGATCCATGCCATTCTTCGTAGTATACCTGAC
K1093           CATATTGTACCGGAATGGTATTTCCTACCGATCCATGCCATTCTTCGTAGTATACCTGAC
Wallone Despa   CATATTGTACCGGAATGGTATTTCCTACCGATCCATGCCATTCTTCGTAGTATACCTGAC
nr.5            CATATTGTACCGGAATGGTATTTCCTACCGATCCATGCCATTCTTCGTAGTATACCTGAC
Pi531291        CATATTGTACCGGAATGGTATTTCCTACCGATCCATGCCATTCTTCGTAGTATACCTGAC
Ames23224       CATATTGTACCGGAATGGTATTTCCTACCGATCCATGCCATTCTTCGTAGTATACCTGAC
Ames26033       CATATTGTACCGGAATGGTATTTCCTACCGATCCATGCCATTCTTCGTAGTATACCTGAC
Ames22531   CATATTGTACCGGAATGGTATTTCCTACCGATCCATGCCATTCTTCGTAGTATACCTGAC
Ames22532       CATATTGTACCGGAATGGTATTTCCTACCGATCCATGCCATTCTTCGTAGTATACCTGAC

                         |----------cob-3F----------→
VL52            AAAGCGGGAGGTGTAGCCGCAATAGCACCAGTTTTTATATGTCTGTTGGCTTTACCTTTT
K1093           AAAGCGGGAGGTGTAGCCGCAATAGCACCAGTTTTTATATGTCTGTTGGCTTTACCTTTT
Wallone Despa   AAAGCGGGAGGTGTAGCCGCAATAGCACCAGTTTTTATATGTCTGTTGGCTTTACCTTTT
nr.5            AAAGCGGGAGGTGTAGCCGCAATAGCACCAGTTTTTATATGTCTGTTGGCTTTACCTTTT
Pi531291        AAAGCGGGAGGTGTAGCCGCAATAGCACCAGTTTTTATATGTCTGTTGGCTTTACCTTTT
Ames23224       AAAGCGGGAGGTGTAGCCGCAATAGCACCAGTTTTTATATGTCTGTTGGCTTTACCTTTT
Ames26033       AAAGCGGGAGGTGTAGCCGCAATAGCACCAGTTTTTATATGTCTGTTGGCTTTACCTTTT
Ames22531   AAAGCGGGAGGTGTAGCCGCAATAGCACCAGTTTTTATATGTCTGTTGGCTTTACCTTTT
Ames22532       AAAGCGGGAGGTGTAGCCGCAATAGCACCAGTTTTTATATGTCTGTTGGCTTTACCTTTT

                                                ←----------cob-3R----------|
                                          ←----------cob-2R----------|
VL52            TTTAAAAGTATGTATGTACGTAGTTCAAGTTTTCGCCCGATTCACCAAGGAATATTTTGG
K1093           TTTAAAAGTATGTATGTACGTAGTTCAAGTTTTCGCCCGATTCACCAAGGAATATTTTGG
Wallone Despa   TTTAAAAGTCTGTATGTACGTAGTTCAAGTTTTCGCCCGATTCACCAAGGAATATTTTGG
nr.5            TTTAAAAGTCTGTATGTACGTAGTTCAAGTTTTCGCCCGATTCACCAAGGAATATTTTGG
Pi531291        TTTAAAAGTCTGTATGTACGTAGTTCAAGTTTTCGCCCGATTCACCAAGGAATATTTTGG
Ames23224       TTTAAAAGTCTGTATGTACGTAGTTCAAGTTTTCGCCCGATTCACCAAGGAATATTTTGG
Ames26033       TTTAAAAGTCTGTATGTACGTAGTTCAAGTTTTCGCCCGATTCACCAAGGAATATTTTGG
Ames22531   TTTAAAAGTCTGTATGTACGTAGTTCAAGTTTTCGCCCGATTCACCAAGGAATATTTTGG
Ames22532       TTTAAAAGTCTGTATGTACGTAGTTCAAGTTTTCGCCCGATTCACCAAGGAATATTTTGG
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Supplemental Fig. 6-2 Alignment of the complete *cob* amplicon sequences (698 bp) of the 9 *Cichorium* species: Two SNPs (grey) were detected at 193 and 620 bp, which discriminated the cultivated chicory plasmotypes ('VL52', 'K1093') from the endive ('Wallone Despa', 'nr.5') and the wild type plasmotypes ('Pi531291', 'Ames23224', 'Ames26033', 'Ames22531', 'Ames22532'). Three primer pairs for HRM analysis are depicted above the sequences (arrows).

```
VL52            TACTTGTATTGATTCTATTTCTTTGTTCGTTGGATTCTTAGGAATTCCTTTCACTCAAGA
K1093           TACTTGTATTGATTCTATTTCTTTGTTCGTTGGATTCTTAGGAATTCCTTTCACTCAAGA
Wallone Despa   TACTTGTATTGATTCTATTTCTTTGTTCGTTGGATTCTTAGGAATTCCTTTCACTCAAGA
nr.5            TACTTGTATTGATTCTATTTCTTTGTTCGTTGGATTCTTAGGAATTCCTTTCACTCAAGA
Pi531291        TACTTGTATTGATTCTATTTCTTTGTTCGTTGGATTCTTAGGAATTCCTTTCACTCAAGA
Ames23224       TACTTGTATTGATTCTATTTCTTTGTTCGTTGGATTCTTAGGAATTCCTTTCACTCAAGA
Ames26033       TACTTGTATTGATTCTATTTCTTTGTTCGTTGGATTCTTAGGAATTCCTTTCACTCAAGA
Ames22531   TACTTGTATTGATTCTATTTCTTTGTTCGTTGGATTCTTAGGAATTCCTTTCACTCAAGA
Ames22532       TACTTGTATTGATTCTATTTCTTTGTTCGTTGGATTCTTAGGAATTCCTTTCACTCAAGA

VL52            CGTTGATATATTATCCAAATGGTTAACTCCCTCTATAAATCTTTTACATAAAAATTCAAA
K1093           CGTTGATATATTATCCAAATGGTTAACTCCCTCTATAAATCTTTTACATAAAAATTCAAA
Wallone Despa   CGTTGATATATTATCCAAATGGTTAACTCCGTCTATAAATCTTTTACATAAAAATTCAAA
nr.5            CGTTGATATATTATCCAAATGGTTAACTCCGTCTATAAATCTTTTACATAAAAATTCAAA
Pi531291        CGTTGATATATTATCCAAATGGTTAACTCCCTCTATAAATCTTTTACATAAAAATTCAAA
Ames23224       CGTTGATATATTATCCAAATGGTTAACTCCCTCTATAAATCTTTTACATAAAAATTCAAA
Ames26033       CGTTGATATATTATCCAAATGGTTAACTCCCTCTATAAATCTTTTACATAAAAATTCAAA
Ames22531       CGTTGATATATTATCCAAATGGTTAACTCCCTCTATAAATCTTTTACATAAAAATTCAAA
Ames22532       CGTTGATATATTATCCAAATGGTTAACTCCCTCTATAAATCTTTTACATAAAAATTCAAA

VL52            CAATTTGATAGATTGGTATGAATTTTCTAAAGATGCATTTTTTTCAGTCAGTATAGCTTC
K1093           CAATTTGATAGATTGGTATGAATTTTCTAAAGATGCATTTTTTTCAGTCAGTATAGCTTC
Wallone Despa   CAATTTGATAGATTGGTATGAATTTTCTAAAGATGCATTTTTTTCAGTCAGTATAGCTTC
nr.5            CAATTTGATAGATTGGTATGAATTTTCTAAAGATGCATTTTTTTCAGTCAGTATAGCTTC
Pi531291        CAATTTGATAGATTGGTATGAATTTTCTAAAGATGCATTTTTTTCAGTCAGTATAGCTTC
Ames23224       CAATTTGATAGATTGGTATGAATTTTCTAAAGATGCATTTTTTTCAGTCAGTATAGCTTC
Ames26033       CAATTTGATAGATTGGTATGAATTTTCTAAAGATGCATTTTTTTCAGTCAGTATAGCTTC
Ames22531       CAATTTGATAGATTGGTATGAATTTTCTAAAGATGCATTTTTTTCAGTCAGTATAGCTTC
Ames22532       CAATTTGATAGATTGGTATGAATTTTCTAAAGATGCATTTTTTTCAGTCAGTATAGCTTC

VL52            TTTCGGAATATTTATAGCATTTTTTTTATATAAACCTGTTTATTCATCTTTTCAAAATT
K1093           TTTCGGAATATTTATAGCATTTTTTTTATATAAACCTGTTTATTCATCTTTTCAAAATT
Wallone Despa   TTTCGGAATATTTATAGCATTTTTTTTATATAAACCTGTTTATTCATCTTTTCAAAATT
nr.5            TTTCGGAATATTTATAGCATTTTTTTTATATAAACCTGTTTATTCATCTTTTCAAAATT
Pi531291        TTTCGGAATATTTATAGCATTTTTTTTATATAAACCTGTTTATTCATCTTTTCAAAATT
Ames23224       TTTCGGAATATTTATAGCATTTTTTTTATATAAACCTGTTTATTCATCTTTTCAAAATT
Ames26033       TTTCGGAATATTTATAGCATTTTTTTTATATAAACCTGTTTATTCATCTTTTCAAAATT
Ames22531       TTTCGGAATATTTATAGCATTTTTTTTATATAAACCTGTTTATTCATCTTTTCAAAATT
Ames22532       TTTCGGAATATTTATAGCATTTTTTTTATATAAACCTGTTTATTCATCTTTTCAAAATT

                |----------ndhF-2F---------->
                           |-----------ndhF-1/3F---------->
VL52            TGGATTTCCTTAATGCATTTGTTAAAATGGGTCCTAATCGAATTTTTTATGACAAAATAAA
K1093           TGGATTTCCTTAATGCATTTGTTAAAATGGGTCCTAATCGAATTTTTTATGACAAAATAAA
Wallone Despa   TGGATTTCCTTAATGCATTTGTTAAAATGGGTCCTAATAGAATTTTTTATGACAAAATAAA
nr.5            TGGATTTCCTTAATGCATTTGTTAAAATGGGTCCTAATAGAATTTTTTATGACAAAATAAA
Pi531291        TGGATTTCCTTAATGCATTTGTTAAAATGGGTCCTAATAGAATTTTTTATGACAAAATAAA
Ames23224       TGGATTTCCTTAATGCATTTGTTAAAATGGGTCCTAATAGAATTTTTTATGACAAAATAAA
Ames26033       TGGATTTCCTTAATGCATTTGTTAAAATGGGTCCTAATAGAATTTTTTATGACAAAATAAA
Ames22531       TGGATTTCCTTAATGCATTTGTTAAAATGGGTCCTAATAGAATTTTTTATGACAAAATAAA
Ames22532       TGGATTTCCTTAATGCATTTGTTAAAATGGGTCCTAATAGAATTTTTTATGACAAAATAAA

                                  <----------ndhF-1/2R----------|
VL52            AAATGCTATATATGATTGGTCATATAATCGGGGTTACATAGATGCCTTTTATGGAAGATT
K1093           AAATGCTATATATGATTGGTCATATAATCGGGGTTACATAGATGCCTTTTATGGAAGATT
Wallone Despa   AAATGCTATATATGATTGGTCATATAATCGGGGTTACATAGATGCCTTTTATGGAAAATT
nr.5            AAATGCTATATATGATTGGTCATATAATCGGGGTTACATAGATGCCTTTTATGGAAAATT
Pi531291        AAATGCTATATATGATTGGTCATATAATCGGGGTTACATAGATGCCTTTTATGGAAGATT
Ames23224       AAATGCTATATATGATTGGTCATATAATCGGGGTTACATAGATGCCTTTTATGGAAGATT
Ames26033       AAATGCTATATATGATTGGTCATATAATCGGGGTTACATAGATGCCTTTTATGGAAGATT
Ames22531       AAATGCTATATATGATTGGTCATATAATCGGGGTTACATAGATGCCTTTTATGGAAGATT
Ames22532       AAATGCTATATATGATTGGTCATATAATCGGGGTTACATAGATGCCTTTTATGGAAGATT

VL52            CTTAACTGCGGGGATGAGAAAATTGGCCGACTTCGCTCATTTTTTTGATAGACGAATAAT
K1093           CTTAACTGCGGGGATGAGAAAATTGGCCGACTTCGCTCATTTTTTTGATAGACGAATAAT
Wallone Despa   CTTAACTGCGGGGATGAGAAAATTGGCCGACTTCGCTCATTTTTTTGATAGACGAATAAT
nr.5            CTTAACTGCGGGGATGAGAAAATTGGCCGACTTCGCTCATTTTTTTGATAGACGAATAAT
Pi531291        CTTAACTGCGGGGATGAGAAAATTGGCCGACTTCGCTCATTTTTTTGATAGACGAATAAT
Ames23224       CTTAACTGCGGGGATGAGAAAATTGGCCGACTTCGCTCATTTTTTTGATAGACGAATAAT
Ames26033       CTTAACTGCGGGGATGAGAAAATTGGCCGACTTCGCTCATTTTTTTGATAGACGAATAAT
Ames22531       CTTAACTGCGGGGATGAGAAAATTGGCCGACTTCGCTCATTTTTTTGATAGACGAATAAT
Ames22532       CTTAACTGCGGGGATGAGAAAATTGGCCGACTTCGCTCATTTTTTTGATAGACGAATAAT
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                ←----------ndhF-3R----------|
VL52            TGATGCAATTCCAAATGGAGTTGGTCTTATGAGTTTCTTTCTAGCAGAGGTTATTAAATC
K1093           TGATGCAATTCCAAATGGAGTTGGTCTTATGAGTTTCTTTCTAGCAGAGGTTATTAAATC
Wallone Despa   TGATGCAATTCCAAATGGAGTTGGTCTTATGAGTTTCTTTCTAGCAGAGGTTATTAAATC
nr.5            TGATGCAATTCCAAATGGAGTTGGTCTTATGAGTTTCTTTCTAGCAGAGGTTATTAAATC
Pi531291        TGATGCAATTCCAAATGGAGTTGGTCTTATGAGTTTCTTTCTAGCAGAGGTTATTAAATC
Ames23224       TGATGCAATTCCAAATGGAGTTGGTCTTATGAGTTTCTTTCTAGCAGAGGTTATTAAATC
Ames26033       TGATGCAATTCCAAATGGAGTTGGTCTTATGAGTTTCTTTCTAGCAGAGGTTATTAAATC
Ames22531       TGATGCAATTCCAAATGGAGTTGGTCTTATGAGTTTCTTTCTAGCAGAGGTTATTAAATC
Ames22532       TGATGCAATTCCAAATGGAGTTGGTCTTATGAGTTTCTTTCTAGCAGAGGTTATTAAATC

VL52            GGTAGGGGGTGGGCGTATTTCTTCCTATCTGTTCTTTTATTTTTCGTATGTAGCAATTTT
K1093           GGTAGGGGGTGGGCGTATTTCTTCCTATCTGTTCTTTTATTTTTCGTATGTAGCAATTTT
Wallone Despa   GGTAGGGGGTGGGCGTATTTCTTCCTATCTGTTCTTTTATTTTTCGTATGTAGCAATTTT
nr.5            GGTAGGGGGTGGGCGTATTTCTTCCTATCTGTTCTTTTATTTTTCGTATGTAGCAATTTT
Pi531291        GGTAGGGGGTGGGCGTATTTCTTCCTATCTGTTCTTTTATTTTTCGTATGTAGCAATTTT
Ames23224       GGTAGGGGGTGGGCGTATTTCTTCCTATCTGTTCTTTTATTTTTCGTATGTAGCAATTTT
Ames26033       GGTAGGGGGTGGGCGTATTTCTTCCTATCTGTTCTTTTATTTTTCGTATGTAGCAATTTT
Ames22531       GGTAGGGGGTGGGCGTATTTCTTCCTATCTGTTCTTTTATTTTTCGTATGTAGCAATTTT
Ames22532       GGTAGGGGGTGGGCGTATTTCTTCCTATCTGTTCTTTTATTTTTCGTATGTAGCAATTTT

VL52            TTTATTAATTTACTACTTTTTTAATCTTTAATCTTGTTG
K1093           TTTATTAATTTACTACTTTTTTAATCTTTAATCTTGTTG
Wallone Despa   TTTATTAATTTACTACTTTTTTAATCTTTAATCTTGTTG
nr.5            TTTATTAATTTACTACTTTTTTAATCTTTAATCTTGTTG
Pi531291        TTTATTAATTTACTACTTTTTTAATCTTTAATCTTGTTG
Ames23224       TTTATTAATTTACTACTTTTTTAATCTTTAATCTTGTTG
Ames26033       TTTATTAATTTACTACTTTTTTAATCTTTAATCTTGTTG
Ames22531       TTTATTAATTTACTACTTTTTTAATCTTTAATCTTGTTG
Ames22532   TTTATTAATTTACTACTTTTTTAATCTTTAATCTTGTTG
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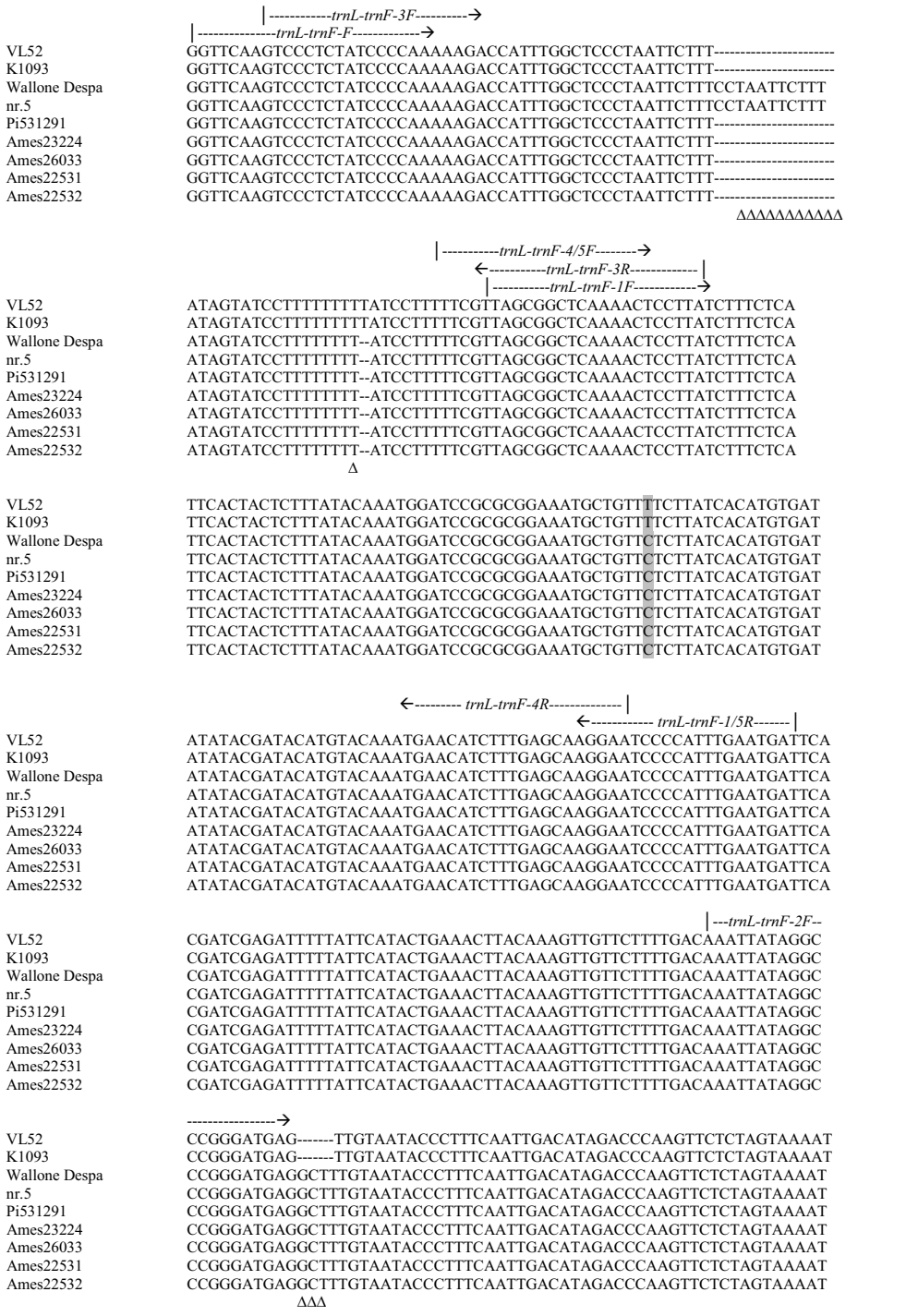
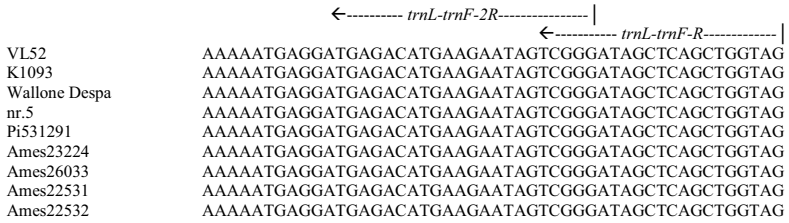
Supplemental Fig. 6-3 Alignment of the complete *ndhF* amplicon sequences (579 bp) of the 9 *Cichorium* species. Three SNPs (grey) were detected: SNPs at 91bp and 357 bp discriminated the endives ('Wallone Despa', 'nr.5') from the others species. The SNP at 278 bp discriminated the industrial chicory plasmotypes ('VL52', 'K1093') from the endive and wild type plasmotypes ('Pi531291', 'Ames23224', 'Ames26033', 'Ames22531', 'Ames22532'). Three primer pairs for HRM analysis are depicted above the sequences (arrows).


trnL-trnF-3F
trnL-trnF-F
trnL-trnF-4/5F
trnL-trnF-3R
trnL-trnF-1F
trnL-trnF-4R
trnL-trnF-1/5R
trnL-trnF-2F
VL52
K1093
Wallone Despa
nr.5
Pi531291
Ames23224
Ames26033
Ames22531
Ames22532



Supplemental Fig. 6-4 Alignment of the complete *trnL-trnF* amplicon sequences (395 bp) of the 9 *Cichorium* species. Three INDELs (triangles) and one SNP (grey) were detected. The SNP at 153 bp discriminated the industrial chicory plasmotypes ('VL52', 'K1093') from the endive ('Wallone Despa', 'nr.5') and the wild type plasmotypes ('Pi531291', 'Ames23224', 'Ames26033', 'Ames22531', 'Ames22532'). Six primer pairs for HRM analysis are depicted above the sequences (arrows).

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Curriculum Vitae Dieter Deryckere

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Degree:

Bioscience Engineer: Cell and Gene Biotechnology (2008)

Education:

Thesis — *Production of transgene and hybrid cell lines and analysis of the saponin biosynthesis in* Maesa *sp* (Department Plant Production University Ghent, 2008).

University — Bioscience Engineer Cell and Gene Biotechnology (2005-2008) University Ghent

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Work Experience:

01/11/2008 – 14/12/2012: Scientific Researcher (Institute for Agricultural and Fisheries Research, Plant Sciences Unit, Applied Genetics and Breeding).
Project: Introduction of CMS and genetic variation in industrial chicory.

Including the practical and technical knowledge of :

- Protoplast fusion and regeneration
- *In vitro* culturing
- FCM (flow cytometry)
- Microscopic analysis
- Fluorescent marker techniques

- Genomic and cytoplasmic DNA marker techniques (PCR, AFLP, RAPD, μsat)
- Chromosome spreads and karyotyping
- GISH/FISH
- Computer analysis: MicroMeasure, Sigmaplot
- DNA Sequencing
- High Resolution Melting (HRM) analysis

Nov./Dec. 2010: Coach Effective Scientific Communication: Coaching PhD students in oral presentation skills. In cooperation with Jean-luc Doumont, Principiae.

Educational guidance of students:

- Simon Deré: Protoplast fusion and regeneration on *Chrysanthemum* and *Spathiphyllum* and detection of *Cichorium* fusion products through HRM, KATHO Roeselare (BE) 2011-2012.
- Aurélie Tredé: Symmetric and asymmetric protoplast fusion on *Cichorium* species for obtaining CMS regenerants, Hogeschool Ghent (BE) 2009-2010.
- Dana Demedts: The use of protoplasts in the breeding of chicory and chrysant, KATHO Roeselare (BE) 2009-2010.
- Senne Degroote: Optimalisation of protoplast and cell suspension culture in chrysant and chicory, KATHO Roeselare (BE) 2008-2009.

Additional Education:

Advanced Academic English: Conference Skills – Leen De Boom, UCT – 2010, Certificate obtained at the University Language Centre (UCT) Ghent in 2011

Advanced Academic English: Writing Skills – Tom De Moor, UCT – 2009, Certificate obtained at the University Language Centre (UCT) Ghent in 2010

Effective Scientific Communication – Jean-luc Doumont, Principiae – 2010

Coaching Oral Presentation Skills – Jean-luc Doumont, Principiae – Nov, 2010

Applied Creative Thinking – Karl Raats (.com) – 2011

Project Management – Tom Jacobs – 2010

Dealing with Conflict – Dan Steer, High Performance People (hipepe.be) – 2010

Personal Effectiveness – Tom Jacobs – 2009

Microscopic Techniques for Molecular and Cellular Analysis - Prof. Erik Manders (NL) - University Ghent – 2010-2011

Applied Plant Biotechnology - Prof. Frank Van Breusegem - University Ghent – 2009-2010

Molecular Techniques in Plant Breeding - Prof. dr. Isabel Roldán-Ruiz - University Ghent – 2008-2009

Developmental Biology of Plants - Prof. Tom Beeckman - University Ghent – 2008-2009

Conference Contributions:

18-22/09/2011: IVCHB 2011 Biotechnological advances in *in vitro* horticultural breeding, Ghent (BE). Oral Presentation: "Optimisation of somatic hybridisation in *Cichorium* species"

05/05/2011: Knowledge for Growth, Ghent (BE). Poster Presentation: "Increasing the inulin production in industrial chicory through somatic hybridization"

20/12/2010: 16th Symposium on Applied Biological Sciences, Ghent (BE). Poster Presentation: "Screening of fusion products after protoplast fusion of industrial chicory with wild chicory and endive"

03/12/2010: BPBA Scientific meeting: "Plant stress biotechnology, Leuven (BE). Poster Presentation: "Screening of fusion products after protoplast fusion of industrial chicory with wild chicory and endive"

13/11/2009: BPBA Scientific meeting: "Plant hormones: new insights for biotechnology", Gembloux (BE). Poster Presentation: "Plant regeneration from leaf mesophyll protoplasts of *Cichorium intybus sativum*"

06/11/2009: 15th PhD Symposium on Applied and Biological Sciences, Leuven (BE). Poster Presentation: "Plant regeneration from leaf mesophyll protoplasts of *Cichorium intybus sativum*"

Articles:

Peer reviewed A1 article in international journal with impact factor:

Deryckere, D., De Keyser, E., Eeckhaut, T., Van Huylenbroeck, J. and Van Bockstaele, E. (2012). High-resolution melting analysis as a rapid and highly sensitive method for *Cichorium* plasmotype characterization. Plant Molecular Biology Reporter

Deryckere, D., Eeckhaut, T., Van Huylenbroeck, J. and Van Bockstaele, E. (2012). Low melting point agarose beads as a standard method for plantlet regeneration from protoplasts within the *Cichorium* genus. Plant Cell Reports 31, 2261-2269 (DOI) 10.1007/s00299-012-1335-8.

Proceedings:

Deryckere, D., Eeckhaut, T., Van Huylenbroeck, J. and Van Bockstaele, E. (2012). Optimisation of somatic hybridization in *Cichorium* species. Acta Horticulturae 961, ISHS 2012, Proc. 7th IS on In vitro culture and horticultural breeding, Ed. D. Geelen.

Peer reviewer:

For Plant and Cell Physiology:
Mehdi Farshad Ashraf, Maheran Abd Aziz, Mihdzar Abdul Kadir and Johnson Stanslas (2012) Microtuberization of *Chlorophytum borivilianum* Sant & Fern (Safed Musli) as influenced by sucrose, CCC and culture systems.

Languages

Dutch	langue maternelle
English	langue professionelle
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