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Agrobacterium-Mediated Transformation of Wheat: General Overview and New Approaches to Model and Identify the Key Factors Involved

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1. Introduction

Wheat is the world's second largest crop, supplying 19% of human calories; the largest volume crop traded internationally and grown on approximately 17% of the world's cultivatable land (over 200 million hectares) (Jones, 2005; Atchison et al., 2010). However, probably due to climate change, some adverse environmental conditions have caused a downward trend in world wheat production (FAO, 2003; 2011). In this context, developing new higher yielding wheat varieties more tolerant or resistant to abiotic and/or biotic stress, using all available plant biotechnology technologies available, should be considered as the major challenge.

The scientific community has made considerable efforts to understand and improve the goal of the integration of an exogenous T-DNA in the genome of a host plant cell and, subsequently, the regeneration into a whole plant. The most extended method for plant genetic transformation uses the *Agrobacterium* bacteria as the biological vector to transfer exogenous T-DNA into the plant cell. Although, *Agrobacterium*-mediated transformation became widely available for the routine transformation of most crops, cereals initially have been recalcitrant to this system, since these crops were not naturally susceptible to *Agrobacterium* sp (Potrykus, 1990, 1991). However, by the mid-1990s, improvements in technological development in *Agrobacterium*-mediated genetic transformation led to the desirable transformation of wheat (Cheng et al., 1997; Peters et al., 1999; Jones et al., 2007). These results "open the avenue" by avoiding the usage of gene direct transfer methods, such as biolistic, which is widely found more disadvantageous compared to *Agrobacterium*-mediated transformation (Jones, 2005; Jones et al., 2007; Khurana et al., 2008).

Developing an appropriate method for genetic *Agrobacterium*-mediated transformation is a highly complex task, because it is essential to understand the effect of all the factors

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influencing the T-DNA delivery into the tissue from which whole plant can be regenerated. After plant regeneration, further analyses were required to check the integration and stability of the T-DNA and to obtain the final transformation efficiency parameter. Artificial intelligence technologies are very successful in establishing relationships, in complex processes, between multiple processing conditions (variables or factors) and the results obtained, using networks approaches. Recently, several studies have demonstrated the effectiveness of artificial neural networks and neurofuzzy logic in modelling and optimizing different plant tissue culture processes. Neurofuzzy logic is a useful modeling tool that has been introduced to help the handling of complex models and to data mining. Data mining can be defined as the process of discovering previously unknown dependencies and relationships in datasets. It is a hybrid technology combining the strength and the adaptive learning capabilities from artificial neural networks (ANNs) and the ability to generalize rules of fuzzy logic. Neurofuzzy logic technology generates understandable and reusable knowledge in the way of IF (conditions) THEN (observed behavior) rules helping the researchers to understand the process or the phenomena they are studying (Gallego et al., 2011).

In this chapter we overview the recent advances in *Agrobacterium*-mediated transformation of the wheat, but we also proposed the utility of artificial intelligence technologies as a modeling tool used to understand the complex cause–effect relationships between the most common parameters used in *Agrobacterium*-mediated transformation of the cereals too. That information should help cereal researchers to gain in knowledge on the transformation process, which means determining the factors that favour the interaction between *Agrobacterium* and cereal plants in order to improve the transfer of T-DNA and afterwards to regenerate whole plants from transformed cells, improving final transformation efficiency. Moreover, in a near future, this technology could be easily adapted to the rest of cereals or even any crop.

2. Agrobacterium-mediated transformation: Main factors

From the early 1990s many efforts were carried out in order to achieve stable transformation of wheat via *Agrobacterium*-mediated transformation (Bhalla et al., 2006; Vasil, 2007). This methodology presents several advantages over other approaches including the ability to transfer large segments of DNA with minimal rearrangement of DNA, fewer copy gene insertion, higher efficiency and minimal cost.

Several factors were identified as influencing the efficiency of T-DNA delivery: primary source materials; *Agrobacterium* strains; plasmids vectors; *Agrobacterium* density; medium composition; transformation conditions such as temperature and time during pre-culture, inoculation and co-culture; surfactants or induction agents in the inoculation and co-culture; and antibiotics or selectable markers, among others (Jones et al., 2005; Bhalla et al., 2006; Opabode, 2006; Kumlehn & Hensel, 2009).

2.1 Plant material

A summary of the different plant sources reported as main factors for *Agrobacterium*-mediated transformation of wheat can be found in Table 1. Wheat recalcitrance to *in vitro* culture is one of the most important crucial steps for *Agrobacterium* mediated transformation

protocols and directly correlated with the wheat source material. It was assessed that *in vitro* regeneration can be highly influenced by different factors such as plant growth regulators. In fact, auxins, polyamines and cytokinins were considered as essential to enhance the efficiencies on target explant and genotype (Khanna & Daggard, 2003; Przetakiewicz et al., 2003; Rashid et al., 2009).

2.1.1 Wheat genotype

Transformation and regeneration of the infected explants are highly genotype-dependent, the plant genotype has been revealed as a major factor influencing transformation efficiency. Indeed, the largest transformation efficiency compared to any other commercial wheat germplasm was reported when the highly regenerable wheat breeding line "Bobwhite" was used (Table 1).

The *Triticum aestivum* Spring "Bobwhite" is the most representative cultivar representing over 25% of the data reported of *Agrobacterium*-mediated transformation of wheat (Table 1), becoming "the genotype model" (Fellers et al., 1995; Sears & Deckard, 1982; He et al., 1988). It has a good response in tissue culture with a high rate of callus induction and regeneration (Janakiraman et al., 2002) making it a suitable cultivar for transformation, since a high ratio for both transformation and regeneration can be achieved. However, it would be highly desirable to transform genotypes other than the model ones (Kumlehn & Hensel, 2009) with much better agronomical and grain quality traits.

Other *T. aestivum* lines, cultivars or varieties such as "Turbo" (Hess et al., 1990); "Millewa" (Mooney et al., 1991); "Chinese" (Langridge et al., 1992); "Kedong 58", "Rascal" and "Scamp" (McCormac et al., 1998); "Lona" (Uze et al., 2000); "Baldus" (Amoah et al., 2001); "Fielder" (Weir et al., 2001); "Florida" and "Cadenza" (Wu et al., 2003); "Vesna" (Mitic et al., 2004); "Veery-5" (Khanna & Daggard, 2003; Hu el al., 2003) and so on (see the complete list in Table 1) were also tested.

Finally, some other commercial *Triticum sp* (different to *T.* aestivum) such as *Triticum dicoccum* (Chugh & Khurana, 2003), *Triticum durum* (Patnaik et al., 2006) or *Triticum turgidum* (Wu et al., 2008; Wu et al., 2009; He et al, 2010) were also being successfully used for Agrobacterium-mediated wheat transformation (see Table 1).

2.1.2 Target explants

The primary source of material is one of the main constraints for *Agrobacterium*-mediated wheat transformation. Regeneration is performed from highly regenerant tissues with active cell division. In these tissues embryogenic calli are induced and regeneration leads to the recovery new formed transgenic plants. Two types of explants are typically used for the recovery of fertile transgenic plants: immature inflorescences and the scutellum of immature zygotic embryos. Although other explants (Table 1) have been used for the same purpose such as reproductive-derived material (Hess et al., 1990; Liu et al., 2002), seeds (Zale et al., 2004); leaf (Wang & Wei, 2004) or shoot meristems (Ahmad et al., 2002), none of them were capable of reliably production of fertile adult transgenic wheat adult plants.

Wheat (Variety / Cultivar)	Explant Type	Strain / Plasmid	Promoter / Reporter Gene	Promoter / Selectable gene	Transformation efficiency (%)	Reference / Remarks
Triticum aestivum						
Turbo (Spring)	SPK	C58C1 / pGV3850:1103neo	-	*nos / nptII*	1	Hess et al., 1990 / No regeneration
Millewa (Spring)	IE	C58C / pGV3850:1103neo	-	*nos / nptII*	1 – 2 (based on kanamycin selection)	Mooney et al., 1991 / Gene integration was not demonstrated
Chinese (Spring)	SPK	LBA4404 / pPCV6NFHyg A281 / pPCV6NFHyg C58C1 / PCV6NFHyg GV3101 / pPCV6NFHyg	*CaMV35S / gus*	*nos / nptII* *CaMV35S / hpt*	0.8 – 4.7 (based on kanamycin selection)	Langridge et al., 1992 / No regeneration
Bobwhite (Spring)	IE PCIE IEdC	ABI / pMON18365	*CaMV35S / gus*	*CaMV35S / nptII*	1.12 (IE) 1.56 (PCIE) 1.55 (IEdC)	Cheng et al., 1997 / Salt strength test, surfactants & explants types
Bobwhite (Spring)	SDS	EHA105 / pIG121Hm	*CaMV35S / gus*	*nos / nptII* *CaMV35S / hpt*	28 foci/seed (*GUS*)	Trick et al., 1997 / Sonication test. Transient GUS expression
Rascal (Spring) Scamp (Spring) Kedong 58 (Winter)	IEdC	EHA101 / pBECKS.red	*CaMV35S / gus* *CaMV35S / gfp* *CaMV35S / Lc/C1*	*nos / nptII*	40 - 70 (based on reported genes)	McCormac et al., 1998 / *gfp* and *Lc/C1* gene reporters
Chinese (Spring)	MSdC IEdC	GV3101 / pMVTBP GV3101 / pNFHK1 GV3101 / p35S*GUS*INT	*CaMV35S / gus*	*CaMV35S / nptII*	1.2 - 2.2 (*GUS*)	Peters et al., 1999 / Use of modular vector
Several (Chinese)	IEdC	AGL1 / pUNN2	-	*ubi1 / nptII*	3.7 – 5.9	Xia et al., 1999 / Stable transformation
Bobwhite (Spring) Lona (Spring)	PCIE	LBA4404 / pBin9UG EHA105 / pBin9UG C58C1 / pBin9UG LBA9402 / pBin9UG	*ubi1 / gus*	-	20 foci/callus (*GUS*)	Uze et al., 2000 / Several factors studied for transformation
Baldus (Spring)	INFdC	AGL1 / pAL154-pAL156 AGL1 / pAL155-pAL156 AGL1 / pSoup-pAL186	*ubi1 / gus*	*ubi1 / bar*	14 – 64 (*GUS*)	Amoah et al., 2001 / Inflorescence tissue. Sonication and vacuum infiltration
Fielder (Spring)	PCIE	AGL0 / pTO134	*CaMV35S / gfp*	*CaMV35S / bar*	1.8 PCIE	Weir et al., 2001 / Several factors studied for transformation
Nongda 146 (Spring)	IE PCIE	AGL1 / pAL155-pAL156	*ubi1 / gus*	*ubi1 / bar*	90 (*GUS*)	Ke et al., 2002/ Transient GUS expression
Sohag 2 (Durum)	SPK	LBA4404 / pBI-P5CS	*CaMV35S / gus*	*nos / nptII*	0.9	Sawahel and Hassan, 2002 / *In planta* transformation
Sourav (Spring) Gourav (Spring) Kanchan (Spring) Protiva (Spring)	IE ME IEdC MEdC	EHA105 / pCAMBIA1301	*CaMV35S / gus*	*nos / nptII*	75 – 85 (IE) 60 - 65 (ME) 80 – 87 (IEdC) 67 - 73 (MEdC)	Sarker & Biswas, 2002 / Transient GUS expression
Bobwhite (Spring)	PCIE IEdC	ABI / pMON18365	*CaMV35S / gus*	*CaMV35S / nptII* *CaMV35S / aroA:CP4*	3.1 (PCIE -glyphosate) 6.1 (PCIE-paromomy.) 10.5 (EC-paromomycin)	Cheng et al., 2003 / Large scale experiments
Bobwhite (Spring)	PCIE	C58C1 / pPTN115	*CaMV35S / gus*	*CaMV35S / nptII*	0.5 – 1.5	Haliloglu and Baenziger (2003) / Several factors were studied
Bobwhite (Spring)	PCIE	ABI / pMON30120 ABI / pMON30174 ABI / pMON30139	-	*act1 / aroA:CP4* *CaMV35S / aroA:CP4* *ScBV / aroA:CP4*	4.4	Hu et al., 2003 / Large-scale production. Roundup ready wheat
Veery5 (Spring)	IEdC	LBA4404 / pHK22 LBA4404 / pHK21	*ubi1 / gus*	*ubi1 / bar*	1.2 – 3.9	Khanna and Daggard, 2003 / Use of spermidine in regeneration
Bobwhite (Spring)	PCIE EC	n.d / PV-TXGT10	-	*act1 / aroA:CP4* *CaMV35S / aroA:CP4*	-	Zhou et al., 2003 / Roundup ready wheat
Florida (Winter) Cadenza (Winter)	IE	AGL1 / pAL154-156	*ubi1 / gus*	*ubi1 / bar*	0.3 – 3.3	Wu et al., 2003 / Several factors studied for transformation
CPAN1676 (Bread) PBW343 (Bread)	MSdC	LBA4404 / pCambia3301	*CaMV35S / gus*	*CaMV35S / bar*	6.7 – 8.7	Chugh & Khurana, 2003 / Herbicide Resistance. Use of basal segment calli as target tissue
Vesna (Spring)	IE	LBA4404 / pTOK233 AGL1 / pDM805	*CaMV35S / gus* *act1 / gus*	*ubi1 / bar* *nos / nptII* *CaMV35S / hpt*	0.13 (LBA) 0.41 (AGL)	Mitic et al., 2004 / Use of super-binary vectors. Only PCR test
Kontesa (Winter) Torka (Winter) Eta (Winter)	IE	AGL1 / pDM805 EHA101 / pGAH LBA4404 / pTOK233	*CaMV35S / gus* *act1 / gus*	*ubi1 / bar* *nos / nptII* *CaMV35S / hpt*	1 (AGL1) 0.2 – 8.1 (EHA101) 0.2 – 2.3 (LBA4404)	Przetakiewicz et al., 2004 / Use of super-binary vectors and auxins
Hesheng3 (Winter) Yan103 (Winter) Yanyou361 (Winter)	IEdC	GV3101 / pROK2-AtNHX1	-	*CaMV35S / nptII*	1.3 – 2.9	Xue et al., 2004 / Survival tests in saline conditions. Field trial
Shannong 9956049 (Winter)	IEdC	LBA4404 / pROK2	-	*nos / nptII*	1.18	Bi et al., 2006 / Insect resistance
Yan361 (Winter) Yan2801 (Winter) H11 (Winter)	SDS	EH105 / pBLG	-	*nos / nptII*	8.62 – 11.2	Zhao et al., 2006 / Powdery Mildew resistance
HD2329 (Bread) CPAN1676 (Bread) PBW343 (Bread)	ME MEdC	LBA4404 / pBI101 LBA4404 / pCAMBIA3301	*act1 / gus* *CaMV35S / gus*	*nos / nptII* *CaMV35S / bar*	1.6	Patnaik et al., 2006 / Genotypic independence
Shiranekomugi	SDS	LBA4404 / pIG121Hm LBA4404 / pBI-res used LBA4404 / pBI-res2 used	*CaMV35S / gus*	*nos / nptII* *CaMV35S / hpt*	33 (PCR) 75 (Southern) 40 (plasmid rescue)	Supartana et al., 2006 / TE is referred to t1 progeny instead of inoculated explants
Yangmai158 (Winter)	PCIE	EHA105 / pCAMBIA3300	-	*CaMV35S / bar*	-	Yu and Wei, 2008 / Insect resistance
Een1 (Winter)	SDS	LBA4404 / n.d.	*CaMV35S / gus*	*CaMV35S / nptII*	3 – 31 (GUS)	Yang et al., 2008 / Use of seedling ages and inoculation time
Crocus (Spring)	SPK	C58C1 / pDs(Hyg)35S AGL1 / pBECKSred	*CaMV35S / Lc/C1*	*nos / nptII* *nos / hpt*	0.44	Zale et al., 2009 / *In planta* transformation
Certo (Winter)	IE	LBA4404 / pSB187	*ubi1 / gfp*	*CaMV35S / hpt*	2 – 10	Hensel et al., 2009 / Detailed protocols for transformation
EM12 (Chinese)	PCME	LBA4404 / pBI121	*CaMV35S / gus*	*nos / nptII*	0.27 - 2.5	Ding et al., 2009 / Optimization of transformation protocol

Wheat (Variety / Cultivar)	Explant Type	Strain / Plasmid	Promoter / Reporter Gene	Promoter / Selectable gene	Transformation efficiency (%)	Reference / Remarks
Triticum aestivum						
Bobwhite (Sring) Yumai66 (Winter) Lunxuan208 (Winter)	ME, PCME	C58C1 / pUbiGN	*ubi1 / gus*	*nos / nptII*	0.06 – 0.89	Wang et al., 2009 / Use of mature embryos
Inqilab-91 (Bread)	ME	EHA101 / pIG121Hm	*CaMV35S / gus*	*nos / nptII* *CaMV35S / hpt*	6.25 – 15.62	Rashid et al., 2010 / Effect of AS & bacterial culture density
Triticum turgidum						
Durum (ofanto)	IE	AGL1 / pAL156-pAL154 AGL1 / pAL156-pAL155	*ubi1 / gus*	*ubi1 / bar*	0.6 – 9.7 (pAL154) 2.1 – 3.9 (pAL155)	Wu et al., 2008 / Super-binary vectors. First time durum transf
Durum (Stewart)	IE	AGL1 / pAL156- pAL154	*ubi1 / gus*	*ubi1 / bar*	2.8 - 6.3	He et al, 2010 / Effect super-binary vectors, AS & picloram
Triticum dicoccum						
DDK1001 (Emmer)	MSdC	LBA4404 / pCambia3301	*CaMV35S / gus*	*CaMV35S / bar*	6.9	Chugh & Khurana, 2003 / Herbicide Resistance. Use of basal segment calli as target tissue
Triticum durum						
PDW215 (Pasta)	ME	LBA4404 / pCAMBIA3301	*CaMV35S / gus*	*CaMV35S / bar*	3	Vishnudasan et al., 2005 / Nematode resistance
PDW215 (Pasta) PDW233 (Pasta) WH896 (Pasta)	ME MEdC	LBA4404 / pBI101	*act1 / gus* *CaMV35S / gus*	*nos / nptII* *CaMV35S / bar*	1.28	Patnaik et al., 2006 / Genotypic independence

Table 1. Summary of wheat materials, *Agrobacterium* strains and vectors, and marker genes used to investigate wheat transformation. Explant type: IE (immature embryo); PCIE (pre-cultured immature embryo); IEdC (immature embryo derived calli); ME (mature embryo); PCME (pre-cultured mature embryo); MEdC (mature embryo derived calli); INF (inflorescence); INFdC (inflorescence derived calli); SPK (spikelet); SDS (seedling); MSdC (mature seed derived calli). Promoters: *CaMV35S* (cauliflower mosaic virus); *ubi1* (maize ubiquitin); *act1* (rice actin); *nos* (nopaline synthase gene); *ScBV* (sugarcane bacilliform virus). Reporter genes: *gus* (β-glucuronidase); *gfp* (green fluorescent protein); *Lc/C1* (anthocyanin-biosynthesis regulatory). Selectable gene: *nptII* (neomycin phosphotransferase II) and *hpt* (hygromycin phosphotransferase) antibiotic resistance and *bar* (phosphinothricin acetyltransferase) and *aroA:CP4* (5-enolpyruvylshikimate-3-phosphate synthase (EPSPS)) herbicide resistance.

By far, the main target explant used to transform wheat was from immature embryos (IE). Concretely, the immature scutellum was used, a specialised tissue that forms part of the seed embryo, and it was recommended that embryo isolation was performed 11-16 days post-anthesis (Jones, 2005). Freshly isolated IE, pre-cultured IE or IE derived callus had been widely included in experiments to obtain transgenic wheat plants. Cheng et al. (1997) reported, for the first time, the success of *Agrobacterium*-mediated transformation in wheat using IE (freshly isolated and pre-cultured) and embryogenic calli producing fertile transgenic plants despite the experiments being limited to small-scale. Later, many attempts were carried out by several authors (McCormac et al., 1998; Xia et al., 1999; Uze et al., 2000; Ke et al., 2002, Sarker & Biswas, 2002) but no stable transgenic plants were reported until Weir et al. (2001), who confirmed results obtained previously by Cheng et al. (1997), transformed pre-cultured immature embryos, 9 day old. Large-scale experiments were carried out using immature embryos as the initiation tissue for both genetic transformation and plant regeneration (Cheng et al., 2003; Hu et al., 2003; Vasil, 2007; Jones et al., 2007; Rashid et al., 2009).

Immature inflorescences were also easier to isolate and can be collected earlier from younger plants in comparison to immature embryos. However, these explants present more specific-genotype requirements for its *in vitro* culture regeneration (Jones, 2005 and references therein). Seeds were also used as started explant for wheat in plant transformation (Trick & Finer, 1997; Supartana et al., 2006; Zhao et al., 2006; Yang et al., 2008; Razzaq et al., 2011) but only Supartana et al. (2006) and Zhao et al. (2006) demonstrated stable gene inheritance and integration in progeny by Southern blot analysis

(Table 1). Other initiation explants were also tested as tissue for wheat *Agrobacterium*-mediated transformation: mature embryo (ME) either freshly isolated, pre-cultured or derived calli (Sarker & Biswas, 2002; Vishnudasan et al., 2005; Patnaik et al., 2006; Ding et al., 2009; Wang et al., 2009; Rashid et al., 2010), inflorescence or inflorescence derived calli (Amoah et al., 2001) and mature seed derived calli (Peters et al., 1999; Chugh & Khurana, 2003). Mature embryos offer some advantage over the typically used immature embryos, as a low-cost procedure because immature embryos must be recollected from plants grown under a controlled environment, moreover the extraction of the embryos in a narrow developmental stage (i.e. 0.8–1.5 mm in diameter) is required (Wu et al., 2009; Wang et al., 2009).

In the early 1990s transgenic wheat materials were generated by inoculating florets with *Agrobacterium* at or near anthesis (Hess et al., 1990; Langridge et al. 1992) produced similar results since both failed to demonstrate gene integration in successive plant generations or successful plant regeneration (Table 1). Using the same protocol but changing the *Agrobacterium* strain and the plasmid construction, a floral dip efficient transformation of wheat was achieved by Sawahel & Hassan (2002). More recently (Zale et al., 2009) by performing transformation at an earlier stage of floral development than previously (i.e., Hess et al., 1990; Langridge et al. 1992; Sawahel & Hassan, 2002) successful transgene integration and expression were obtained when wheat ovules were used as target explants.

2.2 *Agrobacterium* and plasmids

It has been widely described in the literature that the combination of highly competent *Agrobacterium* strain with effective and suitable plasmid construction leading to improved successful wheat transformation efficiencies (Khanna & Daggard, 2003; Cheng et al., 2004). The most used *Agrobacterium* strains and plasmids are summarized in Table 1.

2.2.1 *Agrobacterium* strain

Cereals are not natural hosts for *Agrobacterium* and many studies have been carried out to match host strains with wheat genotypes (Jones et al., 2005). Mainly, only three strains of *Agrobacterium tumefaciens* are currently used in wheat transformation (Table 1) thus from the 41 reports reviewed: 44% used LBA4404, followed by C58C1 (24%) and AGL1 (24%). While other strains has been used with a less frequency (10%) including other *A. tumefaciens* strains such as: A281, GV3101, ABI, EHA101, EHA105, AGL0, M-21 and *A. rhizogenes* LBA9402 and Ar2626. Interestingly, most of those *Agrobacterium* strains share only two chromosomal backgrounds: the C58 type (C58C1, AGL1, GV3101, ABI, EHA101, EHA105 and AGL0) and TiAch5 (LBA4404) (Hellens et al., 2000; Jones et al., 2005).

The infection process of *Agrobacterium* include several chromosome-encoded genes involved in the attachment of bacteria to plant cells and Ti plasmid-encoded *vir* genes, that function in trans, helping the transfer and integration of T-DNA into the plant genome (Wu et al., 2008). Some of the above strains also contain a binary or helper plasmids, carrying further copies of virulence genes. Therefore, depending on agro construction, "standard or low virulent" strains as LBA4404 and C58C1 or "hyper-virulent strains" such as AGL have been designed to successful transformation of wheat.

Although rare, also some a-virulent *A. tumefaciens* mutant strain has also been used for wheat transformation studies as a reliable marker of transformation (Table 1). As an example, Supartana and co-workers (2006) employed the M-21 *Agrobacterium* mutant, in which the *iaaM* gene (tryptophan monooxygenase gene) - involved in IAA (indole acetic acid) biosynthesis in the T-DNA region - is destructed by transposon5 (Tn5) insertion. As a consequence, this mutant strain was capable of integrating its T-DNA into chromosomes of host plants, but no galls were produced. Wheat transformants obtained by the M-21 mutant strain were expected to synthesize a high cytokinin level (since all other genes including the *ipt* gene – involved in cytokinin biosynthesis in the T-DNA region – were intact and fully functional), resulting in a high altered phenotype due to hormone imbalance which can be easily detected (Supartana et al., 2006).

2.2.2 Plasmid and virulence

As stated previously, wheat is not a natural host for *Agrobacterium*, for this reason only a few genotypes (such as Bobwhite) can be transformed with standard strains, such as LBA4404 and binary vectors (Cheng et al., 1997; Hu et al., 2003). When other genotypes were tested, no successful transformation was obtained, only their virulence was increased by adding an extra binary plasmid (such as pHK21) with extra *vir* genes (Khanna & Daggard, 2003) that enhance the transformation.

Many other Ti vectors and helper plasmids, known as binary plasmids, which can include an extra copy of virulence genes in the namely "super-binary" vectors, have been incorporated in the selected *Agrobacterium* strain to enhance infection. Several combinations regarding virulence are possible: from a-virulent to hyper-virulent *Agrobacterium* strain.

The most common *Agrobacterium* strains used in wheat transformation below to hyper-virulent group and is the disarmed plasmid pTiBo542 from *A. tumefaciens* wild strain A281 harbouring additional virulence genes usually *vir* B, C and G, which confer the hyper-virulence character (Komari et al., 1990).

Two different constructs have been widely employed to carry extra *vir* region (Table 1): first, using the helper plasmid pAL155 which is a derivative of pSoup modified by the addition of *vir* G (Amoah et al., 2001; Ke et al., 2002; Wu et al., 2008); and second, using different plasmids as pAl154, pAL186 or pTOK233 carrying "15 kb Komari fragment" containing set of *vir* B, C and G (Amoah et al., 2001; Wu et al., 2003; Mitic et al., 2004; Przetakiewicz et al., 2004; Wu et al., 2008; Wu et al., 2009; He et al., 2010).

2.2.3 Promoters

Regarding the promoters (see Table 1), the most common were the constitutive "*CaMV35S*" (cauliflower mosaic virus) and "*ubi1*" (maize ubiquitin). Other promoters such as "*act1*" (rice actin promoter); "*nos*" (nopaline synthase gene) or "*ScBV*" (sugarcane bacilliform virus) (Hu et al., 2003) were also used with much less frequency.

A great challenge will be to identify specific promoters that would direct the expression of genes in a tissue-specific manner. This can be used not only with reporter genes in studies to optimize the *Agrobacterium*-meditated transformation protocols but also with agronomical importance genes, such as quality improvement, disease resistance or drought tolerance.

2.2.4 Reporter genes

Three reporter marker genes have been used to establish expression and/or integration of foreign DNA into wheat material (See Table 1).

The most usual one is *gusA* (*uidA*) gene encoding the enzyme β-glucuronidase (GUS); although *gfp* (green fluorescent protein) gene, (McCormac et al., 1998; Weir et al., 2001; Hensel et al., 2009) and *Lc/C1 (*anthocyanin-biosynthesis regulatory) genes, that results in the accumulation of anthocyanin so creating the "red cell" phenotype (McCormac et al., 1998; Zale et al., 2009), were also used.

2.2.5 Selectable and interest genes

Antibiotic and herbicide resistance is by far the most widely used selection system in *Agrobacterium*-mediated transformation of wheat (See Table 1). As the selectable marker gene, the most common one is "*nptII*" (neomycin phosphotransferase II) gene (Table 2), which confers resistance to kanamycin antibiotic, although "hpt" (hygromycin phosphotransferase) gene conferring hygromycin B resistance has been recently employed (Zale et al., 2009; Rashid et al., 2010), which may be due to cereals being more sensitive to hygromycin B than to kanamycin (Janakiraman et al., 2002 and references therein).

Selectable marker gene	Encoded enzyme	Selective agent	Mode of action
nptII	neomycin phosphotransferase II	Aminoglycoside antibiotics: -kanamycin -neomycin -hygromycin - G418 (geneticin) - paromomycin	Binds 30S ribosomal subunit, inhibits translation
hpt	hygromycin phosphotransferease	Aminoglycoside antibiotics: -hygromycin	Binds 30S ribosomal subunit, inhibits translation
bar (pat)	phosphinothricin acetyl transferase	Herbicides: -phosphinothricin (PPT) -glufosinate ammonium -bialaphos (tripeptide antibiotic)	Inhibits glutamine synthase
aroA:CP4	5-Enolpyruvylshikimate-3-phosphate synthase	Herbicides: -glyphosate	Inhibits aromatic acid biosynthesis (EPSPS)

Table 2. Selectable marker genes most commonly used in wheat *Agrobacterium*-mediated transformation.

The other most popular selectable gene is "*bar*" (also called "pat", phosphinothricin acetyl transferase) gene that confers herbicide resistance to phosphinothricin (PPT) and glufosinate ammonium, the active ingredient being the herbicide Basta® by Hoechst AG and Liberty by AgroEvo®, respectively (Table 2; Rasco-Gaunt et al., 2001). Also, other resistance marker genes for wheat transgenic plants selection have been described (Table 2), such as" *aroA:CP4*" (5-enolpyruvylshikimate-3-phosphate synthase) gene that confers tolerance to glyphosate, the active ingredient of the RoundupReady® herbicide (Zhou et al., 2003; Hu et al., 2003).

2.3 Transformation conditions

Many variables have been pinpointed, and extensively reviewed (Janakiraman et al., 2002; Sahrawat et al., 2003; Bhalla et al., 2006; Jones, 2005), as the key factors in the *Agrobacterium*-mediated transformation process of wheat. Here, those variables are listed in Table 3 under heading that describe the factor, the type or stage studied, the range tested and the optimal value proposed for the highest transformation efficiency together with the main references related. Latter on those data are discussed step by step and we divided the *Agrobacterium*-mediated transformation protocol in four separates stages: preculture, inoculation, coculture and selection.

Factors	Type	Range tested / Higher efficiency	Some references
Time	Pre-culture	From 4 to 21 days. Optimal conditions varied among source explants	Haliloglu & Baenziger, 2003; Weir et al., 2001; Ding et al., 2009; Amoah et al., 2001
	Inoculation	From 30 min to 12 h. Optimal conditions at 30 min and 3 h.	Yang et al., 2008; Wu et al., 2003; Ding et al., 2009
	Coculture	From 1 to 5 days. Optimal conditions at 3 days.	Wu et al., 2003; Uze et al., 2000
Temperature	Inoculation	From 22 to 28 ºC. Optimal condition at 24-25ºC	Wu et al., 2003; Wu et al., 2008; Mitic et al., 2004
	Coculture	From 21 to 27ºC. Optimal condition at 24-25ºC.	Amoah et at., 2001; Weir et al., 2001; Khanna & Daggard, 2003; Xue et al., 2004; Wu et al., 2008
Auxins	Picloram	From 1 to 10 mg/L. Optimal conditions around 2- 2.2 mg/L	Weir et al., 2001; Ding et al., 2009; He et al., 2010; Jones et al., 2005
	2,4 D	From 0,5 to 10 mg/L. Optimal conditions at 0,5 and 2 mg/L.	Cheng et. al, 1997; Hu et al., 2003; Razzaq et al., 2011
Surfactans	Pluronic F68	From 0.01 to 0.05 %. Optimal conditions at 0.02%	Cheng et al., 1997; Cheng et al., 2003; Khanna & Daggard, 2003; Zhou et al., 2003
	Silwet L-77	From 0.001 to 0.5 %. Optimal conditions at 0.01-0.02%.	Cheng et al., 1997; Wu et al., 2003; Zale et al., 2009; Haliloglu & Baenziger, 2003
Sugars	Maltose	From 40 to 80 g/L. Optimal conditions at 40	He et al., 2010
	Glucose	From 10 to 36 g/L. Optimal conditions at 10-20 g/L.	Cheng et al., 1997; Khanna & Daggard, 2003
Optical Density		From 0.5 to 2 Optimal conditions at 0.6	Sarker & Biswas, 2002; Amoah et al., 2001; Ke et al., 2002; Haliloglu & Baenziger, 2003; Bi et al., 2006
Phenolic inducers	Acetosyringone	From 100 to 400 µM. Optimal conditions at 100-200 µM.	Cheng et al., 1997; McCormac et al., 1998; Amoah et al., 2001; Wu et al., 2003; Patnaik et al., 2006; He et al., 2010
Salt strength		From 0.1 to 2. Optimal conditions at 0.1 – 1 MS salts strength	Cheng et al., 1997; Ding et al., 2009

Table 3. Summary of current published data on main factors with positive effect on wheat *Agrobacterium*-mediated transformation efficiency.

2.3.1 Preculture

Most reports on *Agrobacterium*-mediated transformation include a first stage called "preculture" to increase the transformation efficiency. For example, survival rate was higher in explants precultured before inoculation than in freshly isolated explants (Cheng et al., 1997). Moreover, Uze et al. (2000) reported the highest T-DNA delivery ratio, based on transient GUS assay, of immature wheat embryos "Bobwhite" when precultured during 10 days; Amoah et al. (2001) found that inflorescence tissue precultured during 21d had the highest GUS activity and finally, Ding et al. (2009) obtained the best transformation rate when mature embryos were precultured for 14 days. However, other authors (Jones et al., 2005) described a successful protocol without pre-culture period or special inoculation treatments.

Some plant growth regulators, such as synthetic auxins picloram (4-amino-3, 5, 6-trichloropicolinic acid) and 2,4-D (2,4-dichlorophenoxyacetic acid), are commonly added to the preculture medium to increase regeneration and the recovery of transgenic explants. Przetakiewicz et al. (2004) demonstrated the promotion effect of 2,4-D for obtaining a higher number of transgenic plants than picloram, whereas, picloram promotes a higher regeneration frequency than 2, 4-D in other report (Ding et al., 2009). Taken into account those results, picloram and 2,4-D or both together have been widely employed in wheat transformation via *Agrobacterium* (Table 3).

2.3.2 Inoculation

The second step of any *Agrobacterium* mediated process is the inoculation of wheat explants in an *Agrobacterium* suspension during a quite variable period of time: 30 minutes to 12 hours (see references in Table 3) and several factors have been proposed as key for inoculation such as included as the most important inoculation stage such as: time, temperature, media strength or *Agrobacterium* optical density as well as some inducers of stable transformation, such as acetosyringone, sugars, auxins or surfactans.

Several authors (Amoah et al.; 2001; Yang et al., 2008) have described a direct relationship between increase of inoculation time and decrease in transformation efficiency after 2-3 h and there is a general consensus that the optimal time of inoculation for T-DNA delivery (Jones et al., 2005; Wu et al., 2008; Ding et al., 2009) should be around 3 h.

Although in the literature reviewed (Table 3), a wide range of inoculation temperatures have been tested: 22 – 28ºC (Peters et al., 1999; Cheng et al., 2003; Mitic et al., 2004; Supartana et al., 2006) however, no clue on the optimal ones or significant differences has been clearly reported. Moreover, most reports do not indicate the inoculation temperature and it is assumed that room temperature has been applied (c.a. 25ºC).

The use of surfactants and phenolic inducers in the media were widely assessed by different researchers (Table 3). Surfactants, like pluronic acid F68 and Silwet L-77, were first studied by Cheng et al. (1997) finding that either Silwet or pluronic enhance transient GUS expression, especially on the immature embryos because it is believed that the surface-tension-free cells favour the *A. tumefaciens* attachment. Several studies reported an optimal concentration for Silwet around 0.01% (Wu et al., 2003; Jones et al., 2005) and for pluronic around 0.02% (Cheng et., 1997). On the contrary, other authors (Haliloglu & Baenziger, 2003) have described that the presence of a surfactant in the inoculum medium makes no

difference in terms of T-DNA delivery efficiency, even when concentrations as higher as 0.05% of Silwet have been used.

Acetosyringone was always pointed out to be the key factor in T-DNA delivery in a range of concentration from 100 to 400 μM (McCormac et al., 1998; Xue et al., 2004; He et al., 2010). Its presence, at 200 μM concentration, clearly increased transformation efficiency (Wu et al., 2003; Amoah et al., 2001).

The addition of some sugars, like maltose or glucose to the inoculation medium was essential to achieve efficient T-DNA delivery; in fact T-DNA delivery efficiency was significant reduced in the freshly isolated immature embryos when acetosyringone and glucose were absent in the inoculation media (Cheng et al., 1997, Wu et al., 2003).

Agrobacterium optical cell density at 600 nm around 0.5-0.6 (Cheng et al., 2003; Haliloglu & Baenziger, 2003; Bi et al., 2006); close to 1.0 (Khanna & Daggard, 2003; Jones et al., 2005) or even higher, such as 1.3 (Amoah et al., 2001) during inoculation were found to be crucial for transformation efficiency. However when *Agrobacterium* is inoculated at high density or when is cocultured with the explant at high temperatures or for long period conditions an overgrowth can occurs promoting the death of the explants. Several antibiotics can be used after coculture and the selection stage to control *Agrobacterium* overgrowth or to eliminate it completely, such as timentin (Hensel et al., 2009, Wu et al., 2009), carbenicillin (Cheng et al., 1997) and cefotaxime (Bi et al 2006, Chugh & Khurana, 2003).

2.3.3 Coculture

The third stage of any wheat *Agrobacterium*-tumefaciens transformation protocol starts, after the removal of excess of bacteria from the previous stage, when the explants are cocultivated for a period of 1-5 days (Table 3) in dark conditions at 23 -27ºC. Again, during this period virulence inductors such as acetosyringone, osmoprotectors such as proline, carbon sources such as sugars, and plant growth regulators are added to the medium

Several studies have focused on time, temperature and media composition variables as important factors, during cocultivation stage, to transform wheat successfully. For example, Wu et al. (2003) found that a long cocultivation time (5d) promoted a reduction on the capacity of the transformed immature embryos to form embryogenic callus and regenerate when cocultivation was assessed for 1–5 days. Short periods (2-3 days) have been proposed as optimum for high transformation efficiency (Cheng et al., 1997; Amoah et al., 2001; Wu et al., 2003; Ding et al., 2009).

Also, the temperature during the cocultivation period could play an important role. Weir and coworkers (2001) obtained 83.9 and 81.4% of GFP expression at 21 and 24ºC, respectively and concluded that transient GFP expression is not significantly affected by co-cultivation temperature. Although, an elegant assay demonstrated that coculture at two temperatures (1d at 27ºC and 2d at 22ºC) reduced the damage to the soft callus tissue due to the common overgrowth of *Agrobacterium* during coculture (Khanna & Daggard, 2003). More information about it can be found in 2.3.2 section.

As stated previously for inoculation condition, the addition of acetosyringone 200μM is also critical in the coculture media to increase the efficiency on T-DNA delivery (Cheng et al., 1998; Wu et al., 2003).

Finally, it has been described (Table 3) that the salt strength in both, the inoculation and co-culture media, had a significant influence on the T-DNA delivery. For example, transient GUS expression was higher on freshly isolated immature embryos when one tenth-strength MS salts were used than the full-strength MS salts (Cheng et al., 1997). Several medium strength 2x, 1x, 0.5x, and 0.1x media concentration were also assessed elsewhere (Khanna & Daggard, 2003) but no main conclusion has been drawn and MS media 1x has been generally employed in *Agrobacterium* mediated transformation of wheat (Weir et al., 2001; Ke et al., 2002; Sarker & Biswas, 2002; Wu et al., 2003; Patnaik et al., 2006; Ding et al., 2009)

2.3.4 Selection

Due to the most common selectable marker genes being *nptII, hpt* and *bar*, the most widely selected agents, to discriminate transformed explants , and not to transform explants, were kanamicyne, hygromycin and phosphinothricin (PPT) and their analogues G418 (geneticin) and paromomycin for *nptII* gen and Bialaphos when *bar* gene was used as selectable marker gene.

3. Agrobacterium-mediated genetic transformation: Time to model

As described in the previous section, plant genetic transformation is a really complex process to understand and, subsequently, to optimize. The reason behind this is the important number of variables (factors) involved in the whole process (plasmid or *Agrobacterium* strain, type of plant explant, preculture, inoculation, coculture and selection conditions, etc) together with the different scales of biological organization concerned (molecular, genetic, cellular, physiological and whole plant). Moreover, different kinds of data are generated in those studies: binary data (transformed- non transformed; alive–dead); discrete or categorical (number of GUS spots); continuous (length, weight, …); image data (GUS or GFP) or even fuzzy data (callus colour: brown, brownish, yellowish and so on).

Traditionally, the effect of those variables on genetic transformation studies and particularly, wheat *Agrobacterium*-mediated transformation, is determined by analysis of variance (ANOVA). According to statistical theory (Mize et al., 1999), only continuous data normally or approximately normally distributed should be analysed with ANOVA. Discrete and binomial data should be analysed using Poisson and logistic regression, respectively. This type of methodology makes, the analysis of the results complicated and specialized, the biologist often being helped by statisticians. Finally, although statistics can be used for making predictions, normally this feature is not used in plant transformation studies.

Because of these limitations, plant genetic transformation studies include, usually, a small number of variables at the same time. Often, one variable at a time is studied; for example to study the effect of a variable (eg. effect of acetosyringone) on a selected response (eg. GUS transient expression), the experiments are performed at different concentrations (0, 100, 200 and 300 μM) keeping the rest of the variables constant. This "one-factor at a time" procedure is time consuming and has clear limitations when the best conditions for *Agrobacterium*-meditated transformation of wheat need to be achieved. The main limitation is that this

procedure ignores the possible interactions between variables (the addition of acetosyringone can have a positive or negative interaction with any other variable kept constant during a particular experiment).

Finally, this kind of methodology enables the researcher to select the best combination of factors between the performed experiments and not to predict the best possible combination of factors or, in other words, to optimize the whole procedure.

The *Agrobacterium*-mediated transformation process is difficult to describe accurately by a simple stepwise algorithm or a precise formula and require a network (multivariable) approach using computational models. For developing a model several steps need to be followed: first, a clear identification of the process (including all kind of variables/factors) to be simulated, controlled and/or optimized; secondly, the selection of variables, and the definition of what the model is for; thirdly, the creation of the database with the most accurate and precise data of each variable and the selection of the type of model and finally, the model validation, to check if the distances between the observed and predicted data is low enough (Gallego et al., 2011).

To establish the key factors affecting the quality of an *Agrobacterium*-mediated transformation process an Ishikawa diagram can be developed (Fig. 1) using data from literature (Tables 1, 2 and 3). This cause-effect diagram helps in identifying the potential relationships among several factors, and provides an insight into the whole process. The main factors (causes) can be selected and grouped into major categories such as plant material, *Agrobacterium*, transformation conditions and selection conditions.

Initially both *Agrobacterium* characteristics (strain, plasmid, extra virulence gene, promoters, reporter and selectable marker gene) and plant material (genus and species, variety/cultivar/line and type of explant) should be defined. Within the transformation conditions (preculture, inoculation and coculture) several variables as process conditions (temperature and time); chemical properties as media composition (type, strength, vitamins, sugars, plant growth regulator (PGR) such as synthetic auxins) and/or transformation inductors (acetorysingone and surfactans) should be considered and interrelated. Finally, selection conditions (antibiotics and/or herbicides) need to be established.

From this diagram, it can be deduce that there are an enormous amount of variables involved in the transformation process. Moreover, variables of different types: numerical data (temperature, time, etc.) or nominal (strain, explant, etc.) should be considered. Once the key or main variables (inputs) are identified, their effects over the defined parameters (outputs) should be studied by the appropriate experimental design or model.

Different models and/or networks have been used to integrate all kind of biological components (Yuan et al., 2008). Both networks and model have become more and more accurate (and better at predicting outcomes of the complex biological process) by using new experimental and modelling tools (Giersch, 2000). Recent studies have pointed out the effectiveness of different artificial intelligence technologies, such as artificial neural networks (Gago et al., 2010a, 2010b, 2010c) combined with genetic algorithms and neurofuzzy logic (Gago et al., 2010d; 2011) in modelling and optimizing the complex plant biology process (Gallego et al., 2011).

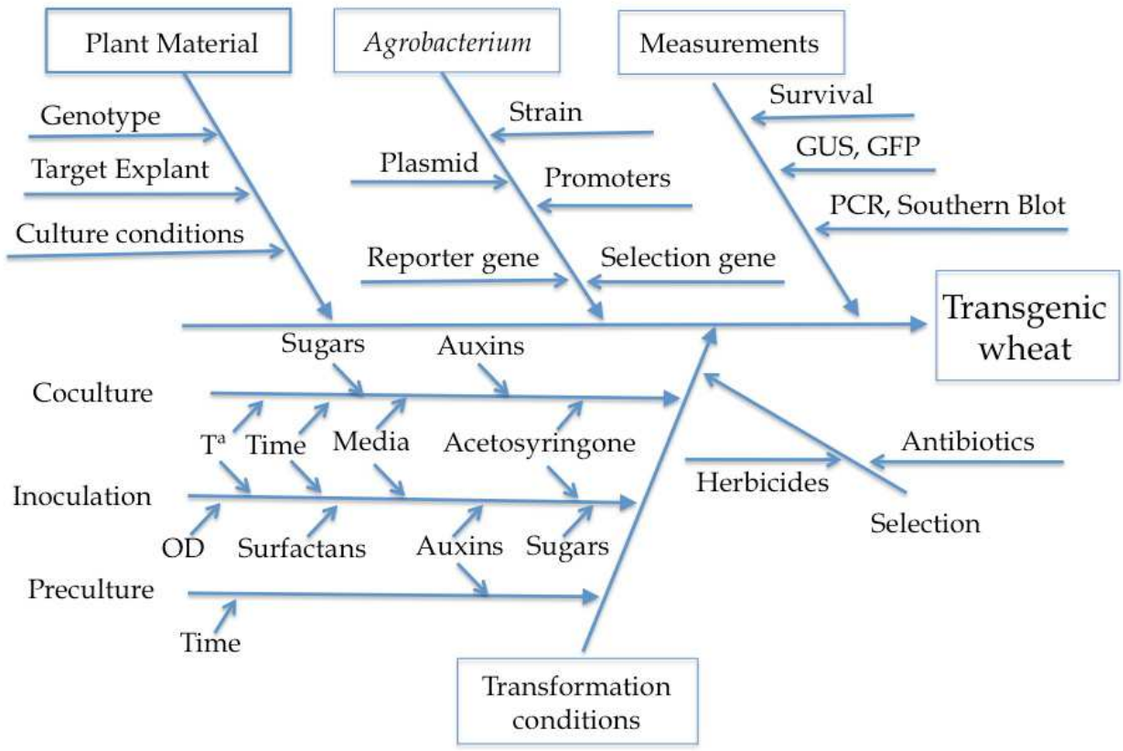

Fig. 1. Ishikawa diagram identifying the potential key variables of a wheat *Agrobacterium*-mediated transformation process.

4. Artificial Intelligence: A novel approach to model, understand and optimize cereals genetic transformation

Artificial intelligence approaches are based on the use of computational systems that simulate biological neural networks. They have been used not only for many industrial and commercial purposes since the 1950s (Russell & Norvig, 2003) but they have also been applied to fields more often related to biology, such as agricultural, ecological and environmental sciences (Jimenez et al., 2008; Huang, 2009). More detailed information about these technologies (Rowe & Roberts, 2005), and their applications to plant biology (Prasad & Dutta Gupta, 2008; Gallego et al., 2011) can be found elsewhere. Herein, we will briefly describe some relevant aspects of three of those technologies: Artificial Neural Networks (ANNs), genetic algorithms and neurofuzzy logic, which have been employed in plant science for modelling and optimizing different processes, in order to facilitate the understanding of its future applicability in cereal genetic transformation studies.

4.1 Artificial neural networks

Artificial Neural Networks (ANNs) are computational systems inspired in the biological neural systems. Information arrives to biological neurons through the dendrites. The neuron soma processes the information and passes it on via axon (Figure 2). In a similar way, ANNs use the processing elements called "artificial neurons", "single nodes" or

"perceptrons", that is, simple mathematical models (functions). Every perceptron receives information (inputs) from "neighbouring" nodes, then processes the information (either positive or negative) by multiplying each input by their associated weight (it is a measure of the strengths of the connection between perceptrons) giving a new result, which is adjusted by a previously assigned internal threshold (to simulate the output action), and produces an output to be transmitted to the next node. The perceptrons are organized into groups called layers. By connecting millions of perceptrons complex artificial neural networks can be achieved. The most used network architecture is called "multilayer perceptron" and consists in three simple layers: input, hidden and output layer (Rowe & Roberts, 2005).

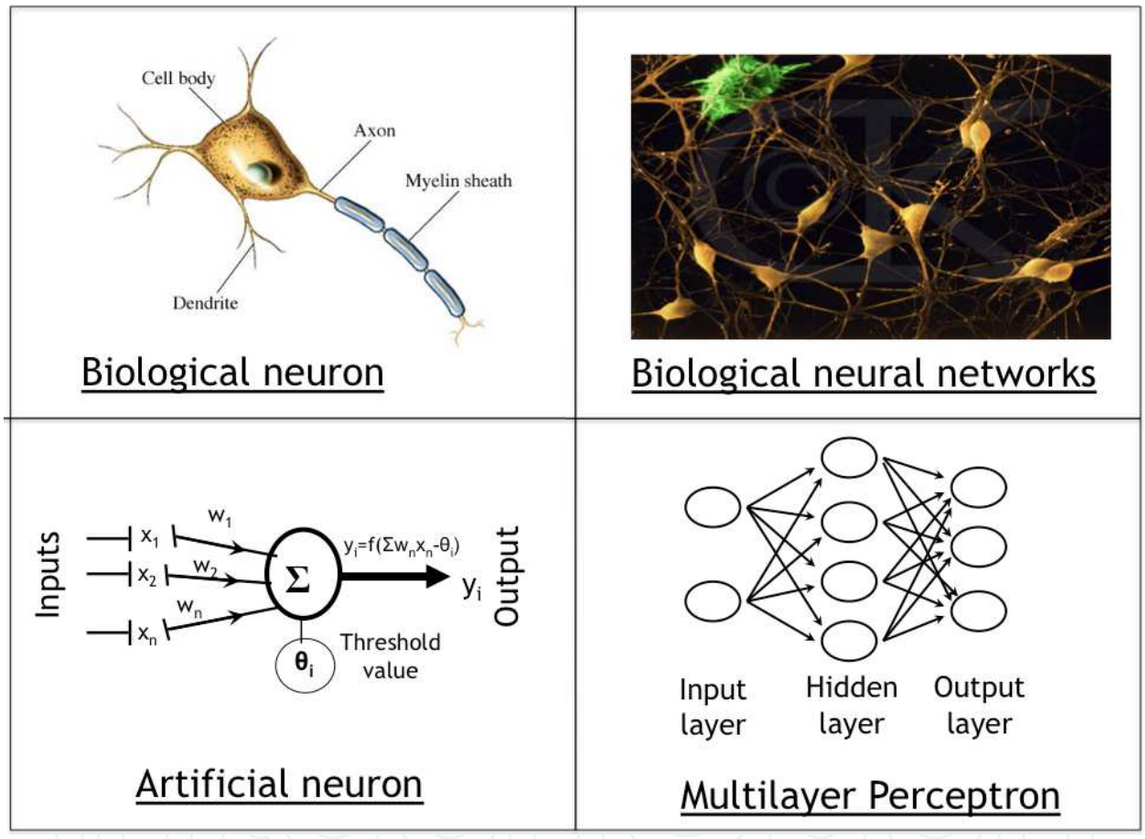

Fig. 2. Comparative schemes of biological and artificial neural system. X= input variable; W= weight of i_n input; θ= internal threshold value; f=transfer function.

Advantageously, while most conventional computer programs are explicitly programmed for each process, ANNs are able to learn, using algorithms designed to optimize the strength of the connections in the networks. For the network to learn it is necessary to use an example dataset (a collection of inputs and related outputs). Between 60 and 80% of the total data are chosen randomly, to perform the "training". In this process ANNs are able to search for a set of weight values that minimize the squared error between the data predicted by the model and the experimental data in the output layer. Furthermore, almost all the rest of the data set (10-20%) is used to "test" the model. Performance and predictability of the

model can be demonstrated by statistical parameters like the correlation coefficient (R^2) and the f value of the ANOVA of the model. Values of both training and test sets over 75% and f values over the f critical value for the corresponding degrees of freedom are indicative of high predictability and good performance (Colbourn & Rowe, 2005; Shao et al., 2006). Validation of the model can be performed by using a set of unseen data (validation data set) After a validation of the model, the ANNs is able to quickly predict accurately the output for a specific never tested combination of inputs or, in other words to answer "what if" questions, saving costs and time. Predictions using ANNs technology have been demonstrated to be more accurate than ones derived from experimental design and traditional statistic methods (Landín et al., 2009; Gago et al., 2010a). In conclusion, the ANNs approach could be useful to data processing, modeling, predicting and optimizing wheat genetic transformation.

ANNs have also some limitations related to the difficulties of interpreting the results when large data sets are used (several inputs and outputs are fitted in the model) and a large number of 2D surface plots or even 3 D graphs are generated by the model. In this case, ANNs can be coupled with other artificial intelligence technologies, such as genetic algorithms or fuzzy logic, creating hybrid systems that help to handle complex models and/or to data mining (Colbourn, 2003).

Sometimes the objective of modelling a specific process is not to predict new results (outputs), such as, when wheat *Agrobacterium*-mediated transformation is used to estimate the transformation efficiency when more amount of acetosyringone is added in the coculture stage. Probably for most researchers the main question could be "how to get" the maximum transformation efficiency, and more generally in those cases the objective is to find the combination of inputs that will provide the "optimum/best/highest" output in other words: optimize the process. This can be achieved combining ANNs and genetic algorithms.

4.2 Generic algorithms

Genetic algorithms (GA) are also a bio-inspired artificial intelligence tool, specially design to select the best solution of a specific problem (optimization). They are based on the biological principles of genetic variation and natural selection (mutation, crossover, selection or inheritance), mimicking the basic ideas of evolution over generations. In a simple way: when combined with ANNs, the genetic algorithms randomly generate a set of inputs and their corresponding predicted outputs using the ANNs model, called "set of candidate solutions" to the problem. Candidate solutions are then selected according to their fitness to previous established criteria; the best ones are used for evolving new solution populations to the problem, using crossover and mutation. After few generations the optimum should be reached because the most suitable candidates have more chance of being reproduced. Using this approach, complex micropropagation processes have been modelled by ANNs and successfully optimized by genetic algorithms (Gago et al., 2010a, 2010b).

4.3 Neurofuzzy logic

Neurofuzzy logic is a hybrid system technology that combines the adaptive learning capabilities from ANNs with the generality of representation from fuzzy logic (Shao et al.,

2006). Fuzzy logic is also an artificial intelligence tool especially useful in problem solving and decisions making, helping with the understanding of the complex cause-effect relationships between variables. When coupled with ANN, it becomes a powerful technique in handling complex models by generating comprehensible and reusable knowledge through simple fuzzy rules: IF (condition) THEN (observed behaviour). This kind of rules facilitates the understanding of a specific process, in a semi-qualitative manner, in a similar way to how people usually analyse the real world (Babuska, 1998; Gallego et al., 2011 and references therein). Many times words are more important for making decisions, drawing conclusions or even solving problems than a collection of accurate data (Fig. 3). Human knowledge is normally built on linguistic tags, and not on quantitative mathematical data, even though sometimes words are imprecise or uncertain.

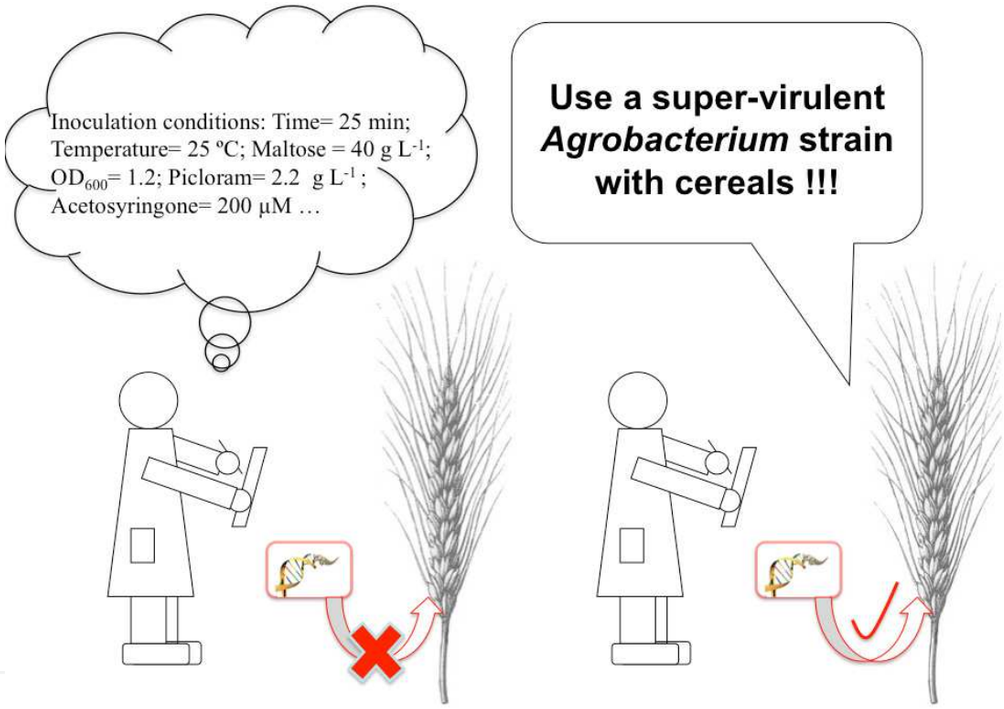

Fig. 3. Precision versus significance in the real world of researchers in the plant genetic transformation field.

The major capabilities of fuzzy logic are the flexibility, the tolerance with uncertainty and vagueness and the possibility of modelling non linear functions, searching for consistent patterns or systemic relationships between variables in a complex dataset, data mining and promoting deep understanding of the processes studied by generating comprehensible and reusable knowledge in an explicitly format (Setnes et al., 1998; Shao et al., 2006; Yuan et al., 2008). The neurofuzzy logic approach has been recently applied in modelling plant processes, such as *in vitro* direct rooting and acclimatization of grapevine (Gago et al., 2010d) or to gather knowledge of media formulation using data mining in apricot (Gago et al., 2011). In those cases, the authors found higher accuracy in identifying the interaction effects among variables of neurofuzzy logic than the traditional statistical analysis.

Moreover, neurofuzzy logic showed a considerable potential for data mining and retrieved knowledge from very large and highly complex databases.

5. Future perspectives

Agrobacterium-mediated transformation of wheat is a complex process although can be understood easily. It involves different scales of biological organization (genetic, biochemical, physiological, etc.) and many factors that influence the process. The storm of information generated by the analysis carried out during those processes would be useless if they could not be analysed together. Nowadays, artificial intelligence technologies give us the opportunity to handle a huge amount of biological data generated during the transformation process, with many advantages over traditional statistics. Artificial Intelligence technologies can solve common problems plant researchers associate to analysing, integrating variable information, extracting knowledge from data and predicting what will happen in a specific situation.

Different artificial intelligence approaches could be used for modeling, understanding and optimizing any *Agrobacterium*-mediated transformation procedure, either for wheat, cereals, fruit trees or any other biological process, giving results at least as good as, and less time consuming, those obtained by traditional statistics . More specifically, ANNs combined with genetic algorithms could predict the combination of variables (inputs) that would yield quality transformed wheat plants.

As a starting point a database can be obtained from historical results in the literature that can be modelled to find the more important variables affecting the *Agrobacterium*-mediated transformation procedure (data mining). On this knowledge, new experiments can be designed and performed and their results added to the database to fulfil the optimization processes (Gago et al., 2010a, 2011).

Great efforts have been made to improve the *Agrobacterium*-mediated transformation process, although the its full optimization is still far from being reached. In the future the application of modelling tools, such as those described here, could add a new insights into discovering the interactions between the variables tested and into understanding the regulatory process controlling molecular, cellular, biochemical, physiological and even developmental processes occurring during wheat *Agrobacterium*-mediated transformation.

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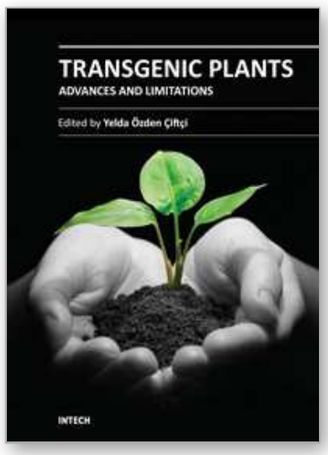

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