Részletes jelentés

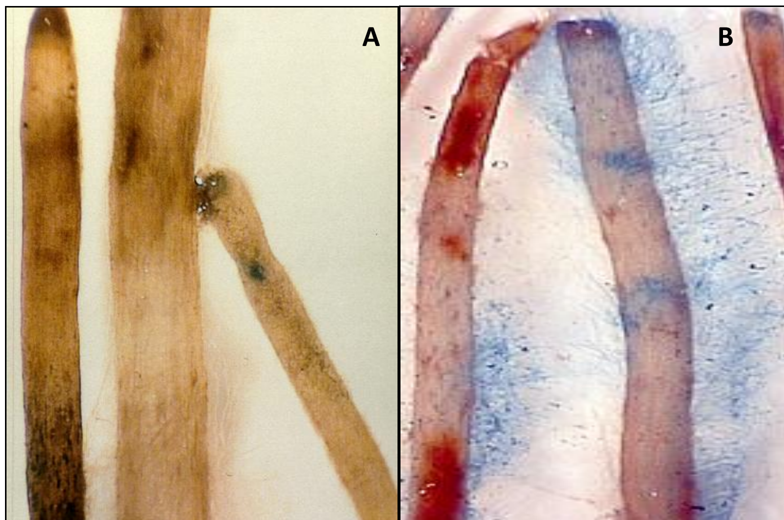
A projekt alapvető célkitűzése kettős (ld. eredeti pályázati leírás 2. része, a projekt munkaterve, 5 old.) : egyrészt hatékony *in vitro* tenyésztési módszerek alkalmazásával egy agrobaktériumon alapuló, lehetőleg *in planta* génátviteli technológia kidolgozása búzában (A), másrészt az *in planta* transzformációhoz speciálisan alkalmas, javított expressziós vektorok előállítása (B).

A. Agrobaktériumos *in planta* transzformáció fejlesztése búzában

Az elért eredményeket az alkalmazott módszerek szerint az alábbi bontásban közöljük:
1. Kalászkakultúrák, és az agrobaktérium kölcsönhatása különböző explantumokkal
2. A növényregeneráció összehasonlítása hazai kereskedelmi búzákon, elsősorban érett magból
3. Az agrobaktériumos transzformáció optimalizálása érett magon alapuló rendszerben
4. Új és gyors *in planta* transzformáció kidolgozása gabonafélék csíranövényeire

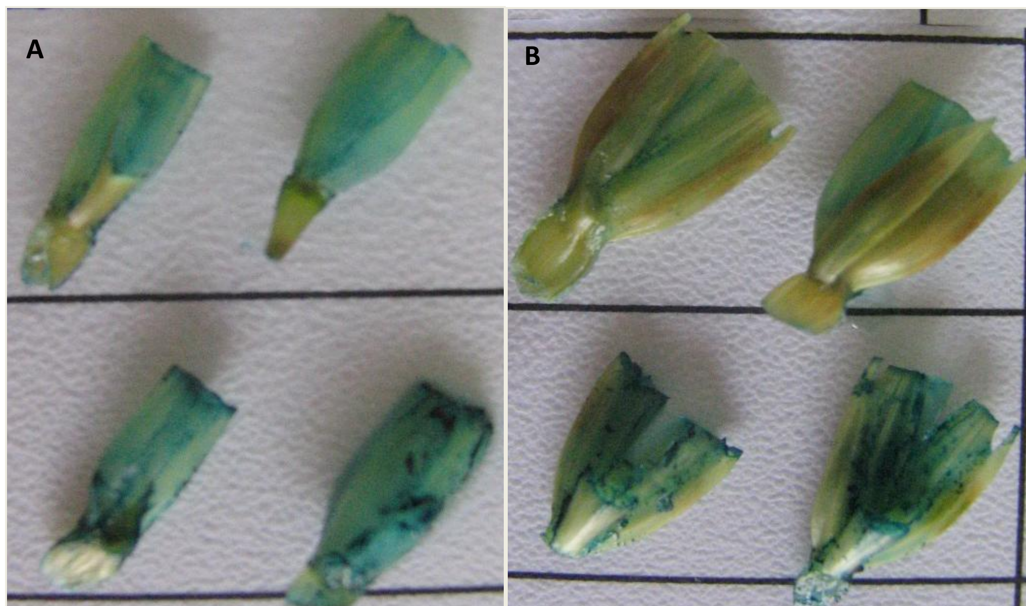
1. Két kenyérbúza (*Triticum aestivum* L. 'Mv Toborzó' és 'Chinese Spring'), valamint egy tönkölybúza (*T. spelta* L.) fajtából indítottunk kalászkatenyészetet, melyek a szemtermés mesterséges érlelésére alkalmasak, és így az *in planta* transzformáció *in vitro* szimulációjaként szolgálhatnak. A korábban publikált módszert (Barnabás és Kovács 1992. Sexual Plant Reprod 5: 286) követve azonban a közölt vagy használható magfogási gyakoriságot (tapasztalatok alapján 30-40% közti értéket tűztünk ki) nem tudtuk reprodukálni: a tenyésztett kalászkákban a magfogás mértéke megbízhatóan 10% alatt maradt mindegyik vizsgált fajtában, és a kapott magok csírázóképessége is igen alacsony volt. A kapott eredményekben szerepet játszhattak a szabadföldi körülmények (fertőzöttség, évjárathatás), valamint a szuboptimális nevelési feltételek üvegházban. Ez a gyakoriság és hatásfok azonban csak hosszadalmas optimalizálás után vezethetett volna használható módszerhez, ezért más alternatívákat kerestünk (ld. 2-4. pontok).

A kalászkatenyészetekkel párhuzamosan az agrobaktériumos transzformáció természetben is lezajló első lépéseit, a baktériumok megkötődését és a *vir* gének indukcióját 3 rendszerben tanulmányoztuk. A fent leírt kalászkatenyészeteket (2 kenyérbúza fajta, ld. fent) alkalmaztuk a kötődési tesztekhez, míg a *vir* gének aktivációját az érett mag (3 búza fajta: 'Mv Emese', 'Mv Toborzó' és 'Chinese Spring') mellett éretlen embriókon (2 búza fajta: 'Mv Emese' és 'Cadenza') is vizsgáltuk a célra kifejlesztett rekombináns baktériumtörzsekkel.
Az agrobaktérium tapadását az A1020G és a Chry5RG törzsek alkalmazásával mutattuk ki. Az A1020G törzs a *chvB* génre (ez egy, a ciklikus β-1,2-glikán szintézisét katalizáló citoplazmatikus membránfehérjét kódol) mutáns, és a növényi sejthez nem képes kapcsolódni, ezért vizes mosással könnyen eltávolítható a növényről. Ez a törzs a pTiB6806/pATC58 plazmidokat hordozza, és a kromoszómája az (intron nélküli) *gus*A markergént tartalmazza. A Chry5RG egy rifampicin rezisztens törzs, mely a pTiChry5 plazmidot hordozza, és kromoszómájába szintén az (intron nélküli) *gus*A markergén van beépítve. A *gusA* gén által kódolt β-glükuronidáz (GUS) enzim detektálásával az A1020G mutáns törzsben a nem tapadt baktériumok kimosásának hatásfokát, míg a Chry5RG törzzsel a tényleges fizikai kötődés mértékét lehet kimutatni (1. ábra).



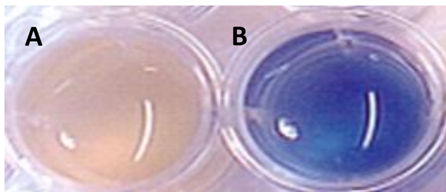
1. ábra. Az agrobaktérium kötődési tesztjének bemutatása. Az agrobaktériumok effektív kimosását a tapadásra nem képes A1020G mutáns törzs negatív GUS reakciója (A), míg a kimosás utáni fizikai kötődést a Chry5RG törzzsel kapott pozitív GUS reakció (B) igazolja.

Eredményeink szerint a vizsgált 2 búzafajtánál az agrobaktérium kötődése megtörténik az izolált kalászkákon, és ennek mértéke különösen az első pollenmitózistól emelkedik és a megtermékenyítés stádiumában is erőteljes (2. ábra).



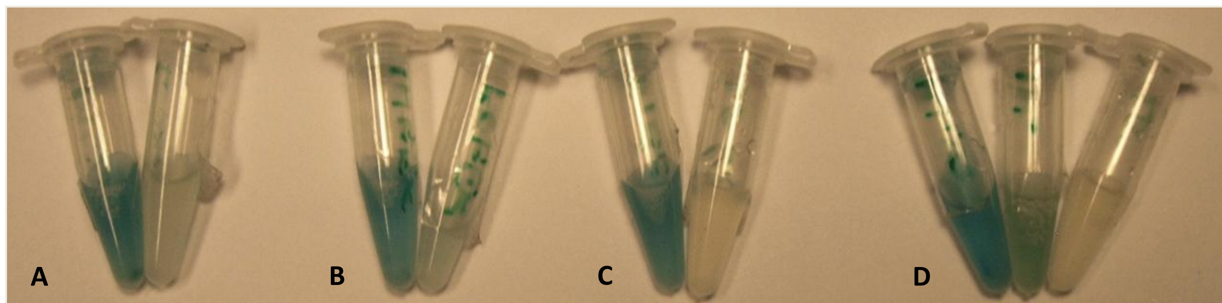
2. ábra. Az agrobaktérium kötődése a ’Mv Toborzó’ őszi búza izolált kalászkáihoz az első pollen mitózis (A) és a virágzás (B) stádiumában. A felső sorok a kontroll reakciót (A1020G), az alsó sorok a kötődési reakciót (Chry5RG) mutatják. Látható, hogy a korai stádiumban a kontroll is ad némi pozitív reakciót (A, felső sor), ami arra utal, hogy a vizes kimosás nem teljesen hatékony, de ezt a csekély háttért lényegesen meghaladja a fizikai kötődés mértéke. A kontroll háttér reakciójának oka a pelyvaszőrök sűrűségében kereshető a fiatalabb kalászkákon.

Az agrobaktérium *vir* indukciója kimutatásához a pSM358cd plazmidot használtuk, ami a β-galaktozidáz (GAL) enzimet kódoló *lacZ* riporter gén és az oktopin típusú pTiA6 plazmid *virE* génjének fúzióját tartalmazza. A *virE* a legerősebben expresszált virulencia gén, így a génfúzió révén könnyen detektálható, és a géntermék jelenléte egyúttal utal a T-DNS+fehérje komplex létrejöttére is. A GAL szubsztrátja, 5-bromo-4-kloro-3-indol-β-D-galaktóz (X-Gal) révén a *vir* indukció létrejötte hisztokémiásan meghatározható a célszövetekben, ami a GUS reakcióhoz hasonló jellegzetes indigókék színreakcióban jelentkezik (3. ábra).



3. ábra. Az agrobaktérium *vir* gén indukciós tesztjének bemutatása. Kontroll, nem induktív vagy gátló körülmények mellett a GAL reakció negatív (A), míg a *vir*(E) indukciót a kék színreakció (B) jelzi.

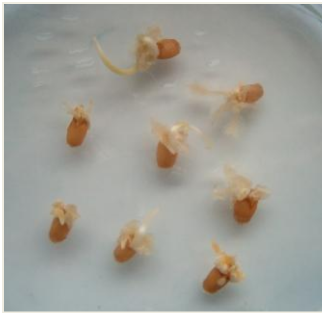
A *vir* indukciós vizsgálatok reprezentatív összefoglalását és értékelését a 4. ábra mutatja be. Az éretlen (4A) vagy érett embrióból (4B) indukált kalluszokat és az izolált érett embriókat (4C) nem indukált baktériumokkal együtt inkubáltuk, majd a GAL reakcióval néztük a *vir* indukció bekövetkeztét és mértékét. A nem indukált baktériumokkal kapott pozitív GAL reakciók (baloldal a pároknál) azt igazolják, hogy a búza explantumok aktiválják a *vir* indukció folyamatát. A nem indukált baktériumok negatív GAL reakciója az explantumok hiányában (jobboldal a pároknál) jelzi, hogy a negatív kontroll rendben van, és az explantumokkal kapott pozitív reakció nem fals háttér reakció eredménye.
A kísérletek azt mutatják, hogy a ’Mv Emese’ kalluszai és a ’Chinese Spring’ érett embriói egyaránt képesek az agrobaktérium *vir* indukciója kiváltására (4. ábra). Hasonló eredményeket kaptunk a ’Mv Toborzó’ és a ’Cadenza’ explantumaival is.



4. ábra. A *vir* gének indukciója éretlen embrióból (A) és érett embrióból (B) indított kallusszal (’Mv Emese’), valamint izolált érett embrióval (C, ’Chinese Spring’) és a kontroll reakciók (D). A párok (A-C) bal oldalán az explantumokkal aktivált, míg jobb oldalán az explantumok hiányában nem indukált baktériumok GAL reakciója látható. A kontroll reakciók (D): indukált pozitív (bal), acetosziringon nélkül kisebb mértékben indukált (középső) és nem indukált negatív (jobb).

A megfelelő kontrollokkal kiegészített kísérletek alapján levonhatjuk, hogy a különböző *in vitro* rendszerekben mind a baktériumok fizikai kapcsolódása, mind pedig *vir* génjeik aktivációja megtörténik, ami a sikeres génátvitel előfeltétele.

2. A kalászkatenyésztés alternatívájaként az érett magból indukált tenyésztéshez megoldottuk az ebben az esetben gyakori és nagyfokú mikrobiális szennyezés oly mértékű csökkentését, hogy a tenyésztés során a táptalajon fellépő fertőzés gyakorisága 10% alatt mozog. A kalluszindukció érett mag esetében igen gyors (3-6 nap, 5. ábra) és gyakorisága – egy-két kivételtől eltekintve – meghaladja a 90 százalékot. A növényregenerációt részletesebben is vizsgálva megállapítottuk, hogy a minél fiatalabb kalluszok átrakása regenerációs táptalajra lényegesen növeli a hajtásképződés gyakoriságát, ami esetenként elérheti a 100 százalékot is.



5. ábra. Kalluszindukció 5 nap alatt *T. monococcum* érett magjából (táptalaj: 2 mg/L dicamba).

Az érett magból indított tenyésztéshez összesen 20 kenyérbúza fajtát (17 őszi és 3 tavaszi) vizsgáltunk több ismétlésben. A legjobb (általános) *in vitro* reakcióval rendelkező genotípusok azonosításához az érett magon kívül további három rendszerben is teszteltük ugyanezt a 20 fajtát: portok (mikrospóra) eredetű haploidindukcióban, éretlen embrióindukcióban és izolált érett embrióindukcióban. Mind a négy rendszerben 5-10 ismétlésben megállapítottuk a kalluszindukció és a zöld (ill. albinó) növények gyakoriságát, melynek alapján a fajtákat összehasonlítólag értékeltük (1. táblázat). A kapott eredmények nemzetközi szaklapban történő publikációja az adatok statisztikai értékelése után folyamatban van (Mészáros et al., csatolva).

1. táblázat. Húsz kenyérbúza fajta regenerációs képessége négy szövettenyésztési rendszerben

Csoport	Fajta	Hatásos teny. rend-szer száma	Portok		Éretlen embrió		Érett szkutellum		Egész érett mag	
1	Mv Regiment	+++	2.1	d	59.4	a	34.0	bc	62.5	bcde
1	Mv Emese	+++	8.4	c	31.1	cd	40.5	b	64.9	bcde
2	Fatima2	++	17.7	a	9.8	hij	63.0	a	29.0	h
2	Mv Béres	++	20.5	a	13.3	fgh	13.3	efghi	72.5	bc
2	Mv 16	++	13.5	b	13.0	ghi	23.6	def	80.0	b
2	Bobwhite26	++	0.9	d	35.5	bcd	NT		95.0	a
2	Mv Mambo	++	0.2	d	39,1	bc	10.5	fghij	54.0	bcde
2	Chinese Spring	++	3.6	d	41.8	b	18.5	efg	48.5	efg
2	Mv Magdaléna	++	1.0	d	7.1	hij	32.5	bcd	54.0	ef
3	Mv Pálma	+	19.3	a	9.8	hij	0.5	j	8.5	i
3	Mv Palotás	+	0.3	d	25.2	de	0.0	j	76.0	b

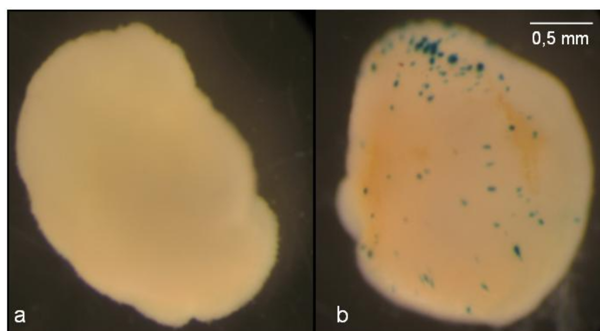
3	Mv Csárdás	+	1.5	d	23.3	de	24.0	cde	36.5	fgh
3	Mv Marsall	+	4.6	d	9.5	hij	7.5	ghij	74.0	bcd
3	Mv Magvas	+	0.1	d	6.5	hij	6.0	hij	63.0	bcde
3	Mv Hombár	+	3.7	d	23.6	ef	3.5	ij	50.0	efg
3	Mv Suba	+	1.8	d	19.0	efg	4.8	hij	55.0	def
3	Mv Süveges	+	NT		2.5	ij	16.8	efgh	55.5	cdef
3	Mv Toborzó	+	2.5	d	0.9	j	8.4	ghij	51.5	efg
3	Cadenza	+	0.8	d	8.0	hij	7.0	ghij	35.0	gh
-	Mv Kolo	-	1.6	d	4.5	hij	NT		NT	
	Averages		**5.5**		**20.6**		**17.5**		**56.1**	

Hatásos tenyésztési rendszer: >30% regenerációs gyakoriság (portok kultúrában >10%). Az eltérő betűk a szignifikánsan (P>5%) különböző regenerációs gyakoriságot jelzik egyazon oszlopon belül. NT, nem tesztelt. Az 1BL/1RS transzlokációt hordozó fajták aláhúzva.

A táblázat alapján is az alábbi következtetések vonhatók le: (i) univerzális fajta, amelyik minden szövettenyésztési rendszerben egyaránt kiválóan teljesít, nincsen, (ii) a leghatékonyabb rendszernek az érett magon alapuló bizonyult (ami egyben a legegyszerűbb is), és ezt követte a hasonló hatásfokú éretlen embriós illetve érett szkutellumos (pajzsocska) rendszer, (iii) az 1BL/1RS transzlokáció jelenléte egyik tenyésztési rendszer hatásfokával sincs szoros kapcsolatban, azaz a genetikai háttér szerepe vagy a kölcsönhatás lehet fontosabb, (iv) a szövettenyésztés szempontjából legsokoldalúbb fajták (a vizsgált szortimentben) a ’Mv Regiment’ és a ’Mv Emese’.

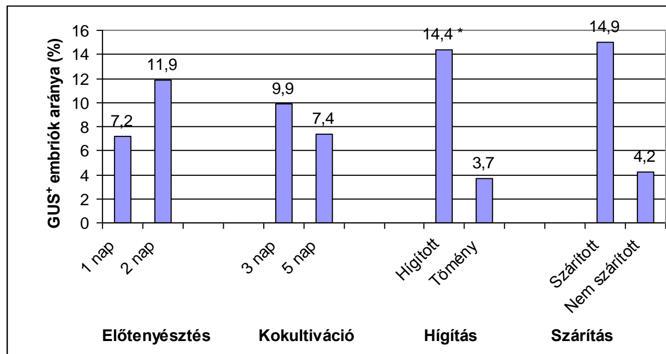
3. Következő lépésnek az érett magon végzett agrobaktériumos génátvitel sikerét befolyásoló tényezők közül célul tűztük ki: (i) az érett búza embrió előtenyésztési ideje, (ii) a baktérium-koncentráció, (iii) a kokultiváció időtartama, és (iv) a kokultiváció alatti szárítás, ill. a T-DNS átviteli hatékonyság közötti kapcsolat vizsgálatát és optimalizálását az intront hordozó $gusA^{INT}$ riporter gén tranziens expressziójával a ‘Mv Csárdás’ őszi búza fajtában, amely közepes reakciót képvisel ebben a szövetttenyésztési rendszerben.

A preparált érett embriókat kalluszindukciós táptalajon 1 vagy 2 napig előtenyésztettük, tömény (OD_{600}=1) vagy 50-szeresére hígított indukált agrobaktériumszuszpenzióval fertőztük, majd 3 vagy 5 napig kokultiváltuk közvetlenül kalluszindukciós táptalajon, vagy a táptalajra fektetett szűrőpapíron (szárítás). A tranziens GUS expressziót a kokultivációt követő második napon GUS hisztokémiai festéssel vizsgáltuk (6. ábra).



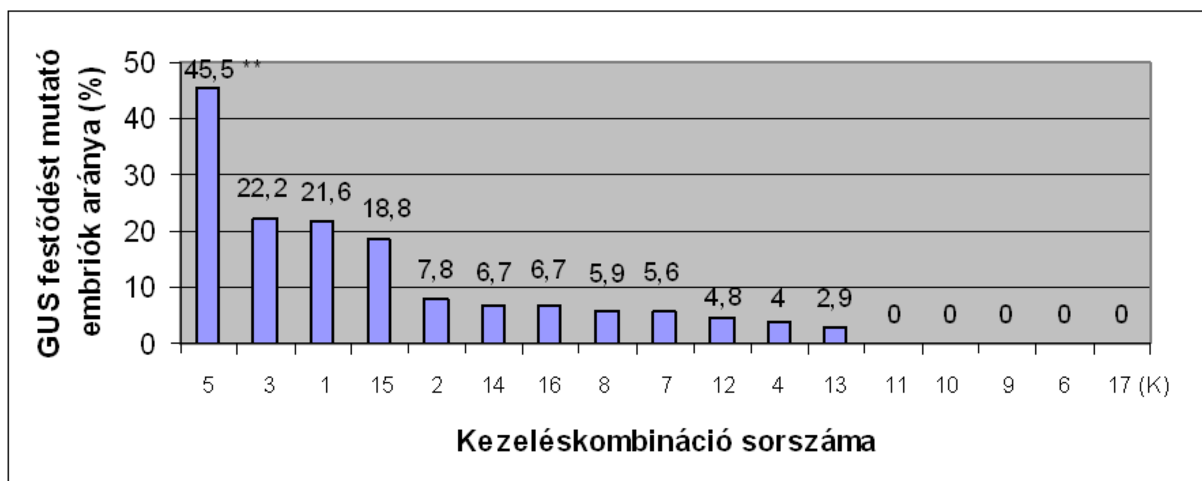
6. ábra. Kontroll (a) és tranziens GUS expressziót mutató (b) ‘Mv Csárdás’ őszi búza érett embrió.

Az érett embriók előtenyésztési ideje és a kokultiváció időtartama nem befolyásolta lényegesen a GUS expressziót, bár a legtöbb kezelés egy napos előtenyésztés után általában hatásosabb volt. A kokultiváció alatti szárítás már jelentősen növelte a GUS-pozitív embriók számát, de a változás (a magas szórás miatt) nem volt szignifikáns (7. ábra).



7. ábra. Különböző kezelések hatása a tranziens GUS expresszió gyakoriságára a 'Mv Csárdás' őszi búza érett embrióiban.
* P=5%-os szinten szignifikáns eltérés. A variációs koefficiens 121% volt.

A baktérium hígítása már önmagában is szignifikáns (P=5%) növekedést váltott ki (7. ábra), de leghatásosabbnak (P=1%) a szárítás és hígítás kombinációja bizonyult, melynek eredményeképp a GUS expresszió átlagos gyakorisága meghaladta a gyakorlatban is elfogadható 45%-ot (8. ábra).



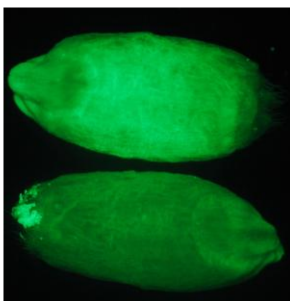
Kezelés-kombináció	5	3	1	15	2	14	16	8	7	12	4	13	11	10	9	6
Hígítás	+	+	+	–	+	–	–	+	+	–	+	–	–	–	–	+
Szárítás	+	+	+	+	–	–	–	–	+	–	–	+	+	–	+	–
Előtenyész-tési idő (nap)	2	1	1	2	1	2	2	2	2	1	1	2	1	1	1	2
Kokultivá-ció idő (nap)	3	5	3	5	3	3	5	5	5	5	5	3	5	3	3	3

8. ábra. A vizsgált kezeléskombinációk listája és hatásuk a tranziens GUS expresszió gyakoriságára a ‘Mv Csárdás’ őszi búza érett embrióiban.
17 (K): kontroll. ** P=1%-os szinten szignifikáns eltérés. A variációs koefficiens 148% volt.

Az optimalizált paraméterek a ’Mv 15’ mikrospóra eredetű embrióin is sikerrel voltak alkalmazhatók, és hatékony tranziens GUS expressziót eredményeztek (9. ábra). Hasonló eredményt értünk el a ’Mv 16’ mikrospóra embrióival is.

9. ábra. Hatékony tranziens génexpresszió a ’Mv 15’ őszi búza mikrospóra eredetű embrióiban egy 35S::*gusA*^{INT} (A) és egy UBI::*gfp*^{INT} (B) konstrukcióval. A kép jobboldalán egy negatív kontroll.

Az *in planta* transzformáció terén több kísérletet végeztünk el, melyek során kontrollált (üvegházi) körülmények között két (egy *gusA*^{INT} és egy *gfp*^{INT} riporter gént hordozó) agrobaktérium vektorkonstrukciót juttattunk be két tavaszi búza (’Chinese Spring’ és ’T Dong’) kalászaiba elsősorban pipettázással a korai egysejtmagvas mikrospóra stádiumban. A kezelt növényekről összesen 124 kalászban sikerült magot fognunk (2-34 szemtermés/kalász), melyek közül a pályázat költségén üzembehelyezett fluoreszcenciás sztereómikroszkóppal (3,67 mFt) a *gfp*^{INT} gént hordozó agrobaktériummal kezelt magok vizsgálata megtörtént.

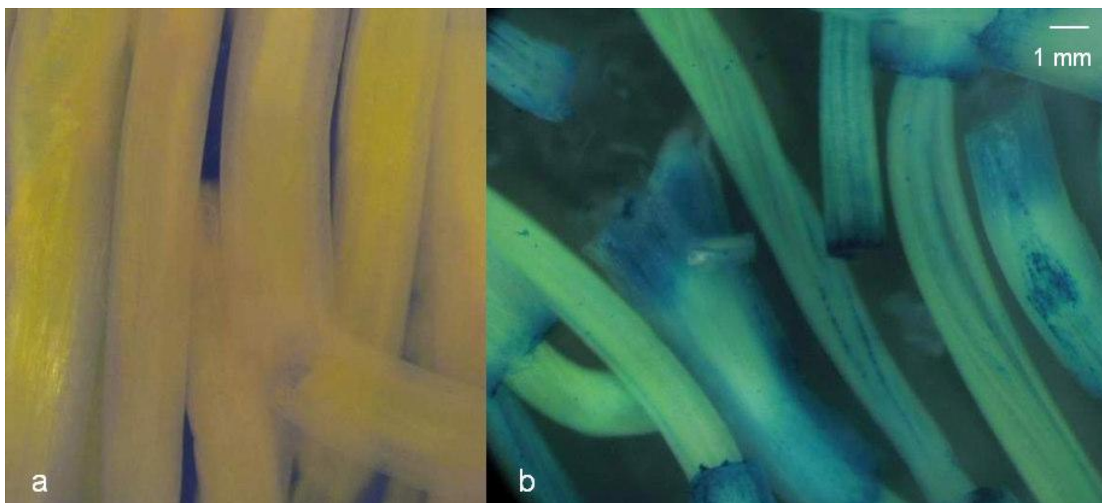


10. ábra. Fluoreszkáló (fent) és kontroll (lent) ’T Dong’ tavaszi búza magok.

Bár az agrobaktériummal kezelt anyagban kettő, a kontrollhoz képest határozottan fluoreszkáló magot ezrelékes gyakorisággal (2/1587 mag) találtunk (10. ábra), az ezekből csíráztatott növények negatívnak bizonyultak, ami arra utal, hogy a fluoreszcenciát valamilyen más jelenséggel (pl. fertőzés) összefüggő anyag okozhatta. Az eredmények alapján a korai generatív fejlődés során az in planta transzformáció igen alacsony hatásfokkal mehet végbe, és a jövőben a megtermékenyítés és a pollentömlő-hajtás időszakára lehet érdemes összpontosítani.

4. A projekt utolsó futaméve előtt a kísérleti növények nevelésére szolgáló üvegházat váratlanul lebontották, és helyére újat építettek. Mivel így az év teljes időszaka elveszett volna a projekt szempontjából, ezért 1 év (költségnélküli) halasztást kértünk és kaptunk. A kiesett időszak alatt és a nehézségek ellenére a projekt célkitűzésével összhangban olyan *in planta* génátviteli eljárást dolgoztunk ki, amihez még üvegházi növénynevelésre sincs szükség. A munkát egyetemi szakdolgozat keretében végeztük el, és egyelőre ebben a formában van közölve (Dőry, 2009). Ezzel a módszerrel gyors és hatékony tranziens génexpressziót értünk el több gabonafajban is.

Első lépésben az agrobaktériumnak a növényi sejtekhez kapcsolódását vizsgáltuk az A1020G és Chry5RG törzsek segítségével. Háromnapos 'Mv Csárdás' csíranövényeket kezeltünk e törzsekkel a korábban leírt módon (ld. 1. pont, 1. old. és 1. ábra). A Chry5RG törzzsel inkubált hajtások intenzív desztillált vizes mosás után is erőteljes GUS festődést mutattak, míg a kezeletlen kontroll és az A1020G mutáns törzs esetében nem vagy alig (0-7%) találtunk GUS festődést (11a ábra): a vizes mosás tehát csaknem tökéletesen eltávolította a fizikailag nem kapcsolódott agrobaktériumokat. A Chry5RG törzs 100%-os festődése (11b ábra) viszont azt igazolta, hogy az adott körülmények között az agrobaktérium jó hatásfokkal és stabilan kötődik a növényi sejtekhez. A hajtás minden részén látható volt a GUS festődés, de legsűrűbben a vágásfelület környékén (11b ábra).

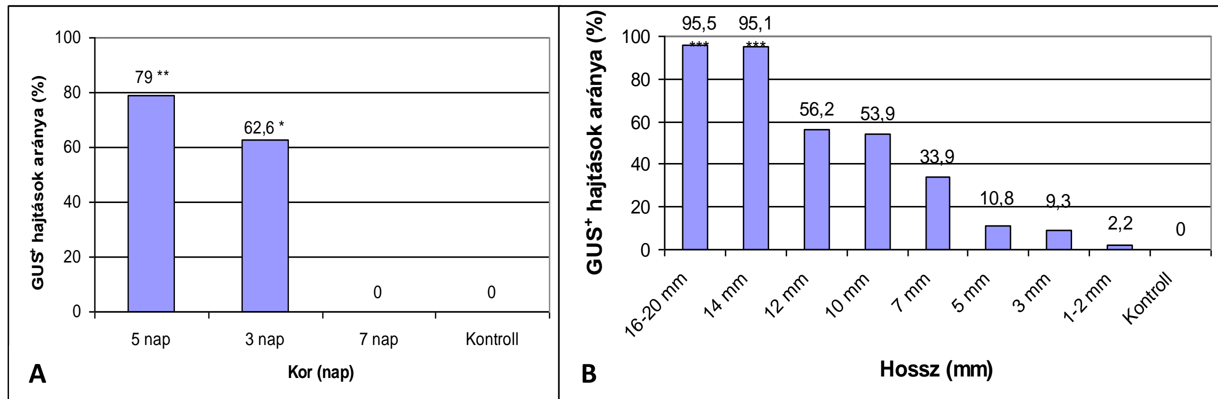


11. ábra. Az A1020G kapcsolódási mutáns (a) és Chry5RG (b) agrobaktérium törzsek kapcsolódása a 'Mv Csárdás' őszi búza hajtásaihoz desztillált vizes mosás után.

A következő lépésben három-, öt- és hétnapos csíranövények ('Mv Csárdás') hajtását a magtól 1-2, 3, 5, 7, 10, 12, 14 és 16-20 mm távolságra elvágtuk egy *gusA*^{INT} gént hordozó vektorral történő transzformáció előtt agrobaktériumos vagy steril szikével. Ezután az explantumokat *vir*-indukált agrobaktérium-szuszpenzióban, kvarchomok és/vagy csiszolópapír-darabok jelenlétében 30 percig rázattuk 2200 rpm fordulatszámmal, majd 30 percig inkubáltuk rázatás nélkül. A kezeléseket minimum 20 hajtáson végeztük, és a kísérleteket általában ötször megismételtük. Három nap kokultiváció elteltével hisztokémiai festéssel vizsgáltuk a tranziens GUS expressziót a hajtásokban. Ezek nagy részében vagy teljes egészében – a sebzés módjától függetlenül – kimutatható volt a GUS expresszió, míg a nem-transzformált, kontroll hajtásokban ez elmaradt. Mivel az intron jelenléte a *gusA* gén kódoló régiójában meggátolja a GUS enzim agrobaktériumban való termelődését, a

transzformált hajtások GUS expressziója a bakteriális T-DNS búzasejtekbe történő átvitelének és működésének tulajdonítható.

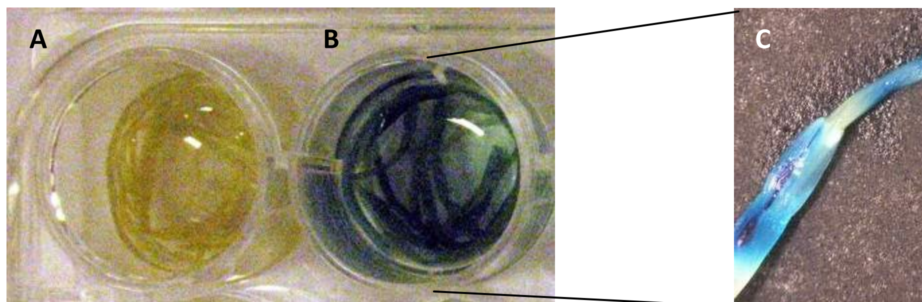
A csíranövények kora és a vágás távolsága szignifikánsan befolyásolta a GUS expresszió hatásfokát: az öt- és háromnapos kor (12A ábra), illetve a 14 és 16-20 mm-es méret (12B ábra) bizonyult a legjobbnak.



12. ábra. A csíranövények korának (A) és a vágás magtól való távolságának (B) hatása a tranziens GUS expresszió gyakoriságára a ‘Mv Csárdás’ őszi búzában.

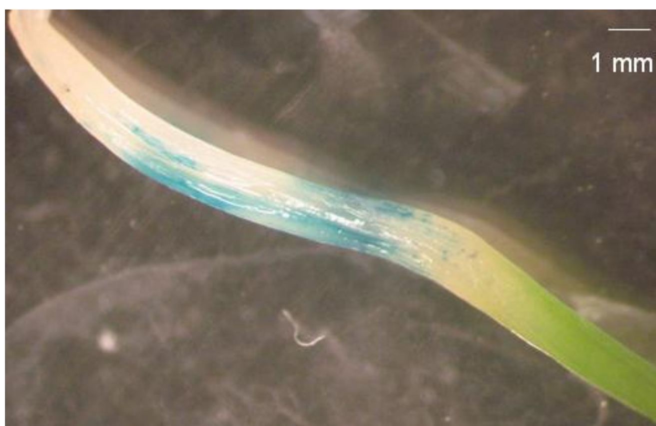
A többi paraméter: A: 16-20 mm hajtások, B: három- vagy ötnapos növények (kvarchomok, agrobaktériumos szike). Az egyes kezeléseket (n=20) öt kísérletben ismételtük. *** P=0,1%-os szinten szignifikáns eltérés, ** P=1%-os szinten szignifikáns eltérés, * P=5%-os szinten szignifikáns eltérés. A variációs koefficiens 48% (A) és 22% (B) volt.

A 14-20 mm-es távolságnál a GUS festődést mutató növények átlagos gyakorisága meghaladta a 95%-ot. Ezeknél a kezeléseknél fordultak elő a legnagyobb méretű festődések is: egyes hajtások teljes felületükön mutattak GUS expressziót (13. ábra).



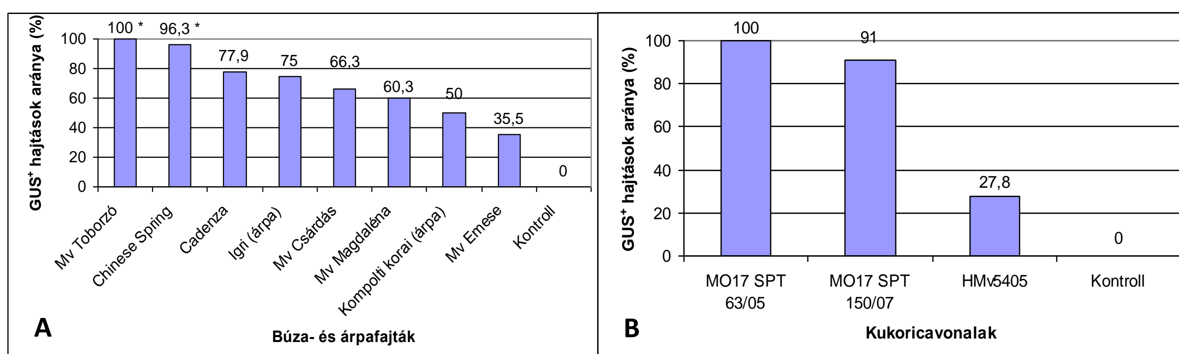
13. ábra. Kontroll (A) és tranziens GUS expressziót (B-C) mutató ‘Mv Csárdás’ őszi búza hajtások.

Megfigyeltük, hogy az 1 mm-nél hosszabb hajtások a kokultiváció ideje alatt túlnyomórészt kihajtottak. Ezért a hajtásokat ezt követően 3 napig kétszeresére hígított MS tápoldattal megnedvesített steril szűrőpapírra, Petri-csészébe helyeztük fényre, majd pedig növénynevelő talajlabdákba ültettük át őket. Egy héttel a transzformáció után a felnevelt ‘Mv Csárdás’ növényekben a stabil GUS expresszió gyakorisága még mindig 14,7% (5/34 növény) volt (14. ábra). Ez már mindenképpen stabil GUS expressziónak tekinthető, mert a GUS enzim féléletideje növényi sejtekben kb. 50 óra, tehát a pozitív reakció nem lehet a tranziens GUS génműködés eredménye.



14. ábra. Stabil GUS expressziót mutató első levél 1 hetes ‘Mv Csárdás’ növényen.

A módszer kevéssé genotípusfüggő, mert különböző őszi és tavaszi búza- és árpafajtákon, valamint kukoricavonalakon is sikerrel alkalmaztuk (15. ábra).



15. ábra. A tranziens GUS expresszió gyakorisága különböző búza- és árpafajtákban (A), illetve kukoricavonalakban (B).
Az egyes kezeléseket (n=20) két kísérletben ismételtük. * P=5%-os szinten szignifikáns eltérés. A variációs koefficiens 15% (A) és 19% (B) volt.

A kidolgozott módszerek felhasználhatók többek között különböző génexpressziót szabályozó elemek (pl. promóterek és poliA régiók) tesztelésére, vagy gének (pl. rezisztenciagének) tranziens – és stabil – csendesítésére vagy túlexpresszálására. A leírt eljárások hozzájárulhatnak a búza és a gabonafélék jelenleginél egyszerűbb és hatékonyabb genetikai módosításához.

B. Expressziós vektorok készítése

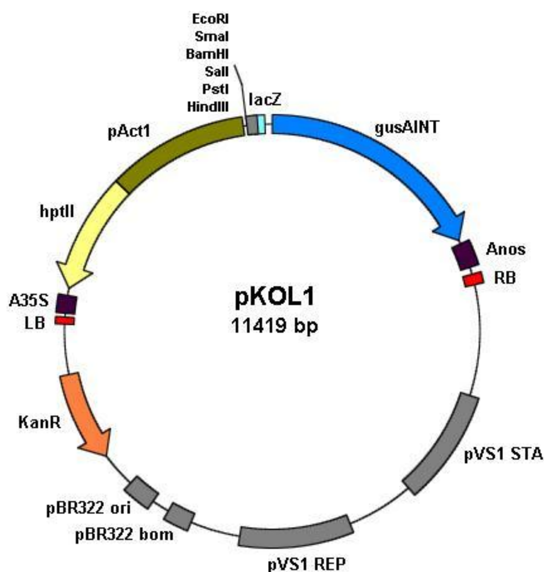
Az *in planta* transzformáció sajátossága, hogy a szövettenyésztés kiiktatásával megy végbe, ezért a transzgénikus események gyors kimutatása vagy korai szelekciója során előnyt jelent a bevitt géneknek speciálisan a magban történő hatékony expressziója. Ennek érdekében két meglévő expressziós vektort (pLS24 és pLS25) úgy módosítottunk, hogy közös szelekciós markergénjük, a hygromicin foszfotranszferáz enzimet kódoló gén (*hpt*II) búzában nem elég aktív (karfiolmozaik vírus 35S RNS) promóterét erős konstitutív promóterrel (Act1) cseréltük fel (pKOL1), ill. a riporter génekhez konstitutív (Ubi1) promótert kapcsoltunk (pKOL2 és pKOL3), majd azt kicseréltük egy endospermium-specifikus promóterre (pKOL4 és pKOL5). Összeállítottunk továbbá két olyan transzformációs kazettát, melyek már nem riporter gént tartalmaznak, hanem a búzaliszt minőségét befolyásoló egyik nagy molekulatömegű (HMW) glutenin alegységfehérje (1Ax2*B) génjét (pKOL6), vagy pedig egy az állatgyógyászatban alkalmazható antigén fehérje génjét a 'Porcine Epidemic Diarrhea' vírusból (pKOL7).

Összesen hét transzformációs kazettát készítettünk agrobaktérium-közvetítette növény-transzformációra a pCAMBIA1391z jelzésű vektorból (GenBank: AF234312) kiindulva. Ez a vektor a bal és jobb határszekvenciák (LB ill. RB, ld. lenti ábra) által kijelölt T-DNS régión kívül tartalmazza: (i) a kanamicin antibiotikum rezisztencia gént (KanR) a plazmid baktériális szelekciójához, (ii) a pBR322 plazmid replikációs origóját (pBR322 ori) és mobilizációs régióját (pBR322 bom = 'basis of mobility') *E. coli*ban történő szaporításhoz, illetve agrobaktériumba történő transzkonjugációhoz, és végül (iii) a *Pseudomonas aeruginosa* pVS1 plazmid replikációs (pVS1 REP) és stabilizációs (pVS1 STA) régióit, melyek a proteobaktériumokban (pl. agrobaktérium) történő stabil fenntartáshoz szükségesek.

Az alábbiakban az elkészített konstrukciók sematikus leírása és térképei találhatók.

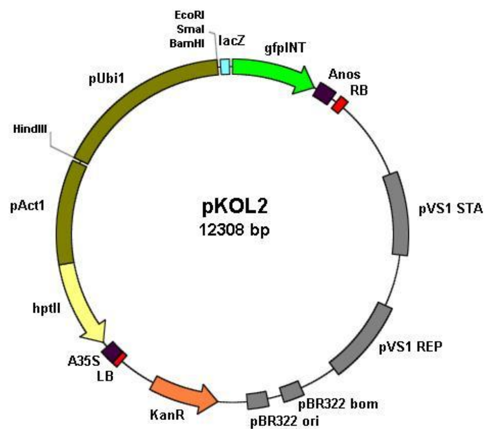
pKOL1

rizs aktin1 (*act1*) gén promótere és első intronja (pAct1)::*hpt*II::karfiolmozaik vírus 35S RNS poliA régió (A35S) – (nincs promóter):: intront tartalmazó *E. coli* béta-glükuronidáz enzimet kódoló kimérikus gén (*gus*AINT*)*:: nopalin szintáz gén poliA régió (Anos)



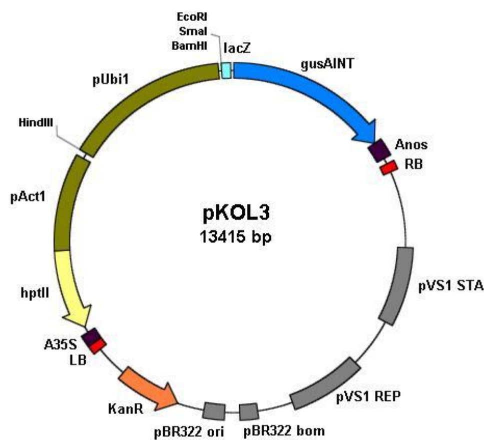
pKOL2

pAct1::*hpt*II::A35S – kukorica ubiquitin1 gén promótere (pUbi1)::intront tartalmazó S65T mutáns *Aequorea victoria* zölden fluoreszkáló fehérjét kódoló kimérikus gén (*gfp*INT)::Anos



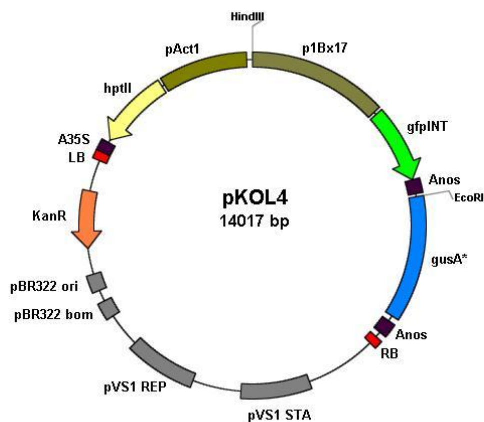
pKOL3

pAct1::*hpt*II::A35S – pUbi1::*gus*AINT::Anos



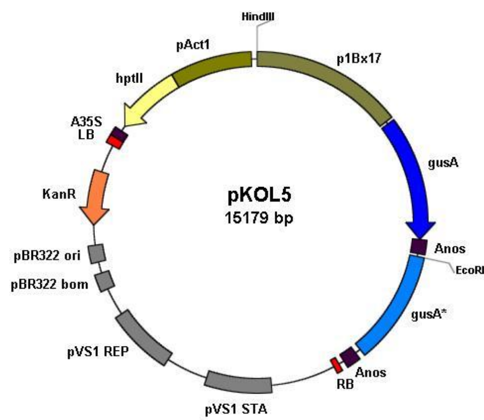
pKOL4

pAct1::*hpt*II::A35S – 1Bx17 búza HMW glutenin alegységfehérje gén (endospermium-specifikus) promótere (p1Bx17)::*gfp*INT::Anos (*gus*A*: a pKOL1-be történt beillesztés után az eredeti *gus*AINT génből visszamaradt darab)



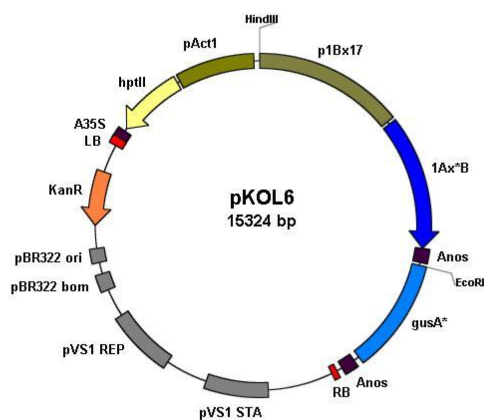
pKOL5

pAct1::*hpt*II::A35S – p1Bx17::(intron nélküli) *gus*A::Anos (*gus*A*: a pKOL1-be történt beillesztés után az eredeti *gus*AINT génből visszamaradt darab)



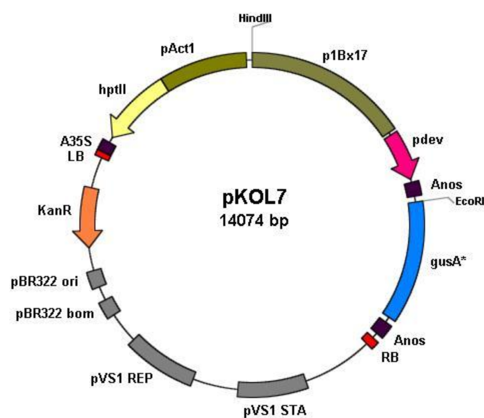
pKOL6

pAct1::*hpt*II::A35S – p1Bx17 (+*act*1 intron)::1Ax2*B búza HMW glutenin alegységfehérje génje (1Ax2*B)::Anos (*gus*A*: a pKOL1-be történt beillesztés után az eredeti *gus*AINT génből visszamaradt darab)



pKOL7

pAct1::*hpt*II::A35S – p1Bx17 (+*act*1 intron)::'Porcine Epidemic Diarrhea' Vírus antigén (*pedv)*::Anos (*gus*A*: a pKOL1-be történt beillesztés után az eredeti *gus*AINT génből visszamaradt darab)



Az egyes konstrukciók elkészítéséhez alkalmazott klónozási stratégia konkrét lépései a következők voltak:
A **pKOL1** kazetta abban különbözik a pCAMBIA1391z transzformációs kazettától, hogy a *hpt* szelekciós marker gén standard karfiolmozaik vírus 35S RNS promóterét a búzában sokkal hatékonyabb rizs aktin1 génjének első intronját is tartalmazó promóterével (pAct1) helyettesítettük. Ehhez a rendelkezésünkre álló pAct1F plazmidból PCR módszerrel felszaporítottuk a promóter részt úgy, hogy a DNS fragment 5' végére *Hin*dIII, míg a 3' végére *Nco*I restrikciós endonukleáz enzim által felismert nukleotid szekvenciákat tettünk a primerek révén. A megsokszorozott DNS darabot PCR klónozó kit (pCR2.1, Invitrogen) segítségével klónoztuk. A RAP-pTOPO nevű plazmidot tisztítottuk és az inszertet ellenőrzésképpen szekvenáltuk M13F és M13R primerek segítségével. A szelekcióra használt *hpt* gént a poliA régiójával, valamint a pCAMBIA kazettában lévő T-DNS-hez tartozó bal oldali határszekvencián túli rövid szakasszal együtt, PCR módszerrel felszaporítottuk úgy, hogy a gén 5' végére *Bsp*HI restrikciós endonukleáz enzim által felismert nukleotid szekvenciát tettünk további klónozás céljára. A DNS fragment 3' végét nem módosítottuk, vagyis a kazettában meglévő *Sac*II restrikciós endonukleáz enzim felismerő helyet tartalmazta. A megsokszorozott DNS fragmentet a fentebb leírt módon klónoztuk és szekvenáltuk. A klón neve HPT-pTOPO. Mivel a két klónozott szekvenciát (RAP ill. HPT) a pCR2.1 plazmidban nem tudtuk összefűzni, a RAP szekvenciát szubklónoztuk pET22b plazmidba. A RAP-pET plazmidot kinyitottuk *Nco*I és *Xba*I restrikciós endonukleáz emésztéssel, s a HPT nevű DNS darabot beillesztettük, melyet előtte *Bsp*HI és *Xba*I restrikciós endonukleázokkal emésztettünk. Az erős konstitutív promóter által irányított szelekciós markergént tartalmazó DNS fragmentumot a pET plazmidból *Hin*dIII és *Sac*II restrikciós endonukleáz emésztés, valamint agaróz gélelektroforézist követő tisztítás után klónoztuk a megfelelő enzimekkel kinyitott, gélelektroforézis után tisztított, pCAMBIA1391z vektorba.

A pKOL2 kazettát egy korábban elkészített pLS24 nevű pCAMBIA1391z alapú transzformációs vektor segítségével állítottuk össze, melyben benne volt az intront tartalmazó GFP fehérje génje (*gfp*INT) a kukorica ubiquitin1 gén promóterének (pUbi1) irányítása alatt. Ezt a kazettát meghasítottuk *Hin*dIII és *Sac*II restrikciós endonukleázokkal. Agaróz gélelektroforézissel elválasztottuk a megfelelő DNS szálat, a DNS-t megtisztítottuk, majd összekapcsoltuk a fentebb leírt módon előállított, megtisztított szelekciós markergént is tartalmazó DNS fragmenttel.

A **pKOL3** kazettát ugyancsak egy korábban elkészített pLS25 nevű pCAMBIA1391z alapú transzformációs vektor segítségével állítottuk össze, melyben benne volt az intront tartalmazó béta-glükuronidáz enzim génje (*gusA*INT) az ubiquitin fehérjegén (pUbi1) promóterének irányítása alatt. Ezt a kazettát meghasítottuk *Hin*dIII és *Sac*II restrikciós endonukleázokkal. Agaróz gélelektroforézissel elválasztottuk a megfelelő DNS szálat, a DNS-t megtisztítottuk, majd összekapcsoltuk a fentebb leírt módon előállított, megtisztított szelekciós markergént is tartalmazó DNS fragmenttel.

A **pKOL4** kazetta alapját a pKOL1 transzformációs kazetta adta. A pKOL1 plazmidot *Hin*dIII és *Mfe*I restrikciós endonukleáz enzimekkel emésztettük, agaróz gélelektroforézis után a megfelelő DNS darabot tisztítottuk, s ebbe beépítettük egy szövetspecifikus promóter által irányított *gfp*INT gént tartalmazó (1Bx17-GFP-NOS), agaróz gélben szintén tisztított, megfelelő (*Hin*dIII és *Eco*RI) „ragadós" végekkel rendelkező DNS fragmentumot.

Ezt a plazmid konstrukciót úgy állítottuk össze, hogy a S65T mutáns, zölden fluoreszkáló fehérjét kódoló *gfp*INT gént egy pLMNC95 nevű plazmidból felszaporítottuk úgy, hogy a DNS szakasz 5' végéhez *Bam*HI, míg a 3' végéhez *Kpn*I restrikciós endonukleáz enzim felismerő helyet adtunk. A PCR terméket a fentebb már említett módon klónoztuk, és a gén nukleotid sorrendjét szekvenálással ellenőriztük. A GFP-pTOPO plazmidból *Bam*HI és *Kpn*I enzim hasítás után kivágtuk a számunkra szükséges DNS darabot, és a korábban elkészített TLZ-pUC18 plazmidba szubklónoztuk azonos helyekre agaróz gélben való elválasztás és tisztítás után. A TLZ-pUC18 plazmid tartalmaz egy endospermium-specifikus promótert, mely a búza HMW glutenin alegységfehérjék közül az 1Bx17 jelű fehérjegén promótere. Ebben a plazmidban van még egy nopalin szintáz poliA (Anos) szekvencia is. Az elkészült, ellenőrzött plazmid neve 1Bx17-GFP-NOS-pUC18.

A **pKOL5** kazetta alapját szintén a pKOL1 transzformációs kazetta adta. A pKOL1 plazmidot a fentebb leírt módon hasítottuk, választottuk el és tisztítottuk. Ehhez a DNS darabhoz kapcsoltuk az azonos „ragadós" végeket adó restrikciós endonukleázokkal (*Hin*dIII, *Eco*RI) emésztett, a béta-glükuronidáz enzim (GUS) génjét is tartalmazó DNS fragmentet (1Bx17act1-GUS-NOS), melyet előtte agaróz gélben választottunk el a nem szükséges plazmid darabtól, és megfelelő kittel tisztítottunk.
A pKOL4 kazettában található endospermium-specifikus promóter és a pKOL5 kazettában lévő promóter szekvencia között az a különbség, hogy az utóbbi egy kimérikus promóter. Az endospermium-specifikus 1Bx17 HMW glutenin fehérjegén promóterének 3' végéhez hozzáragasztottuk a rizs aktin1 gén első intronját, mely bizonyítottan javítja az expressziót szövetspecifikus promóter esetében is. A kiméra promótert (p1Bx17) és nopalin szintáz poliA (Anos) szekvenciát tartalmazó plazmid neve pTSI. Ennek *Nco*I és *Bam*HI helyére klónoztuk a PCR módszerrel (lásd fent) felszaporított intron nélküli *gus*A gént, melyet megfelelő restrikciós endonukleáz felismerő helyekkel (*Bsp*HI, *Bam*HI) láttunk el. A ligálás, transzformálás, ellenőrzés után kapott plazmid neve 1Bx17act1-GUS-NOS-pUC18.

A **pKOL6** kazetta elkészítéséhez a pKOL1 plazmidba a fentebb leírt enzimes hasítás és tisztítás után ligáltuk az 1Ax2*B jelű HMW glutenin alegységfehérje génjét és endospermium-specifikus kiméra promótert is tartalmazó, megfelelő ragadós végekkel rendelkező DNS fragmentet (1Bx17act1-1Ax2*B-NOS).
Előtte a pTSI plazmidba klónoztuk a PCR módszerrel felszaporított, restrikciós endonukleáz felismerő helyekkel ellátott, szekvenciájában ellenőrzött búza tartalékfehérje gént. A plazmid neve 1Bx17act1-1Ax2*B-NOS-pUC18.

A **pKOL7** kazetta elkészítéséhez a pKOL1 plazmidba a korábban leírt enzimes hasítás és tisztítás után ligáltuk a sertésekben vírusos hasmenést okozó PEDV koronavírus ellen hatékony, ehető vakcina előállítására alkalmas fehérje génjét, valamint az endospermium-specifikus kiméra promótert is tartalmazó DNS szakaszt (1Bx17act1-PEDV-NOS).
A PEDV fehérje megfelelő restrikciós endonukleáz felismerő helyekkel ellátott génjét PCR módszerrel felszaporítottuk, szekvenciájában ellenőriztük a korábban leírt módon, majd a pTSI plazmidba építettük. A plazmid neve 1Bx17act1-PEDV-NOS-pUC18.

A fenti transzformációs kazettákat az AGL0 standard agrobaktérium törzsbe transzformáltuk, s így használhatók a további növénytranszformációs kísérletekben. A munka során összesen kb. 24,3

kilobázist építettünk be a különböző vektorokba, melynek több mint felét, kb. 14 kilobázist nukleotidszekvenálással is ellenőriztünk és igazoltunk.

A pályázattal kapcsolatos közlemények

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* csatolva (a fenti sorrendben), OTKA – támogatás jelölve, IF – impakt faktor, FH – független hivatkozás

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ARTICLE

Regeneration ability of wheat (*Triticum aestivum* L.) embryos after bombardment with a particle gun

Boglárka N. Kisgyörgy*, Cecilía Tamás, Mariann Rákszegi, László Sági, László Láng, Zoltán Bedő

Agricultural Research Institute of the Hungarian Academy of Sciences, Martonvásár, Hungary

ABSTRACT The plant regeneration ability of the spring wheat variety Cadenza from the UK and the winter variety Mv Emese from Hungary was studied over the course of two years. The calli were regenerated with 10 hours illumination in the first year and 14 hours illumination in the second, and the external environment was not completely excluded, light being admitted through a window. The level of plant regeneration was evaluated 7–8 weeks after the isolation of the scutella. Both genotypes exhibited considerable fluctuation in plant regeneration over the two years, but no significant difference was observed in the mean plant regeneration ability of Mv Emese and Cadenza as a function of the two illumination periods
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KEY WORDS

particle bombardment,
bar,
plant regeneration,
Triticum aestivum L

Breeders are able to improve the agronomic traits of wheat (*Triticum aestivum* L.) by exploiting the genetic variability of the species itself and by crossing it with closely related species (Bedő et al. 1998; McIntosh 1998). From the breeding point of view the aim of transformation is to create new varieties with better yield potential, resistance or breadmaking quality by improving these traits in varieties well adapted to the given climatic conditions (Lazzeri et al. 1997; Vasil 1998, Pellegrineschi et al. 2000). The first reports of successful wheat transformation were published by Vasil et al. (1992), who opened up the way for the introduction of genes into the wheat genome which could not be incorporated by natural means. Over the past 15 years many authors have reported on the development of transgenic genotypes.

In many cases the initial material used for biolistic gene transformation is the scutellum isolated from immature embryos, the uncut side of which is bombarded (Sparks and Huw 2004). In cereal species the DNA is either introduced into the plant material a few hours after isolation (Barro et al. 1997; Pellegrineschi et al. 2002) or 2–8-day calli are transformed (Vasil et al. 1992; Patori et al. 2001; Sparks and Jones 2004).

One major criterion for plant transformation is the existence of a tissue culture system with good *in vitro* plant–cell–plant regeneration (Shewry and Jones 2005). The regeneration ability of wheat callus is a quantitative trait, the genes responsible for it being located on various chromosomes (Galiba et al. 1986; Ben Amer et al. 1997). The regeneration ability, and thus the efficiency of transformation, depends to a great extent on the genotype (Fennel et al. 1996; Varsheny and Altpeter 2002), on the plant organ used for callus formation (Barro et al. 1999; Folling and Olesen 2001) and on the tissue culture conditions (He et al. 1989; Barro et al. 1999; Rákszegi et al. 2003). There have been many reports of studies on the tissue culture conditions and the composition of the nutrient media used for plant regeneration (Rasco-Gaunt et al. 1999; Zhang et al. 1999). Depending on the concentration and ratio of the plant hormones applied, either shoot or root regeneration may be initiated first (Dudits and Heszky 2003). The nutrient media most frequently used for species belonging to the *Triticeae* genus are MS (Murashige and Skoog 1962) and variants of this. Shoot regeneration requires a fairly high concentration of cytokinin, with or without auxin (Barro et al. 1998), while 5–10 mg/l zeatin has also been found to have a positive effect on regeneration. Root regeneration takes place on hormone-free medium (Barro et al. 1999; Sparks and Jones 2004). High concentrations of sugars or sugar alcohols in the nutrient medium, or the presence of various metal ion additives (e.g. silver) may increase the efficiency of plant regeneration in the case of bread wheat.

Wheat is the most sensitive to the environmental variables of temperature and photoperiod during the vegetative period (Slafer and Rawson 1994). Various recommendations can be found for the light conditions, ranging from 20 µmol m^{-2} s^{-1} with a 16-h photoperiod (Tamás et al. 2004) to 250 µmol m^{-2} s^{-1} with 12-h daylength (Sparks and Huw 2004). Vectors supplied with a ubiquitin promoter, such as pAHC20 or pAHC25, have a high level of expression in monocotyledonous plants (Alan et al. 1995), so they are widely used for wheat transformation.

Our aim was to study the regeneration reaction of two wheat varieties with different growth habit.

*Corresponding author. E-mail: nemethne@mail.mgki.hu

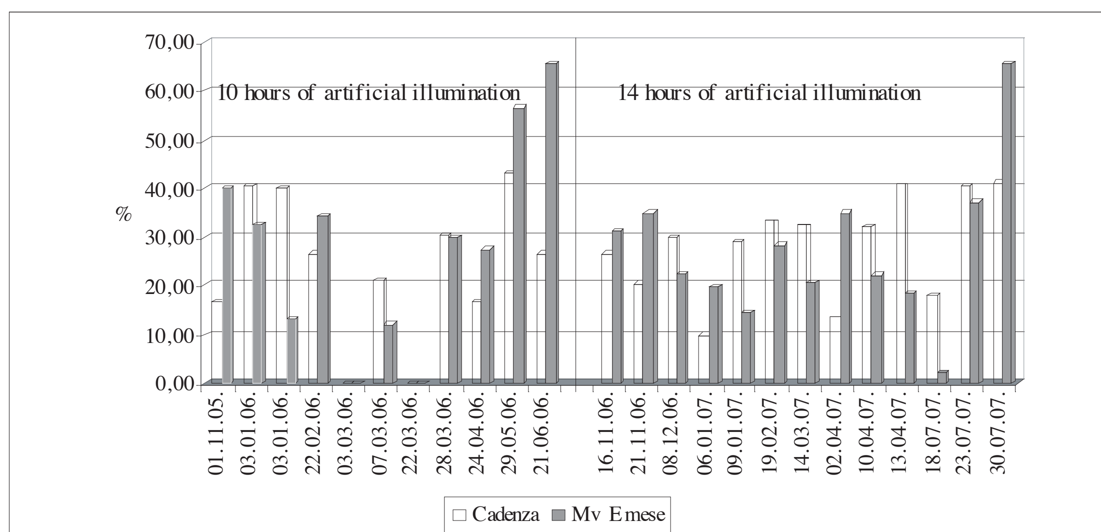

Figure 1. Percentage values of plant regeneration for the winter wheat Mv Emese and the spring wheat Cadenza under 10 and 14 hours of artificial illumination.

Material and Methods

After germination, donor plants of the Cadenza spring variety were vernalised at 4°C for 2 weeks and those of Mv Emese winter variety for 6 weeks. The seedlings were then planted out and raised under constant phytotronic conditions at a day/night temperature of 18/16°C with 16-h daylength. Immature grains were collected 12–14 days after flowering, after which the scutella were isolated and the nutrient media prepared as described by Sparks and Huw (2004). The scutella were placed on medium designed to induce callus formation. After keeping them in the dark for two days, the scutella were transformed using a PDS-1000/He particle gun according to the manufacturer's instructions. A 28 Hgmm vacuum was created in the chamber and the helium gas was injected into the space above the macrocarrier at a pressure of 650 psi. The distance between the Petri dish containing the scutella and the macrocarrier was 5.5 cm. Gold particles with a diameter of 0.6 µm were suspended in distilled water at a density of 20 mg/ml and coated with pAHC25 plasmid DNA as described by Sparks and Huw (2004).

pAHC25 is a complete cassette containing the *bar* selection marker gene coding for the phosphinotricin acetyltransferase (PAT) enzyme, isolated from the microorganism *Streptomyces hygroscopicus*, and the *uidA* reporter gene coding for ß-glucoronidase, isolated from *E. coli*. The PAT enzyme inactivates the total herbicide phosphinotricin (PPT), which was applied to the nutrient media as a selection agent.

Two or three hours after bombardment the scutella were placed in fresh Petri dishes at an equal distance from each other and incubated in the dark at 23°C for three weeks. Scutella exhibiting callus formation were then transferred to shoot regeneration medium and kept in the light for a further three weeks.

Root and shoot regeneration were induced by illumination with cool white light at low intensity (20 µmol m^{-2} s^{-1}) at a constant temperature of 23°C. In the first year of the experiment, between September 2005 and June 2006, a daylength of 10 hours was applied. In the second year (September 2006 to June 2007) this was increased to 14 hours. The external environment was not completely excluded: light was allowed in through a window facing north-east and measuring 0.72 m^2. After the sixth week calli exhibiting plant regeneration were placed on shoot regeneration medium containing 2 mg/l phosphinotricin for selection and the efficiency of plant regeneration was evaluated in the 6th–7th week. Plant regeneration was expressed as the percentage of calli producing shoots compared with the number of embryos isolated. The regeneration values of embryos isolated on the same day were grouped according to the date when they were transferred to the light. In both years approximately 1500 embryos of each genotype were isolated and bombarded.

Results

The plant regeneration ability of the genotypes Mv Emese and Cadenza, isolated at the same time, was evaluated when exposed to 10- and 14-hour illumination (Fig. 1). The values were grouped according to the date when the cultures were placed in the light. In the case of 10-hour illumination the plant regeneration of Mv Emese exhibited greater deviation over the course than that of Cadenza (Fig. 1), with values ranging from 0–65.6% for Mv Emese and from 0–43.0% for Cadenza. Both genotypes had the lowest regeneration ability

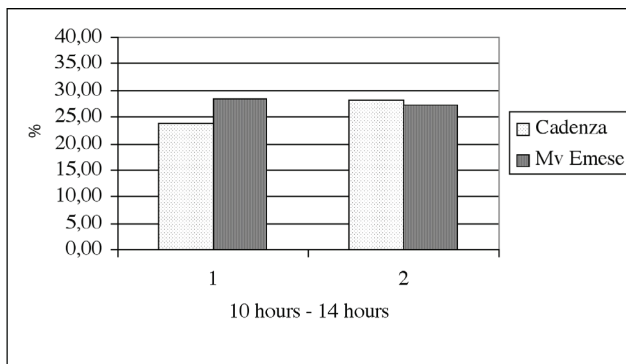

Figure 2. Mean plant regeneration from scutella of the wheat varieties Mv Emese and Cadenza under 10 and 14 hours of artificial illumination.

in March, when no plants were regenerated from the calli in two of the four experiments set up for each variety. The highest percentage of plant regeneration was recorded for Cadenza calli in May and for Mv Emese calli in June.

In the case of 14-hour illumination (Fig. 1) the difference between the minimum and maximum values of plant regeneration was again greater for Mv Emese (2.2–65.6%) than for Cadenza (10.0–41.3%), the lowest values being recorded in July for Mv Emese and in January for Cadenza, while the largest numbers of plants were regenerated from both Mv Emese and Cadenza calli in July. Greater differences were observed between the mean plant regeneration values of the two genotypes in the case of 10-hour illumination than for 14-hour illumination (Fig. 2), with values of 23.8% for Cadenza and 28.4% for Mv Emese at 10 hours illumination and 28.3% for Cadenza and 27.2% for Mv Emese at 14 hours.

Discussion

A comparison was made of the plant regeneration ability of the spring wheat variety Cadenza (UK) and the winter wheat variety Mv Emese (Hungary). Cadenza has been used for years as a donor plant for wheat transformation, while Mv Emese was found by Tamás et al. (2004) to have good plant regeneration ability.

The experiments were carried out under the same conditions in two years, the only difference being the length of illumination. The regeneration ability of the two varieties did not differ significantly at illumination periods of 10 and 14 hours, though under 10 hours of artificial illumination the values recorded for Mv Emese exhibited greater differences than those of Cadenza. Both the maximum and mean plant regeneration values of Mv Emese were higher than those of Cadenza at this level of illumination. A larger number of plants could be regenerated from isolated scutella of Mv Emese wheat than from Cadenza under short-day illumination, when the external environment was not completely excluded. When the artificial illumination was increased to long-day conditions there was an improvement in the plant regeneration of Cadenza, while that of Mv Emese did not change to any great extent. In the case of 10-hour illumination in spring, both genotypes were incapable of plant regeneration in some cases, while this was not observed for 14-hour illumination. Both varieties exhibited maximum regeneration ability in summer in both daylenght.

The greatest level of plant regeneration was observed in June and July for Mv Emese calli and in May and July for Cadenza calli. Data from the literature confirm that plant regeneration is strongly dependent on the genotype (Varsheny and Alpenter 2002).

No significant difference was observed between the plant regeneration levels of isolated, callus-forming scutella of the two varieties despite the fact that one was a winter variety and the other a spring variety. The plant regeneration of both genotypes exhibited mean values of 24–28% in both years.

Cadenza is a more suitable donor plant for transformation as it only requires a few days of vernalisation, compared with six weeks for Mv Emese, which thus lengthens the time required for producing donor plants. Under the climatic conditions of Eastern Central Europe, however, Mv Emese is well adapted, so it can be recommended for use as a donor plant in transformation studies.

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BIOTECHNOLOGY

Development and characterization of a chimaeric tissue-specific promoter in wheat and rice endosperm

Maria Oszvald · Mark Gardonyi · Cecília Tamas · Imre Takacs · Barnabas Jenes · Laszlo Tamas

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Abstract The recently achieved significant improvement of cereal transformation protocols provides facilities to alter the protein composition of the endosperm, for example, to increase or decrease the quantity of one of its protein components or to express foreign molecules. To achieve this goal, strong endosperm-specific promoters have to be available. The aim of our work was to develop a more efficient tissue-specific promoter which is currently used. A chimaeric promoter was assembled using the 5′ UTR (1,900 bp) of the gene coding for the 1Bx17 HMW glutenin subunit protein, responsible for tissue-specific expression and the first intron of the rice actin gene (*act*1). The sequence around of the translation initial codon was optimized. The effect of the intron and promoter regulatory sequences, using different lengths of *1Bx17* HMW-GS promoter, were studied on the expression of *uid*A gene. The function of promoter elements, promoter length, and the first intron of the rice *actin* gene were tested by a transient expression assay in immature wheat endosperm and in stable transgenic rice plants. Results showed that insertion of the rice *act*1 first intron increased GUS expression by four times in transient assay. The shortest *1Bx17* HMW-GS promoter fragment (173 bp) linked to the intron and GUS reporter gene provided almost the same expression level than the intronless long *1Bx17* HMW-GS promoter. Analysis of the stable transformant plants revealed that 173 nucleotides were sufficient for endosperm-specific expression of the *uid*A gene, despite 13 nucleotides missing from the HMW enhancer sequence, a relevant regulatory element in the promoter region.

Keywords Endosperm-specific expression · Rice intron · Transient · Bioreactor

M. Oszvald
Department of Biochemistry and Food Technology,
BUTE University, Budapest, Hungary

M. Gardonyi · L. Tamas (✉)
Department of Plant Physiology and Molecular Plant Biology,
Eotvos Lorand University, Pázmány P. sétány 1/C.,
1117 Budapest, Hungary
e-mail: tamasl@ludens.elte.hu

M. Oszvald · B. Jenes
Agricultural Biotechnology Center, Gödöllő, Hungary

C. Tamas · I. Takacs
Agricultural Research Institute of the HAS,
Martonvásár, Hungary

Introduction

Cereal storage proteins are expressed only in the starchy endosperm during the mid- and late developmental stage of the grain. Endosperm-specific expression of seed storage protein genes is regulated by interactions of multiple *cis*-acting elements in their promoters (Onodera et al. 2001). The first identified sequence which was considered to be necessary for endosperm-specific expression was located around 300 bp upstream of the transcription start site (Kreis et al. 1985). The 30-bp long sequence is called -300 element, prolamin box, or endosperm element and contains two conserved sequences. The prolamin box is not present in high molecular weight (HMW) prolamin gene promoters. HMW prolamin promoters, however, contain a 38-bp regulatory element identified by Thomas and Flavell (1990) and called the HMW enhancer. The location of this

sequence is highly conserved in all HMW prolamin promoters, beginning at position −185 to −189.

HMW glutenin subunit (GS) promoters are currently the most powerful endosperm-specific promoters (Lamacchia et al. 2001; Butow et al. 2004). Bx type HMW glutenin subunits (encoded by *Glu-B1-1* gene) are expressed at the highest level among the HMW-GS proteins (Juhasz et al. 2003). Promoter sequences of Bx type proteins indicated a 54-bp duplication in the sequence, positioned at around 400 bp upstream of the transcription start site and called "cereal-box". Anderson and Greene (1989) suggested a connection between sequence duplication and the higher expression level of Bx7 HMW-GS protein. This promoter is an ideal candidate to drive a high level tissue-specific expression of the transgene in wheat and other cereals.

Storage protein genes do not contain introns. Increasing the gene expression level by the addition of an intron to the promoter region of a heterologous gene has been demonstrated in plants (Humara et al. 1998; Vibok et al. 1999). Studies carried out suggest that introns may improve the efficiency of mRNA processing. In plants, particularly in monocots, introns have been shown to contribute to the enhancement of gene expression in the case of constitutive promoters. McElroy et al. (1991) showed that the rice *act*1 first introns increased the GUS enzyme expression level ten times, transiently, when it was fused to the CaMV 35S promoter. When rice *waxy* first intron was inserted between the CaMV 35S promoter and the *uid*A gene, an increase of about 15-fold was detected in a transient expression assay in rice protoplast (Li et al. 1995). Hwang et al. (2001) studied the effects of the first intron on different tissue-specific promoters and found that the GUS expression level was increased transiently from two to six times.

The effect of the length of the tissue-specific promoter has also been studied. Clarke and Appels (1998) reported that by increasing the length of a tissue-specific rye promoter, the GUS activity was also increased. Experiments carried out on immature rice endosperm have also proved that the length of the tissue-specific rice *Glb* promoter has strong effect on the protein expression level (Hwang et al. 2001).

Our long-term goal is to develop an expression cassette based on an endosperm-specific promoter which can be used for high level recombinant protein expression in a cereal "bioreactor". Here, we report the construction of a chimaeric promoter containing transformation cassette, which is based on the strongest endospermium-specific wheat promoter and the first intron of the *actin* gene of rice. The translation initiator site was also modified, according to the available sequence data information, to get the highest possible level of expression. The results on GUS activity in the endosperm are presented, using wheat transient expression assay and also rice stable transformants.

Materials and Methods

Plasmid constructions. The rice *act*1 first intron was amplified up by polymerase chain reaction (PCR) from rice genomic DNA, using primers called Act1F (5′-GG*CTCGAG*GTAACCACCCCGCCCCTC-3′) and Act1R (5′-GG*CCATGG*CGGTCCTACAAAAAAGCTCCG-3′) and also containing the restriction endonuclease site (XhoI and NcoI, respectively, bold and underlined) as well. PCR cycling conditions were: 5 min initial denaturing at 95°C, followed by 36 cycles consisting of denaturing at 95°C for 15 s, annealing at 55°C for 20 s and extension at 72°C for 30 s. The final extension step was 5 min at 72°C. The reaction mixture contained 10 ng of genomic DNA, 1 µl of 10× PCR buffer, 10 mM of each primer, 1 mM dNTPs and 1 U of Pfu DNA polymerase (Promega). The amplified PCR fragment was cloned behind the *1Bx17* HMW-GS promoter (Reddy and Appels 1993) in a transformation cassette, called pTLZ (Oszvald et al. 2003). The resulting construct was identified as pTSI. The reporter gene *uid*A was amplified by PCR from the pAHC25 plasmid (Christensen and Quail 1996) and was cloned behind the chimaeric wheat promoter to create the transformation cassette, called pTSI-GUS.

To reduce the length of the *1Bx17* HMW-GS promoter, parts of the 5′ end were deleted, using restriction endonuclease enzymes. In a double digestion reaction, *Sph*I and *Hind*III enzymes were applied, and *Mun*I, in a single reaction. Plasmids were re-ligated after purification of the DNA on an agarose gel. The shorter promoter containing cassettes were called pTSIS-GUS and pTSIM-GUS, respectively.

Plant materials. For transient expression a spring wheat (*Triticum aestivum* L.) variety 'Bobwhite', while for stable transformation a rice (*Orysa sativa* L.) variety 'Taipei 309', was used.

Transient expression assay. Immature wheat seeds were harvested 9 days after anthesis and were surface-sterilized. The immature endosperm was carefully excised and cultured on MS (Murashige and Skoog 1962) medium. Endosperm tissues were bombarded with gold particles, coated with 5 µg DNA of the appropriate cassette, using a biolistic gun, called "Genebooster" (Jenes et al. 1996). pAHC25 plasmid was used as a positive, *1Bx17* promoter::HMW-GS::NOS cassette (pTLZ-Ax2^{*B}, Oszvald et al. 2003) as a negative control. The luciferase gene, coupled to the ubiquitin promoter, was also used for bombardment as an internal control to normalize the transfection efficiency. Twenty-five immature endosperms were bombarded on one plate. All of them were collected for one GUS assay and considered as one measurement. The GUS expression level was determined, following the method published by Hwang et al. (2001), using data of five independent measurements.

Rice transformation. Rice transformation was carried out, as it was published by Cho et al. (2004). Embryos were separated from the endosperm after 7 d and were further cultured on N6 culture medium (Chu et al. 1975) for callus production. After 3–4 wk of cultivation, callus was bombarded by the "Genebooster". Transgenic callus was selected on medium containing 50 mg dm^{-3} of hygromycin B. Rapidly growing resistant tissues were moved to MS regeneration medium. Plantlets with 5–6 cm long shoots and well developed roots were transferred to the soil and were grown in the glass-house.

PCR analysis. The putative transgenic rice plants were screened for the selection marker gene and the transgene by PCR. Total genomic DNA was isolated from leaf tissues using the Qiagen Plant DNeasy kit. PCR analysis for the *hpt* gene was carried out using the following primer pair: hptF (5′-CAGAAGAAGATGTTGGCG-3′) and hptR (5′-TTATCGGCACTTTGCATCGG-3′). Transgene analysis was performed with a set of primers specific for the *1Bx17* HMW-GUS promoter (5′-TCCCTATAAAAGCC CATCC-3′) and the *uid*A gene (5′-GGATCCTCATTGT TTGCCTCCCTG-3′). PCR products were analyzed on 1% (*w*/*v*) agarose gel.

Histochemical GUS assay. Expression of the *uid*A gene was assayed in T1 transgenic plants by incubating pieces of plant tissue (leaf, root, seed) overnight at 37°C in X-Gluc buffer as it was published by Wiley et al. (2007). GUS activity was quantified in some plants by the fluorogenic 4-methyl umbelliferyl glucuronide assay, as described by Mori et al. (2007).

Results

Construction of transformation cassettes. Four transformation cassettes were constructed using the pTLZ vector that contains a 1,900-bp long fragment, upstream of the *1Bx17* HMW-GS gene and 300 bp long fragment of the NOS terminator region. To study the effect of the intron on endosperm-specific protein expression, rice actin first intron (*act*1) was chosen. The primer pair used for amplification of the intron sequence was designed according to the published data (McElroy et al. 1990). To design the reverse primer (Act1R), an extended study was carried out to find the best possible sequence for the 5′ UTR preceding the ATG codon, to improve the translation efficiency. The EMBL nucleotide sequence database http://www.srs.ebi.ac.uk/embl) was screened for wheat and rice storage protein sequences. All of them (32 and 21, respectively) were aligned (seven bases upstream from the start codon) and the 50/75 consensus rule, described by Cavener (1987), was applied in each position to find the consensus sequence. These sequences were compared to data published by Joshi et al. (1997) and by Sawant et al. (2001). As a result of this study, we specified a new translation initiator sequence, such as *GACCGCC* for the transformation cassette (*see* Table 1).

Genomic DNA, purified from the variety Taipei 309 was used as template. Sequencing the PCR amplified fragment, we found out that it was 469 bp long, 143 bp longer than we expected according to the published intron sequence (McElroy et al. 1990). The PCR fragment was cloned behind the *1Bx17* HMW-GS promoter in the pTLZ vector, and the new construct was identified as pTSI. Apart from the 1,900-bp long *1Bx17* HMW-GS promoter containing transformation cassette (pTSI-GUS), two shorter constructs were also prepared by digestion. Cassette, called pTSIS-GUS contains a promoter, which is 649 bp shorter. The shortest one (pTSIM-GUS) has only a 251-bp fragment from the 5′ UTR of the storage protein gene (Fig. 1).

Transient GUS expression. Transient expression results showed that the first intron of the rice *actin* gene has a positive effect on protein expression level, increasing GUS activity by four times compared with the intron-less cassette (pTSI-GUS to pTLZ-GUS) (Fig. 2). Deletion of the far upstream end of the promoter (pTSIS-GUS) before E motifs had no significant effect on the *uid*A gene expression level. In contrast, a decrease of about 3.5-fold was observed with the shortest promoter (pTSIM-GUS) when almost all the known specific regulatory elements were deleted. The 172-bp long promoter with intron provided almost the same expression level as the intronless long *1Bx17* HMW-GS promoter.

GUS enzyme expression in stable transgenic rice plants. To find out whether the shortest chimaeric promoter still provides tissue-specific expression of the *uid*A gene and to compare the strength of the chimaeric promoters, stable

Table 1. Nucleotide sequence preceding the coding region in different plant derived genes

Parameters	−7	−6	−5	−4	−3	−2	−1
Bx17HMW sequence	C	A	C	T	G	A	G
Wheat storage proteins	G	A	C	C	A/G	A/C	C/G
Rice storage proteins	C	A	C	A	A/G	C	A/C
Monocots [a]	G	G	C	A/C	A/G	A/C	C
Storage proteins [a]		T	C	C	A	C	C
Highly expressed plants[b]		T	A	A	A	C	A
Optimized sequence present in pTSI-GUS	G	A	C	C	G	C	C

[a] According to Joshi et al. (1997), *[b]* according to Sawant et al. (2001)

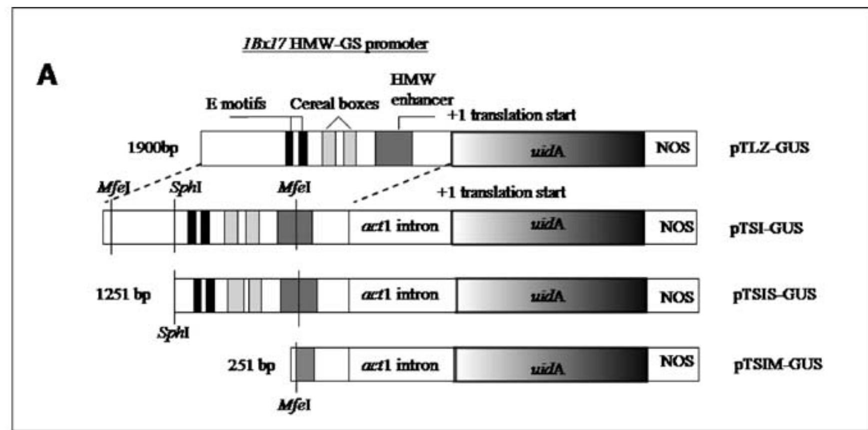

Figure 1. (A) Schematic diagram of transformation cassettes used in this study. Rice *act*1 first intron and *uid*A reporter gene was fused to the 3′ end of the *1Bx17* HMW glutenin promoter fragment. (B) Nucleotide sequence alignment of the HMW enhancer region of the tissue specific *1Bx17* HMW-GS promoter in the pTSI-GUS and pTSIM-GUS transformation cassettes. The perfect HMW enhancer element is *underlined with continuous line*, while the truncated enhancer sequence with *dashed line*. *Mfe*I restriction enzyme digestion site is *boxed*.

transgenic rice plants were produced, using the pTSI-GUS, pTSIM-GUS, and pTLZ-GUS transformation cassettes. Bombardment of approximately 400-400 calli, derived from mature seeds followed by selection on antibiotic containing medium gave rise to seven, four, and six plants, respectively, that were antibiotic tolerant. PCR analysis of the genomic DNA proved that all of them contained the gene of interest. Southern blotting showed (not published) that we had transgenic plants from each transformation event with single insertion of the *uid*A gene. T1 plants were grown in greenhouse, and leaf, root, stem and seed samples were subjected for histochemical detection of GUS enzyme activity. The GUS-specific signal was very high in the endosperm, while leaf, stem, and root samples showed no significant GUS activities (Fig. 3). Results of the GUS assay on stable transgenic rice plants showed that removing 13 nucleotides has no effect on tissue specificity of the promoter. Our data demonstrate that the last 25 nucleotides of the 38-bp long major regulatory element, called HMW enhancer, are enough to provide tissue specificity for a HMW glutenin promoter (Fig. 1*b*).

Fluorometric quantification of the expression level in stable transgenic rice seeds, carrying single insertion of the *uid*A gene, was also performed. (Fig. 3). A comparison of the GUS expression revealed that the long chimaeric promoter provided the highest expression level in the endosperm. The shortest chimaeric promoter showed around 25% of the long promoter's activity. The chimaeric

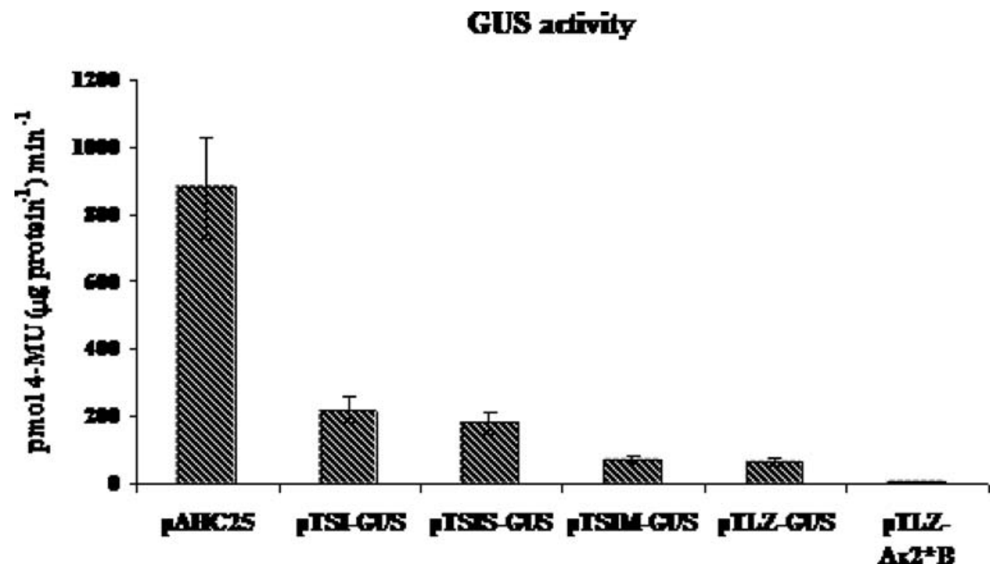

Figure 2. Fluorometric quantification of GUS activity of chimaeric promoters with different length and the *1Bx17* HMW-GS promoter in immature wheat endosperm using a transient expression assay. pAHC25 and pTLZ-Ax2*^{B} cassettes are used as positive and negative controls, respectively. *Values* represent the mean ± SE of triplicates.

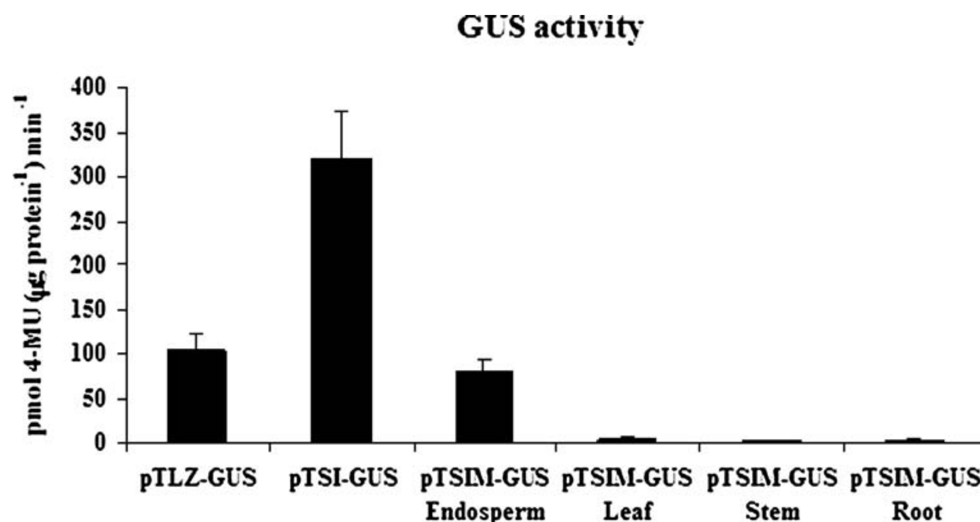

Figure 3. β-Glucuronidase activity of three GUS expression cassettes in the endosperm of stable transgenic rice plants. GUS assay was also performed on leaf, stem, and root samples derived from the pTSIM-GUS transformed rice plant. *Values* represent the mean ± SE of triplicates.

promoter proved to be stronger than the intronless promoter (pTLZ-GUS) showing roughly threefold increase in the GUS expression activity.

Discussion

The recently achieved significant improvement of cereal transformation protocols (Jones 2005; Shrawat and Lorz 2006) provides facilities to alter the protein composition of the endosperm, to increase or decrease the quantity of one of its protein components, or to express foreign molecules. The aim of the present work was to study the effect of the first intron of the rice *actin* gene on a tissue-specific promoter of wheat in a transient expression assay using immature wheat endosperm. A 1.9-kbp long fragment of the 5′ UTR, preceding the translated sequence of the highly expressed *1Bx17* HMW glutenin subunit gene, was chosen to investigate its activity, both in a transient expression assay and in stable transgenic plants. The endosperm-specific promoter and the rice *act*1 first intron were fused to create a chimaeric promoter. To improve the protein expression level, the 3′ end of the PCR-amplified rice DNA fragment was modified, according to the result of our database search and previously published data (Joshi et al. 1997; Sawant et al. 2001). The sequence of the last seven nucleotides was determined as GACCGCC. Four nucleotides are different in this sequence compared with the same segment of the *1Bx17* HMW-GS 5′ UTR. The last double C of this optimized sequence can be used as part of the *Nco*I restriction enzyme cleavage site, allowing site-specific cloning of the gene of interest, starting with ATG for methionine. Depending on the second amino acid of the protein, different enzyme recognition site should be added to the 5′ end of the gene for cloning it into the transformation cassette. If ATG is followed by either G, T, or A, the choice of restriction enzyme is *Nco*I, *Pci*I or *Bsp*HI, respectively.

The length of the amplified first intron of *actin* gene, derived from variety Taipei 309, was 452 bp. Apart from 143 bp insertion, the sequence is in perfect match with the previously published (McElroy et al. 1990) sequence (account number S44221). The insertion started after the 207th nucleotide of the published intron sequence. Because high fidelity polymerase (Pfu) was used for amplification, and the same longer sequence was obtained for all the three independent PCR products in three replicas of sequencing, false result was ruled out. We concluded that this variety should have different *act*1 intron sequence.

The enhancing effect of the first intron of rice *act* and *waxy* as well as maize *adh* genes, on constitutive promoters through GUS enzyme expression has been widely studied (Luehrsen and Walbot 1991; McElroy et al. 1991; Li et al. 1995). It was proven that all of them positively influenced the promoter activity. We studied the effect of *act*1 intron on the endosperm-specific promoter of *1Bx17* HMW-GS gene from wheat. Our work demonstrated that the rice *act*1 intron increased tissue-specific expression of GUS enzyme by four times in the transient system. It contradicts the previous result of Hwang et al. (2001). They did not find detectable increase in GUS expression level in the presence of *act*1 intron, fused to *Glb* or *1Bx7* promoters, in the rice immature-endosperm-based transient assay. McElroy et al. (1991), however, measured a tenfold increase in GUS activity under the control of CaMV 35S promoter in rice cells. One explanation for the different finding could be the sequence of the rice *act*1 intron used in our experiments. Although we did not study the effect of the 143-bp long insertion in the first intron, however, it may have a positive effect on the expression level. The other reason, which may explain the contradiction, is the optimized translation initiation site used in our study. It is well-known from

previous reports that this sequence has a strong effect on the protein expression (Joshi et al. 1997; Sawant et al. 2001).

Apart from the effect of the first intron of the rice *actin* gene on the strength of the tissue-specific promoter, the length of the *1Bx17* HMW glutenin subunit promoter was also studied. Deletion of the far upstream region of the promoter was proved to have no significant effect on the expression level; however, a substantial drop was observed when the length of the 5′ UTR was reduced to 251 nucleotides. Previous studies of the endosperm-specific promoters had identified a primary enhancer sequence, which is considered to be the major regulatory element in the expression of the HMW glutenin subunit genes. This motif starts 13 nucleotides upstream from the CAATTG sequence, recognized by the *Mfe*I enzyme. To test whether the less than complete enhancer fragment containing promoter (pTSIM) is enough for tissue-specific driving of the *uid*A gene expression, the pTSIM-GUS cassette was used for producing stable transgenic rice plants. Deletion of the N and E boxes, and furthermore, the cereal-box and partial sequences of the HMW enhancer (see Fig. 1.) does not appear to affect specificity of the *1Bx17* promoter in transgenic rice. The *1Bx17/act*1 intron chimaeric promoter showed strict tissue specificity, being expressed only in the endosperm with no expression in any other tissues.

The enhancing effect of an intron on gene expression, using constitutive promoters in monocots, has been well documented. In the present paper, we demonstrated, using a transient expression assay that the first intron of the rice *actin* gene has a positive effect on the strength of the *1Bx17* HMW endosperm-specific promoter, increasing the expression level by four times. As transgenic rice experiments revealed, a 172-bp long promoter was sufficient for endosperm-specific expression of the *uid*A gene, even though 13 nucleotides were missing from the consensus sequence of the HMW enhancer (Fig. 1*b*), the regulatory element only remained in the promoter region.

This tissue-specific chimaeric promoter can be used for developing cereal based vaccine (Oszvald et al. 2007) or “bioreactors”, expressing recombinant protein in the endosperm of the transgenic plants.

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GENETIC MODIFICATION OF CEREALS IN THE AGRICULTURAL RESEARCH INSTITUTE OF THE HUNGARIAN ACADEMY OF SCIENCES

L. SÁGI, M. RAKSZEGI, T. SPITKÓ, K. MÉSZÁROS, B. NÉMETH-KISGYÖRGY, A. SOLTÉSZ, F. SZIRA, H. AMBRUS, A. MÉSZÁROS, G. GALIBA, A. VÁGÚJFALVI, B. BARNABÁS and L. C. MARTON

AGRICULTURAL RESEARCH INSTITUTE OF THE HUNGARIAN ACADEMY OF SCIENCES (HAS), MARTONVÁSÁR, HUNGARY

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Research with transgenic plants in the Agricultural Research Institute of the Hungarian Academy of Sciences is primarily related to applications that are essential for the genetic improvement of cereals. The two main directions are connected to wheat and maize breeding and are focused on improving agronomic and nutritional traits. This paper highlights experiments in these areas, which are conducted in national as well as international collaborations. The transparency of this work is ensured by the dissemination of information about approved confined field tests to the public via the internet.

Introduction

One of the basic scientific tasks of the Agricultural Research Institute of the Hungarian Academy of Sciences is to investigate the characteristics of wheat and maize and to reveal their biochemical and genetic backgrounds. The knowledge acquired is then exploited in cereal breeding. In addition to conventional breeding methods, an increasing role has been played over the last ten years by modern biotechnological procedures (e.g. genetic modification or gene transformation) which, among other things, allow the effects of individual genes to be analysed. The development of genetically modified plants is dependent on the availability of reliable gene transfer techniques adapted to local laboratory conditions. This purpose is served by experiments involving reporter genes (e.g. *gusA* and *gfp*) or the *bar* selectable marker gene, where the success of gene transfer is indicated by a simple colour reaction or by the presence of herbicide resistance. "Useful" genes can only be transferred after the optimisation of the methodology, which must be carried out individually for each plant species intended for genetic modification (in the present case wheat, barley and maize). In Martonvásár the aim of genetic modification is partly to improve the agronomic traits and environmental resistance of these plants, and partly to modify breadmaking quality or nutritional value to satisfy the requirements of the processing industry.

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Research on wheat

Among the agronomic traits of wheat, the main focus is on resistance to powdery mildew and frost tolerance. Within the framework of an EU project, in cooperation with the University of Zurich, work is underway on the introduction of a gene responsible for powdery mildew resistance into winter and spring wheat varieties, followed by the monitoring of its incorporation into the wheat genome and its ability to exert a positive effect and make the test plants resistant to powdery mildew.

The cold stress-related gene regulatory pathway is one of the most thoroughly investigated regulatory systems in the plant kingdom. It has been proved that the *CBF* genes, coding for transcription factors, are among the key regulators for low temperature stress response. Recently it has demonstrated that four *CBF* genes are the main regulators in wheat (Vágújfalvi et al., 2005). To directly prove the involvement of these genes in frost tolerance, *Arabidopsis*, rice, wheat and barley plants were considered for transformation.

Due to methodological advantages, the model plant *Arabidopsis* was the first to be transformed with the candidate *CBF* genes (Fig. 1) in collaboration with Corvinus University, Budapest, Hungary. The plants were transformed with three candidate genes using the floral dip method. The T2 generation was subjected to frost tests and several transgenic lines with increased frost tolerance were identified. Plants with increased tolerance will be verified for transgene expression and copy number. Cereals have been transformed in cooperation with the Agricultural Biotechnology Center, Gödöllő, Hungary and the John Innes Centre, Norwich, UK, using the biolistic method in the case of wheat and rice. In these experiments the candidate genes were driven by the constitutive maize ubiquitin promoter, but tests on the effectiveness of a cold-inducible wheat promoter, *WCS120*, are also planned. Currently the transformants are being tested for the presence of the transgenes. Since wheat transformation is time-consuming and labour-intensive, barley plants were transformed using the *Agrobacterium*-mediated method. The verification of successful transformation is now in progress. The participation of the candidate *CBF* genes in the control of frost tolerance is also being screened with the RNAi technique.

In addition to agronomic traits, the breadmaking quality of wheat is also of major importance. This depends chiefly on the composition of the storage proteins (e.g. HMW glutenins and gliadins). It has long been known that better quality products can be prepared if certain glutenin subunits are present in higher quantities. To confirm this observation, spring wheat varieties were transformed with glutenin subunits 1Dx5 and 1Ax1 at Rothamsted Research, UK, after which the breadmaking quality of the transgenic plants was examined in Martonvásár (Rakszegi et al., 2005). As the result of genetic modification the quantity of 1Dx5 glutenin subunit in the flour was found to increase fourfold, leading to extremely strong dough (Fig. 2). This flour can be used in practice by mixing it with other types of flour. The 1Ax1 transformation led to more stable dough which softened at a slower rate. The incorporated wheat genes and the encoded traits proved to be inherited in a stable manner from one generation to the next.

In order to improve the nutritional value of wheat, transformation was carried out (in cooperation with the Plant Physiology Department of Eötvös Loránd University, Budapest) with the *AmA1* gene coding for a seed albumin (Raina and Datta, 1992) containing a high proportion of essential amino acids, which humans are unable to synthesise and which must thus be consumed as food. The gene used for the genetic modification originated from *Amaranthus hypochondriacus*, commonly referred to as the cereal source for people allergic to wheat flour and other foodstuffs. The gene transfer resulted in a substantial rise in the essential amino acid content of the flour, with increases of around 6% for lysine, 2.8% for threonine and 3.8% for tyrosine, thus improving the nutritional value of the wheat.

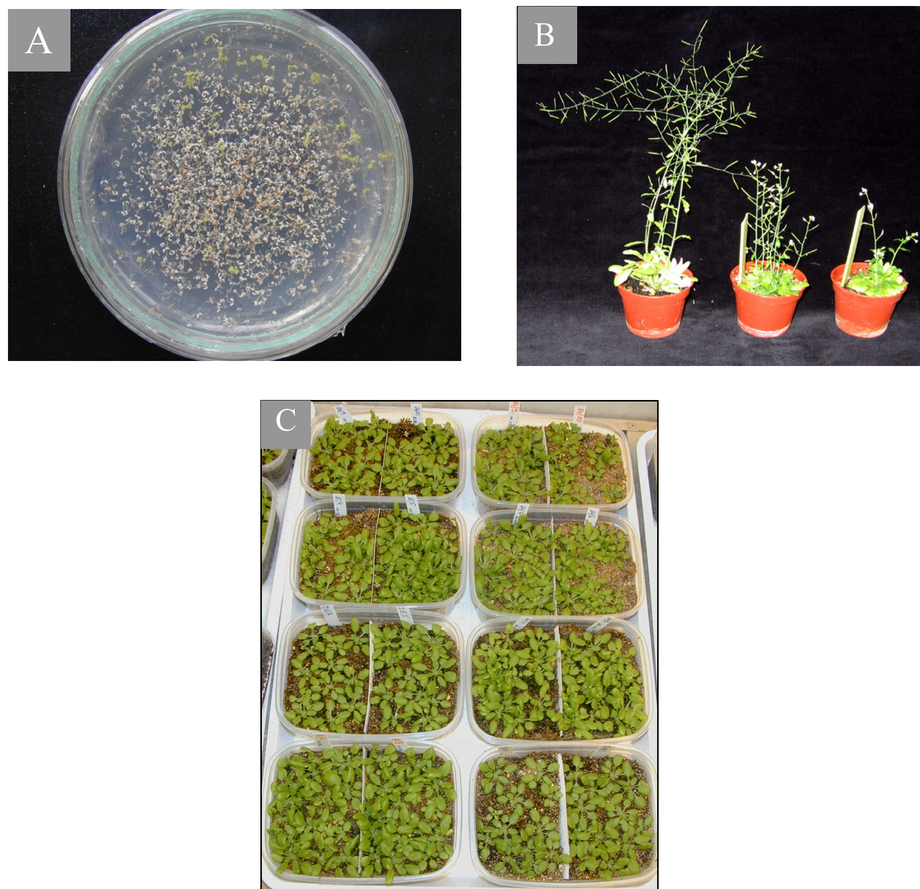

Fig. 1. Arabidopsis transformation. A: selection for *CBF* transformants, B: T1 generation, C: Propagation of the T2 generation

Fig. 2. Effect of 4-fold over-production of the 1Dx5 storage protein subunit on breadmaking quality: bread baked using the control flour (left) and that baked using flour from GM plants (right)

Research on maize

In gene transformation experiments on maize, genes are transferred into calli induced from immature embryos, using a gene gun, or into calli of microspore origin, via cocultivation with *Agrobacterium tumefaciens*. As microspores are haploid, the transgenes in plants regenerated from calli of microspore origin can be made homozygous and non-segregating within a single generation, due to the spontaneous or artificial doubling of the genetic material. The aim of this research is to induce resistance to viral and fungal pathogens. In the case of viruses, a segment of the coat protein of maize dwarf mosaic virus is produced by the cells of transgenic plants, with the consequence that the virus is unlikely to be able to reproduce when the plants are grown in the field. In the case of fungi, a chitin-decomposing enzyme (endochitinase) originating from *Trichoderma hamatum* (Fekete et al., 1996), a fungus that parasitises other fungi and is used in many countries in organic farming, is present in the plant cells and is thus able to decompose fungal hyphae invading the plant tissues. The aim of both projects is to develop maize hybrids resistant to pathogens and to suit them for use in the field.

Field gene transfer is another possibility for producing genetically modified plants. This technique is based on incorporating the transgenes into "model" plant lines ideally suited for gene transformation and then using these plants as crossing partners in the field transformation programme. The donor line carrying the gene is single-crossed with the recipient lines, after which new transgenic lines are developed through several cycles of backcrossing and selection. The advantage of this procedure is that transgenes can be incorporated into maize lines which have commercial value but are not suited to current gene transformation techniques, preventing them from being used for genetic modification up till now.

In Martonvásár the field breeding programme is aimed at the transfer of genes responsible for resistance to western corn rootworm (*Diabrotica virgifera virgifera*) and herbicides. In cooperation with Monsanto Hungária Co. Ltd., the gene coding for the Cry3Bb1 delta-endotoxin protein of the bacterium *Bacillus thuringiensis* subsp. *kumamotoensis* (Donovan et al., 1992) is transformed into Martonvásár maize lines, allowing a specific protein to be produced in the plant cells which destroys both the larvae and imagos of corn rootworm when they attack the plant tissues (Fig. 3). The results of recent biosafety research have shown that this protein exerts no negative effect on non-target organisms in the field (Rauschen et al., 2008). A modified maize gene (*epsps*) is responsible for the herbicide resistance in this programme. This provides protection against glyphosate, the active ingredient in Roundup, a total herbicide capable of destroying practically every weed on the growing area, with the exception of the genetically modified maize.

The field release of the plants arising from these research projects is for experimental purposes, aimed at investigating the expression and inheritance of the incorporated genes. The research institute has a licence to carry out such confined field experiments, details of which are publicly available in the database of authorised GMO releases in Hungary, to be found on the website of the Agricultural Biotechnology Center in Gödöllő (http://biosafety.abc.hu).

Fig. 3. Comparison of the damage caused by western corn rootworm: the resistant GM line is symptom-free (left), unlike the susceptible control (right)

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Corresponding author: L. Sági
Phone: +36-22-569-547
E-mail: sagil@mail.mgki.hu

MARTONVÁSÁRI BÚZAFAJTÁK *AGROBACTERIUM TUMEFACIENS* KÖZVETÍTETTE GENETIKAI MÓDOSÍTÁSÁNAK OPTIMALIZÁCIÓJA: NÖVÉNYREGENERÁCIÓ ÉS TRANZIENS RIPORTER GÉNEXPRESSZIÓ

Mészáros Klára[1], Kiss Tibor[1], Dőry Magdolna[1], Karsai Ildikó[1], Láng László[1], Bedő Zoltán[1] és Sági László[1]
[1]*MTA Mezőgazdasági Kutatóintézete, 2462 Martonvásár, Brunszvik u. 2.*

A nemesítés során mindig központi probléma volt a fajták biotikus stresszrezisztenciájának növelése. Ennek megvalósítására a biotechnológia eszköztárát is felvonultatták. A búza, gazdasági jelentősége ellenére, az utolsók között került a genetikailag módosított gabonafélék közé. Az első sikereket génbelövéssel érték el, ám napjainkban az Agrobacterium tumefaciens *közvetítette génátvitel egyre jobban terjed, valamint az éretlen embrió mellett az érett embriót is egyre gyakrabban alkalmazzák célszervként. Kísérletünkben 19 búzafajta érett embrióból kiinduló regenerációs képességét határoztuk meg. A legjobb válaszadó genotípusok agrobaktériumos transzformációját végeztük el. A génátvitelt befolyásoló tényezők (az előkezelés időtartama, a baktériumszuszpenzió hígítása, a kokultiváció ideje és a kokultiváció alatt alkalmazott embriószárítás) 16-féle kombinációjából leghatékonyabbnak a kokultiváció alatt alkalmazott szárítás és a baktériumszuszpenzió hígítása bizonyult. Ezzel a módszerrel két martonvásári őszi búzafajta sikeres transzformációját végeztük el riporter- és szelekciós génekkel. Ezzel újabb, a gyakorlat számára fontos fajták genetikai módosítására nyílik lehetőség, melyek a nemesítéshez és a genetikai vizsgálatokhoz adnak alapanyagot.*

Az évezredek óta folyó nemesítés hatékonyságának növelésére a múlt századtól a biotechnológia eszköztárát is bevetették az új bőtermő és betegségekkel szemben ellenálló fajták nemesítésére. A kórokozókkal szembeni rezisztencia mindig központi problémája volt a nemesítési programoknak, ezért a rendelkezésre álló eszközök széles skáláját alkalmazták a biotikus stresszrezisztencia fokozására. A rezisztenciagének bevitele azért került gyorsan a géntranszformációs kísérletek célkeresztjébe, mert számos esetben egyetlen vagy kisszámú gén felelős az ellenálló fenotípus kialakításáért. Másfelől ezek a rezisztenciagének gyakran olyan rokon fajokban fordulnak elő, amelyek csak nehezen vagy egyáltalán nem keresztezhetők a termesztett búzával, így a célgén átvitele nem lehetséges hagyományos módszerekkel. A búza egyike a gazdaságilag legjelentősebb szántóföldi növényeknek, mégis az utolsók között került a genetikailag módosított gabonafélék körébe. Az első sikereket génbelövéssel érték el, de napjainkban egyre elterjedtebbé válik az *Agrobacterium tumefaciens* (továbbiakban agrobaktérium) közvetítésével történő génátvitel. Ennek a transzformációs módszernek számos előnye van a közvetlen génátvitellel szemben. A bevinni kívánt gén kisebb kópiaszámban épül be az általában transzkripcionálisan aktív kromoszómarégiókba, továbbá a nagyméretű DNS-szakaszok átrendeződése és törése ritkábban fordul elő.

A búza genetikai módosítása során a legelterjedtebben és legsikeresebben alkalmazott explantum az éretlen embrió. A donor növények üvegházi vagy fitotroni fölnevelése azonban időigényes és költséges, valamint a transzformálható embriók mérete és kora is szűk határok között változik. Ezzel szemben az érett embrió mag formájában tartósan és korlátlan mennyi-

ségben áll rendelkezésre, így előállítása összehasonlíthatatlanul olcsóbb, továbbá az érett embriók fiziológiai állapotában csak minimális variabilitás figyelhető meg, mely egyöntetűbb *in vitro* reakcióhoz vezet. Végül, az érett embrióból szintén hatékonyan lehet növényt regenerálni. Ezért a búza transzformációjához az érett embriók alkalmazása ígéretes alternatívát jelenthet. A transzgenikus növények előállításának azonban továbbra is határt szab az *in vitro* jól regenerálódó genotípusok korlátozott köre. Az első sikereket tavaszi genotípusok transzformációjával érték el, ezek gazdasági értéke azonban nálunk csekély, valamint a genomikai kutatások fejlődése is megkívánja a transzformálható genotípusok körének bővítését, és az ehhez kapcsolódó transzformációs rendszer kidolgozását. Az agrobaktériummal történő transzformációt befolyásoló legfontosabb tényezők a megfelelő genotípus mellett az ideális explantum, a fertőzés és a kokultiváció feltételei (Wu és mtsai 2003).

Munkánk során célul tűztük ki martonvásári nemesítésű őszi búzafajták regenerációs képességének vizsgálatát érett embrióból kiindulva, majd a legjobban regenerálódó fajták kiválasztását és agrobaktériummal történő transzformációját, valamint a transzformáció egyes lépéseinek optimalizását.

Anyag és módszer

Vizsgálatainkat 16 martonvásári nemesítésű őszi búzafajtán végeztük: kontrollként három, a transzformációs kísérletekben leggyakrabban alkalmazott tavaszi búzafajtát ('Bobwhite', 'Cadenza' és 'Chinese Spring') alkalmaztunk *(1. táblázat)*. Az érett és éretlen embriók regenerálása Filippov és mtsai (2006), illetve Jones és mtsai (2005) által kidolgozott módszer alapján történt. A regenerációs képességet a lerakott explantumok arányában határoztuk meg.

A búzafajták agrobaktériumos transzformációját AGL1 törzzsel végeztük, melynek bináris

1. táblázat

A kísérletekben vizsgált fajták felsorolása, származása és életformája

Fajta	Pedigré	Életforma
BOBWHITE-26	AURORA//KALYAN/BLUEBIRD/3/WOODPECKER	Tavaszi
CADENZA	AXONA/TONIC	Tavaszi
CHINESE SPRING	LV-WEST-SICHUAN/LV-SICHUAN	Tavaszi
FATIMA 2	FUNDULEA-29/LOVRIN32	Őszi
MARTONVÁSÁRI-16	MV4/KAVKAZ//P4089/3/ZLATNA-DOLINA/ARTHUR-71/4/RUBIN	Őszi
MV BÉRES	ERYT352/MAGDALÉNA	Őszi
MV CSÁRDÁS	JUBILEJNAJA-50/FUNDULEA-29//MVMA	Őszi
MV EMESE	MVMA/MV12//F2098W2-21	Őszi
MV HOMBÁR	FLEMING/MATADOR	Őszi
MV MAGDALÉNA	JUBILEJNAJA-50/FUNDULEA-29//MVMA	Őszi
MV MAGVAS	CARMEN/MACVANKA-2//MVMA	Őszi
MV MAMBÓ	KALÁKA/MV16//F2076	Őszi
MV MARSALL	MV15-91/KALÁKA//MV15-91/FATIMA-2	Őszi
MV PÁLMA	F797/MV08-82//MV15	Őszi
MV PALOTÁS	MVMA/MV8//F2098W2-21	Őszi
MV REGIMENT	GA901273-46-I/F6038W12-1	Őszi
MV SUBA	ERYT1778/MAGDALÉNA//MAGDALÉNA	Őszi
MV SÜVEGES	MIRONOVSKAJA-29/MAGDALÉNA//MAGDALÉNA	Őszi
MV TOBORZÓ	ERYT336/MAGDALÉNA	Őszi

2. táblázat

Az agrobaktériumos transzformáció vizsgált paraméterei

Kezelés	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Előtenyésztés (nap)	2	2	2	2	2	2	2	2	1	1	1	1	1	1	1	1
Hígítás*	–	–	–	–	+	+	+	+	–	–	–	–	+	+	+	+
Szárítás**	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+
Kokultiváció (nap)	3	3	5	5	3	3	5	5	3	3	5	5	3	3	5	5

*Az indukció előtt az agrobaktérium-szuszpenziót ötszörösére hígítottuk.
**Az embriókat a kokultiváció idejére szűrőpapírra helyeztük. Kontroll = 9. kezelés

plazmidja hordozza a β-glükuronidáz enzimet kódoló és egy intront tartalmazó *uidA* (GUS^{INT}) riporter és a *bar* szelekciós gént. Az 'Mv Csárdás' és 'Mv Emese' őszi búza érett embrióit egy vagy két napig tenyésztettük az agrobaktériumos fertőzés előtt (ötszörös hígítással vagy anélkül), majd három vagy öt napig kokultiváltuk. A tenyészeteket a kokultiváció ideje alatt közvetlenül a táptalajra helyeztük, vagy a táptalajra szűrőpapírt tettünk a deszikkáció előidézéséhez. A fertőzést követő 5. és 7. napon hisztokémiás reakcióval értékeltük a tranziens GUS expressziót. A fenti kezelések 16 kombinációját alkalmaztuk *(2. táblázat).*

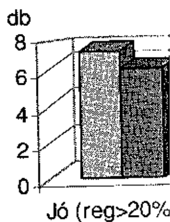
A kísérlet statisztikai elemzését a Breeder-programmal (Kuti és mtsai 2003) végeztük.

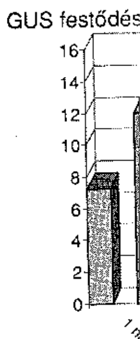
Eredmények és következtetések

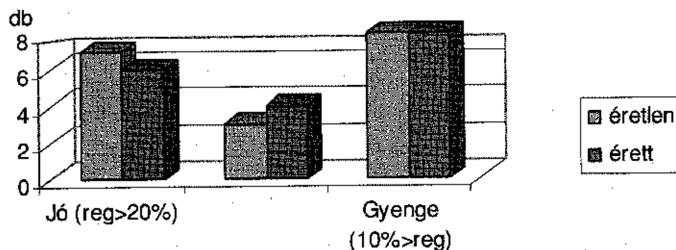
A búzatranszformációs kísérletekben az éretlen embrióexplantum alkalmazása a legelterjedtebb. Ezért első lépésben összehasonlítottuk 16 őszi búzafajta regenerációs képességét érett és éretlen embrióból kiindulva. Az éretlen embrióból kiinduló növényregeneráció értéke az 'Mv Regiment' 59%-os és az 'Mv Toborzó' 0,1%-os értéke között változott. Az éretlen embrió alternatívájaként az érett embrióból történő növenyregenerálást is megvizsgáltuk ugyanazokon a genotípusokon. A legeredményesebb fajtának a 'Fatima 2' bizonyult (63%), de az 'Mv Palotás'-ból nem regenerálódott növényt (0%). A jól regenerálódó (>20%) fajták száma éretlen embrióból kiindulva nyolc ('Mv Regiment', 'Chinese Spring', 'Mv Mambó', 'Bobwhite', 'Mv Emese', 'Mv Palotás', 'Mv Hombár', 'Mv Csárdás'), érett embriót alkalmazva hat volt ('Fatima 2', 'Mv Emese', 'Mv Regiment', 'Mv Magdaléna', 'Mv Csárdás', 'Mv16'). A közepesen regenerálódóké (10–20%) éretlen és érett embrió esetén három ('Mv Suba', 'Mv Béres', 'Mv16'), illetve négy ('Chinese Spring', 'Mv Süveges', 'Mv Béres', 'Mv Mambó') volt. Mindkét explantum alkalmazásakor 8, illetve 9 fajta gyenge regenerációs értéket (<10%) mutatott. 4–4 genotípus növényregenerációs képessége szignifikánsan (p=5%) nagyobb volt, mint a kontrollként alkalmazott 'Cadenzáé' (átlag: 7,05%), amely a transzformációs kísérletek egyik legelterjedtebb modellfajtája. A két explantum egymáshoz képest nem adott szignifikáns különbséget: az érett embrióból kiinduló regeneráció (átlag: 17,4%) nem volt kevésbé hatékony, mint az éretlen embrióból (átlag: 20,5%), csak a genotípusok rangsora volt eltérő.

A további transzformációs kísérleteket a jól regenerálódó 'Mv Csárdás' és 'Mv Emese' fajták érett embrióiból indukált 3, 6 és 9 napos kalluszokon végeztük. Az izolálás után az érett embriók gyors növekedésnek indultak a táptalajon, ez a 6. napig tartott, amikor elérték az 1,5 cm körüli átmérőt. Ez után a növekedés lelassult, amit a 9 és 6 napos kalluszok minimális méretkülönbsége jelzett.

Transzformáció után 3 napos kokultivációt alkalmaztunk, majd áthelyeztük a kalluszokat antibiotikum (Timentin) tartalmú táptalajra, amelyen azonban az agrobaktérium gyorsan elszaporodott, és benőtte a kalluszokat. A további kísérletekben ezért frissen izolált (1 vagy 2 napos) érett embriót használtunk, mivel ennek tömege kisebb, és egyenletesebb a felszíne, így az





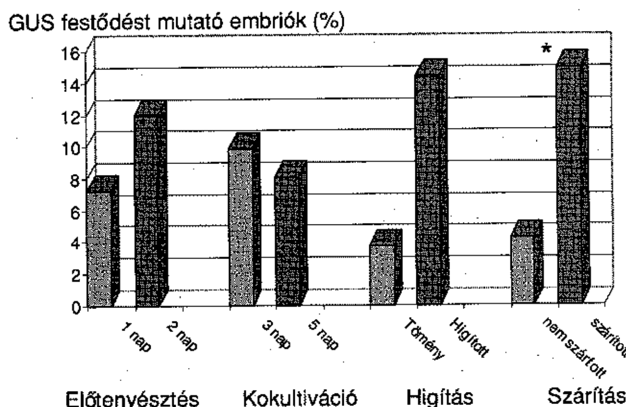


1. ábra. 19 búzafajta növényregenerációs gyakorisága érett és éretlen embrióból kiindulva

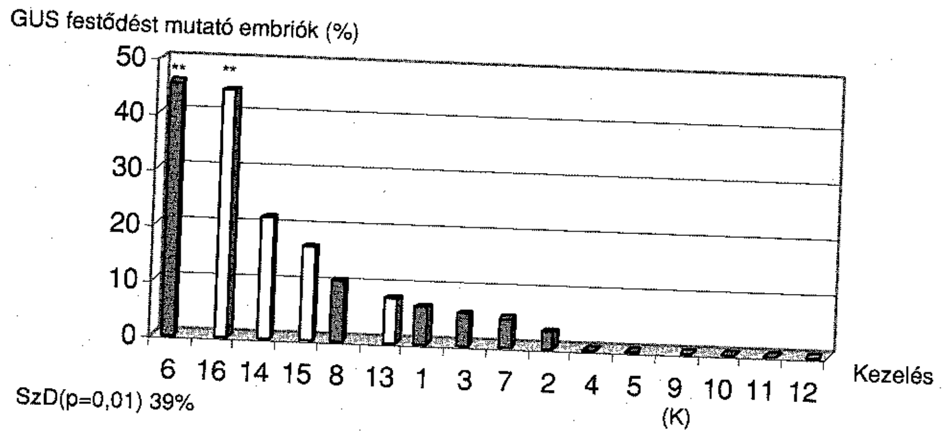
agrobaktérium kisebb mértékben szaporodik fel, valamint az antibiotikum is gyorsabban felszívódik. Ez az időtartam rövidebb, mint más szerzők által leírt optimális előtenyésztési idő. Patnaik és mtsai (2006) *Triticum aestivum* és *T. durum* érett embrión végrehajtott agrobaktériumos transzformációs kísérletében az optimális előtenyésztési időt 2–3 napban határozták meg.

Az 'Mv Csárdás' érett embriós transzformációjának optimalizálását végeztük el az AGL1 agrobaktériumtörzzsel az előtenyésztés, fertőzés és kokultiváció 16 különböző kombinációját alkalmazva. Az érett embriók előtenyésztési ideje és a kokultiváció időtartama nem befolyásolta jelentősen a GUS expressziót, bár a többi kezelés egynapos prekultiváció után általában hatásosabb volt. A kokultiváció alatti szárítás megduplázta a GUS-pozitív embriók számát, bár a változás nem volt szignifikáns. A baktérium higítása már szignifikáns (p=5%) növekedést váltott ki *(2. ábra)*, de leghatásosabbnak (p=0,1%) a higítás és szárítás kombinációja bizonyult, melynek eredményeképp a GUS expresszió átlagos gyakorisága elérte a 45%-ot *(3. ábra)*. A szárítás megnöveli a sejtfal átjárhatóságát, ami elősegíthette a T-DNS-bejutását. Ez a tapasztalat megegyezik a Ding és mtsai (2008) által leírtakkal. Különböző kezeléseket összehasonlítva Cheng és mtsai (2003) szintén azt találták, hogy az előtenyésztett éretlen embriók és embriogén kalluszok szárítása a kokultiváció ideje alatt növelte a T-DNS-átvitel gyakoriságát, és csökkentette az agrobaktérium elszaporodását, ami kedvező hatással volt a növényregenerációra. A higabb baktériumszuszpenzió valószínűleg kisebb stresszt okoz a növényi sejteknek, és így kevesebb hal el programozott sejthalál következtében. E tényezők együttesen elősegíthették a hatékonyabb T-DNS átvitelt.

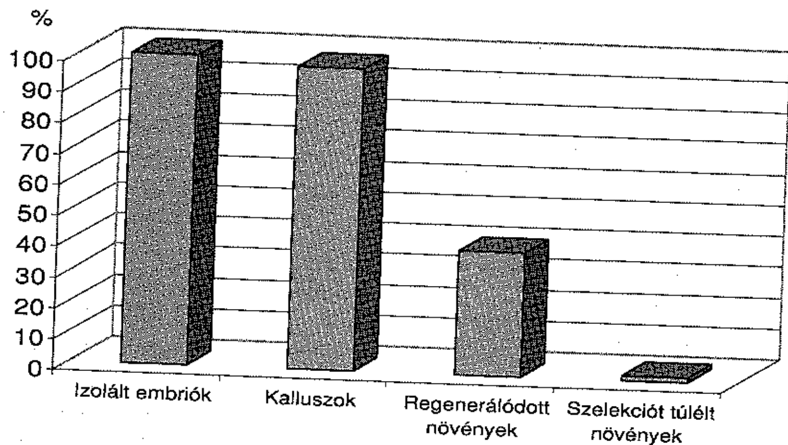
Az optimalizált transzformációs és kokultivációs kezeléssel elvégeztük az 'Mv Emese' 1500 érett embriójának transzformációját az AGL1 agrobaktériumtörzzsel *(4. ábra)*. Az érett embriók kalluszosodása 97% volt, melyekből 40%-os hatékonysággal regeneráltunk növényt. A növényregeneráció hatékonysága nem csökkent a transzformáció hatására a transzformálatlanhoz (40%) képest. A regeneráció után 2×3 hétig tartó, 4 mg/l koncentrációjú foszfinotricin- szelekciót alkalmaztunk. Ezt a regenerált növények 4,2%-a élte túl, ami az izolált embriókra nézve 1,6%-os gyakoriságot jelent. Patnaik és mtsai (2006) búza érett embriójának agrobaktériummal történő transzformációja során 1,3–1,8%-os gyakorisággal regenerálták transzgenikus növényeket. A tranziens génexpresszió gyakorisága 45% volt a kokultiváció alatt alkalmazott szárítás és a baktérium szuszpenzió higítás



2. ábra. A vizsgált kezeléscsoportok hatása a GUS^{INT} riportergén tranziens expressziójára 'Mv Csárdás' érett embriókban
*szignifikáns (p=5%)



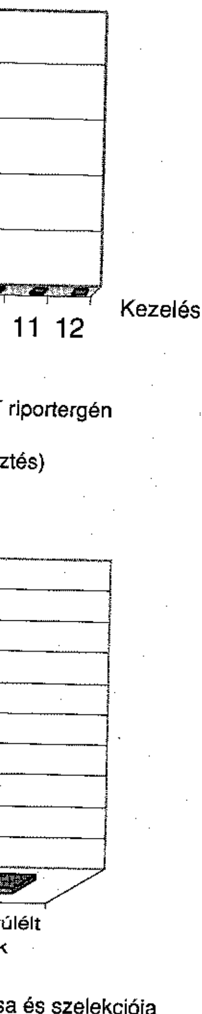
3. ábra. Az egyes kezeléskombinációk (*I. 2. táblázat*) hatása a GUSINT riportergén tranziens expressziójára 'Mv Csárdás' érett embriókban (9 = kontroll, sötét és szürke oszlopok = 2 ill. 1 napos előtenyésztés) **szignifikáns (p=0,1%)



4. ábra. Agrobaktériummal transzformált 'Mv Emese' érett embriók regenerálása és szelekciója

hatására, a növényregeneráció és szelekció után viszont a túlélés gyakorisága már csak 1,6% volt. Ez jelzi azt a problémát, hogy a hatékony génbevitelt (melyet a tranziens génexpresszióval mutatunk ki) a stabil beépülés csekély aránya követi. Ding és mtsai (2009), valamint Wang és mtsai (2009) a nagy tranziens génexpresszió mellett szintén kis stabil génbeépülést tapasztaltak. Ennek megoldása, a stabil beépülés hatékonyságának növelése, további kísérleteket igényel.

Összefoglalva, kísérleteinkben az 1–2 napos érett búzaembriók hígított baktériumszuszpenzióval történő transzformációja, és a deszikkáció kokultiváció alatti alkalmazása bizonyult génátvitel szempontjából a leghatékonyabb kezeléskombinációnak. A szelekció alkalmazása 1,6%-os túlélési gyakoriságot eredményezett, ez megegyezik az irodalomban közölt értékekkel. Ezzel a módszerrel két martonvásári fajta sikeres genetikai módosítását végeztük el, s ez lehetőséget ad nagy nemesítési értékű alapanyagokban akár egyetlen tulajdonság megváltoztatására. Ezzel a módszerrel sikeresen bővíthető a transzformálható genotípusok köre, és értékes alapanyag adható a nemesítés és a genetikai vizsgálatok számára.



sa és szelekciója

alkalmazása bizonyult
a leghatékonyabb ke-
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igot eredményezett, ez
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i értékű alapanyagok-
nság megváltoztatásá-
ikeresen bővíthető a
usok köre, és értékes
esítés és a genetikai

Köszönetnyilvánítás

A dolgozat a 48480 és a 68659 sz. OTKA projektek támogatásával készült.

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OPTIMALIZATION OF AGROBACTERIUM TUMEFACIENS-MEDIATED GENETIC MODIFICATION OF MARTONVÁSÁR WHEAT CULTIVARS

Klára Mészáros[1], **T. Kiss**[1], **Magdolna Dőry**[1], **Ildikó Karsai**[1], **L. Láng**[1], **Z. Bedő**[1] and **L. Sági**[1]
Agricultural Research Institute of the HAS, Martonvásár, P.O. Box 19, H-2462

Improvement of biotic stress resistance has been one of the central problems in plant breeding. In order to reach this aim, biotechnological tools have also been applied. Though wheat is among the most important crop plants, it was one of the last cereals to be transformed, first by particle bombardment. *Agrobacterium*-mediated transformation has now become the more often used method, and the mature embryo proved to be an useful alternative to immature embryo explants. Here, plant regeneration experiments were carried out with 19 wheat cultivars from mature embryo. The most responsive genotypes were then used in transformation experiments. We studied 16 combinations of factors that affect the success of *Agrobacterium*-mediated transformation in mature embryos of wheat: pre-cultivation time, density of bacterial culture, co-cultivation time, and drying (or not) during co-cultivation. Efficiency of T-DNA transfer was positively influenced by bacterial dilution and drying during co-cultivation. Two wheat cultivars, bred in Martonvásár, were transformed with reporter and selection genes using this method, which represents an extended genotype range for genetic modification of wheat, and offers basic material for breeding programs and genetic investigations.

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MOLECULAR FARMING, USING THE CEREAL ENDOSPERM AS BIOREACTOR

L. TAMÁS

DEPARTMENT OF PLANT PHYSIOLOGY AND MOLECULAR PLANT BIOLOGY,
EÖTVÖS LORÁND UNIVERSITY, BUDAPEST, HUNGARY

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Seed is an ideal protein production platform because it is the storage organ of the plant and offers appropriate storage compartments for the deposition of foreign proteins. To achieve high foreign protein expression level in the endosperm tissue, the transformation cassette carried the tissue-specific promoter of the wheat high-molecular-weight glutenin subunit protein 1Bx17, fused to the first intron of rice actin promoter. Transformation protocols were established and optimized in the laboratory for cereals such as rice, barley and wheat using direct DNA delivery and the *Agrobacterium tumefaciens*-mediated transformation system. Both immature (barley) and mature (rice) embryos, and immature inflorescences (wheat) were used as sources of explants. Subunit edible vaccines were produced to introduce the LTB, CTB and fused LTB-PEDV genes into the rice genome. The PEDV gene was also integrated into the barley genome. A project has recently been started to produce a rabbit-derived enzyme in transgenic wheat endosperm to be used by the pharmaceutical industry.

Key words: cereal transformation, edible vaccine, biofermentor, molecular farming

Introduction

Plants have been used by mankind for thousands of years, not only for food and feed, but also as raw materials and medicines. Apart from various plants organs, extracts also served to cure diseases. The systematic investigation of the therapeutic molecules allows these compounds to be used as a natural option in new drug production approaches. To overcome problems arising from inconsistent product quality and environmental effects, DNA technology has been introduced for the possible utilization of plant expression systems. One major achievement was the establishment of efficient plant transformation procedures both for dicots, based on either the nuclear or the plastid genome (Maliga, 2004), and for monocots (Vasil et al., 1992; Jones, 2005). Beyond the

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modification of agronomical traits, and the study of many aspects of gene function or of changes in the functional quality of plant products, the transgenic approach can also be used for the production of recombinant pharmaceutical proteins (Ma et al., 2003; Streatfield, 2007). Plants have become a convenient, safe and cheap alternative for foreign protein expression, replacing microbial or mammalian cell culture methods. Transgenic plant systems offer several advantages, including low energy input, easy control of production scale, and low risk of contamination by human and animal pathogens. The production of plants producing recombinant proteins or chemicals is known as "molecular farming" (Basaran and Rodriguez-Cerezo, 2008).

This technique has the potential to produce molecules in very large quantities for diagnostics, health care, and for the chemical and pharmaceutical industry. Depending on the promoter used in the transformation cassette to drive the transcription of the gene of interest, the recombinant proteins are expressed either constitutively or only in special organs. The use of tissue-specific, strong promoters has the advantage of enhancing the recombinant protein yield in transgenic crops (Twyman et al., 2003). Seed can be one of the most convenient protein production platforms, because it has several subcellular storage compartments for the deposition of the new proteins. Another advantage is the relatively high stability of the recombinant proteins, regardless of the storage temperature. In the case of vaccines or recombinant antibodies deposited in edible parts of the crops, no further processing or purification is required before utilization, providing a cold-chain- and needle-free vaccination process (Nochi et al., 2007)

An overview is given in this article of the efforts carried out at Eötvös Loránd University, in cooperation with national and international laboratories, to use the gene technology approach for producing cereal crops with novel properties for new applications.

Materials and methods

Plasmid constructions

The rice *act*1 first intron was amplified by PCR from rice genomic DNA, using the appropriate primers published earlier (Oszvald et al., 2008b), and fused with the 1Bx17 HMW-GS promoter in a transformation cassette designated as pTLZ (Oszvald et al., 2003). The resulting construct was identified as pTSI.

Rice transformation

Rice transformation was carried out as published by Cho et al. (2004). The embryos were separated from the endosperm after 7 days and were further cultured on N6 culture medium for callus production. After 3–4 weeks of cultivation the callus was bombarded using a "Genebooster". Transgenic callus was selected on medium containing 50 mg dm^{-3} of hygromycin B. Rapidly growing resistant tissues were moved to MS regeneration medium. Plantlets with 5–6 cm shoots and well-developed roots were transferred to soil and grown in a greenhouse.

Barley transformation and tissue culture

Immature barley embryos 1–2 mm in length were isolated from surface-sterilized caryopses. The embryos were placed on a Petri dish containing BCI medium and 0.4 ml *Agrobacterium* suspension (OD_{600}=2) was loaded on the top of them. The suspension was supplemented with 0.015% Silwet L-77. The embryos were transferred after draining onto new BCI medium without antibiotics for co-cultivation for 3 days. On the fourth day, the embryos were placed onto BCI medium supplemented with 75 mg/l hygromycin and 150 mg/l timentin for selection and callus induction. After the callus induction phase, the explants were placed onto DBC medium supplemented with 75 mg/l hygromycin and 150 mg/l timentin for 2–6 weeks, until greening was visible.

PCR analysis

The putative transgenic rice plants were screened for the selection marker gene and the transgene by PCR. Total genomic DNA was isolated from leaf, root and endosperm tissues using the Qiagen Plant DNeasy kit. PCR analysis for the genes was carried out using the appropriate primer pairs, specific for the given gene (either selection marker genes or a particular gene of interest). PCR products were analysed on 1% (w/v) agarose gel.

Immunoblot detection of CTB protein in transformed rice seeds

Total soluble proteins (TSP) were extracted from the mature seeds of transgenic rice plants. Around 100 mg of rice powder was mixed with 500 µl of buffer (200 mM Tris-HCl pH 8.0, 100 mM NaCl, 400 mM sucrose, 10 mM EDTA, 14 mM β-mercaptoethanol, 1 mM phenylmethylsulphonyl fluoride and 0.01% Tween-20) and the homogenate was centrifuged for 15 min at 13,000 g at 4°C. The supernatant containing TSP was then subjected to further analysis. The protein was fractionated by 12% sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto Hybond C membranes (Promega) in transfer buffer using a dry-blot apparatus (Bio-Rad, Hercules, CA). The membranes were incubated after masking with a 1:5,000 dilution of the appropriate antibody, developed in rabbit following the known protocol. Coloured bands were developed using the BCIP/NBT method in TMN buffer.

Quantification of CTB protein level in transgenic rice seeds

The expression of the edible vaccine protein level in the transgenic rice plants was determined by enzyme-linked immunosorbent assay (ELISA) following the method published by Oszvald et al. (2008b). Total soluble protein samples from the transgenic and wild-type plants were coated at 100 µl per well onto 96-well microtiter plates (Dynatech Laboratories, Burlington, MA) together with purified heterologously expressed proteins, and the plates were incubated overnight at 4°C. The plates were washed three times with PBST washing buffer and the background was blocked via incubation in 3% (w/v) bovine serum albumin (BSA). The plates were then incubated for 2 h in TBST containing the appropriate antibody in 1:5,000 dilution. After washing, the plates were incubated in a 1:7,000 dilution of anti-rabbit IgG conjugated with buffer containing alkaline phosphatase (Sigma A-2556) for 2 h at 37°C. After washing with PBST buffer the plates were developed by the addition of TMB substrates (PharMingen 2606 and 2607KC, Fallbrook, CA) at room temperature in darkness. The optical density was measured at 405 nm wavelength in an ELISA reader (Packard Instrument MRA-006, Meriden, CT). The edible vaccine protein expression level in the plant samples was quantified by comparison with known quantities of bacterial protein complex. All measurements were performed in triplicate, and analysis of variance was carried out using the statistical program of Excel (Microsoft Corp., USA).

G_{M1}-binding assay

G_{M1}-ELISA was conducted in an effort to determine the affinity of G_{M1}-ganglioside receptors for the plant-derived edible vaccine proteins following the method published earlier by Oszvald et al. (2008b).

Results and discussion

Research on the wheat endosperm-specific promoter

If seeds are taken as the target of the foreign proteins expressed in transgenic cereals there are several options for deposition. Tissue-specific promoters are available for either embryo (Cuming and Lane, 1979), aleurone (Kalla et al., 1994) or endosperm. Because the endosperm is much larger than the first two, endosperm tissue is the main target for recombinant protein expression. Promoters driving either the transcription of the genes for starch biosynthesis (Rasmussen and Donaldson, 2006) or the storage proteins can be used, but the promoters most frequently utilized in transformation cassettes are the storage protein genes deposited in the protein body. Both wheat prolamin and rice glutelin gene promoters are applied.

In promoter development experiments, the wheat high-molecular-weight (HMW) glutenin subunit promoter was used to construct an expression cassette based on an endosperm-specific promoter useful for high level recombinant protein expression in cereal "bioreactors". HMW glutenin subunit (GS) promoters are currently the most powerful endosperm-specific promoters. Bx type HMW glutenin subunits (encoded by the *Glu-B1-1* gene) are expressed at the highest level amongst the HMW-GS proteins (Juhasz et al., 2003). Storage protein genes do not contain introns. The gene expression level in plants has been proved to be increased by the addition of an intron to the promoter region of a heterologous gene (Vibok et al., 1999). Studies suggest that introns may improve the efficiency of mRNA processing. In plants, particularly in monocots, introns have been shown to contribute to the enhancement of gene expression in the case of constitutive promoters. McElroy et al. (1991) showed that the rice *act*1 first intron transiently increased the GUS enzyme expression level.

As reported earlier (Oszvald et al., 2008a), a chimeric promoter was assembled using the 5' UTR (1900 bp) of the gene coding for the 1Bx17 HMW glutenin subunit protein, responsible for tissue-specific expression and the first intron (456 bp) of the rice actin (*act*1) gene. The sequence around the initial translation codon was optimised. The effect of the intron and promoter regulatory sequences on the expression of the *uid*A gene was studied using different lengths of the 1Bx17 HMW-GS promoter. The functions of promoter elements, promoter lengths and actin first intron were tested by transient expression assay in immature wheat endosperm and in transgenic rice plants. The results showed that the insertion of the rice *act*1 first intron increased GUS expression by four times in transient assay.

Apart from the effect of the first intron of rice actin gene on the strength of the tissue-specific promoter, the length of the 1Bx17 HMW glutenin subunit promoter was also studied. Deletion of the far upstream region of the promoter proved to have no significant effect on the expression level; however, a substantial drop was observed when the length of the 5' UTR was reduced to

237 nucleotides. Previous studies on endosperm-specific promoters identified a primary enhancer sequence, the major regulatory element in HMW glutenin subunit genes. This motif starts 13 nucleotides upstream from the CAATTG sequence, cut by the *Mfe*I enzyme. To test whether the promoter (pTSIM) containing less than the complete enhancer fragment was sufficient for the tissue-specific driving of *uid*A gene expression, the pTSIM-GUS cassette was used to produce stable transgenic rice plants. Deletion of not only the N and E boxes but also the Cereal box and partial sequences of the HMW enhancer did not appear to affect the specificity of the 1Bx17 HMW-GS promoter in transgenic rice. The 1Bx17/*act*1 chimeric promoter showed strict tissue-specificity, being expressed only in the endosperm with no expression in any other tissues.

Research on cereal transformation

The genetic transformation of cereals has been accomplished in the last twenty years through various methods. Biolistics and agro-infiltration have become the most widely used techniques, because the best transformation efficiency can be reached through these approaches. Various type of explants such as mature and immature embryos, or immature inflorescences, can be used for biolistic transformation. Due to the shortage of space in the greenhouse and the time required for immature scutella production and preparation, the option of establishing a method based on mature seed was studied. A successful and efficient method was established to produce transgenic rice plants in the laboratory (Oszvald et al., 2007a). Apart from rice, wheat transformation was also achieved in two collaborating laboratories using the micro-projectile bombardment method (Sági et al., 2008). After intensive studies and the improvement of the tissue culture and regeneration approach (Tamás et al., 2004) a reliable procedure was established (Tamás et al., 2009). The inflorescence-based wheat transformation method also works successfully and can be used to produce transgenic wheat for molecular farming purposes (Jenes et al., unpublished results).

Since Tingay et al. (1997) reported the first *Agrobacterium*-mediated transformation of barley, the method has been set up in many laboratories, and many papers have suggested improvements, particularly in the efficiency of the protocol (Shrawat et al., 2007). A properly working procedure was also established by Eva et al. (2008) based on the variety Golden Promise, using minor modifications to the protocol published by Harwood et al. (2009). A transformation efficiency of 5% was achieved, which is comparable to the 6.7% reached by Shrawat et al. (2007). One reason for the successful transformation could be the use of Silwet-L77 in the inoculation medium and the tissue culture medium optimized by Harwood (personal communication). Silwet L-77 is a strong surfactant, which helps *Agrobacterium* cells to enter the plant tissue. The pre-regeneration medium used in the current work also contained 5 μM Cu^{2+}, which greatly affected the plant regeneration process (Purnhauser, 1991).

Research on edible vaccine production

Plant-based vaccines appear to provide promising examples of a new strategy that combines innovations in medical science and plant biology to generate affordable pharmaceutical products. Several plants have already been studied for their potential use in edible vaccine production and some have reached the phase of clinical trials (Streatfield, 2006). Based on previous intensive studies it can be concluded that vaccine production in plants is a promising cheap alternative in the fight against epidemic diseases, because modified plants could be grown locally, and the administration of the vaccine is safe and easy.

Efforts to generate recombinant proteins in plants have been focused on dicotyledonous plants, mainly including potato, tobacco and alfalfa. These plants, however, have some obvious disadvantages. Green leaf tissues harbour phenolic compounds, as well as a host of other potentially toxic compounds. They are also generally unpalatable, and any useful proteins generated must be extracted and purified before consumption. Cereal grains have a substantial advantage over green tissues. The yields of recombinant proteins tend to be much higher. Unlike proteins synthesized in vegetative plant tissues, seed storage proteins are compartmentalized in protein bodies.

Three GM rice varieties producing epitope vaccine have been produced (Oszvald et al. 2007b; 2007c; 2008b), two of which carry a synthetic sequence, with a sequence modification based on plant-optimised codon usage, coding for the non-toxic B subunit of either the *Escherichia coli* heat-labile enterotoxin (LT) or the *Vibrio cholera* cholera toxin (CT). These sequences were fused to a translation signal (the Kozak sequence) on the 5' end and the ER retention signal, SEKDEL, was added to the C terminus of the protein. The synthetic sequences were inserted into the pTSI plant transformation cassette (Oszvald et al., 2008a) under the control of the chimeric rice actin and wheat Bx17 HMW glutenin promoter. More than twenty hygromycin-b resistant rice lines were regenerated from both transformation events and subjected to further analysis at both the nucleotide and protein levels. The results of RT-PCR revealed that the genes of interest were transcribed neither in the leaves nor in root tissues, while Western blot analysis showed a reasonable level of recombinant proteins in the endosperm tissue.

Functional LTB and CTB proteins were synthesized as monomers, which were subsequently assembled into pentameric structures and deposited in protein bodies. The pentamer formation was confirmed by GM_1-ganglioside binding assay, which is located on the surface of eukaryotic cells. The expression level of both B subunits, measured by quantitative ELISA, varied between 0.5 and 2.7% of the total soluble protein (TSP), which represents about 1.3 mg/g seed recombinant protein. According to Tacket et al. (2004) this level of protein expression is sufficient to generate a sizeable amount of antigen after the consumption of a few milligrams of seeds, and the transgenic rice lines can be used for the production of rice seed-based edible vaccines.

Mucosal vaccines administered either orally or nasally have been shown to be effective in inducing antigen-specific immune responses in both systemic and mucosal compartments. However, some epitopes cannot be delivered directly to mucous membranes, but require a carrier protein to elicit a mucosal response. The Porcine epidemic diarrhoea virus (PEDV) has been identified as a member of the Coronaviridae family of viruses. It has been demonstrated to induce acute enteritis in pigs. The neutralizing epitope of PEDV was identified on the basis of sequence information for the same epitope of the transmissible gastroenteritis virus (TGEV). Efforts were made to develop a plant-based vaccine, produced in either rice or barley endosperm, via the coupling of a synthetic PEDV epitope to a synthetic LTB sequence. Both components of the fusion proteins were detected in the endosperm tissue via Western blot analysis. The fusion protein was demonstrated to assemble into pentamers, as evidenced by its ability to bind to GM_1-gangliosides. An appropriate level of expression (1.9% of TSP) was measured in the transgenic rice endosperm, allowing the successful development of a fusion-type edible vaccine.

Genetically modified cereal plant lines can be integrated into conventional breeding and, after intensive examination and evaluation processes to confirm the safety of the GM-related new lines, the new varieties should contribute to the development of safe edible vaccine production (Bedő, 2002).

Research on natural biofermentors producing enzymes of industrial interest

In recent decades the preparation of enantiomerically pure compounds has received exceptional attention. The synthesis of optically active materials represents a challenge both to academic and industrial chemists. The recognition of the fact that chirality plays a crucial role in nature encouraged tremendous efforts in enantioselective synthesis. According to the results of robust experiments it has been confirmed that enantiopurity is related to biological properties. Opposite enantiomers act differently within an organism and may display various activities. Some of these differences could be life-threatening, as they may result in teratogenic properties. The racemic drug thalidomide caused severe birth defects when taken by pregnant women.

There are a number of ways to produce enantiomerically pure compounds, but they are either very expensive (metal catalysis) or put enormous pressure on the environment (diastereomeric recrystallization). Asymmetric synthesis is generally used in natural product synthesis and in the industrial production of pharmaceuticals, flavours, fragrances, pesticides, etc. Among the variety of methods available for the synthesis of enantiomerically pure compounds, the application of enzymes has become accepted as a routine procedure. Although the concept of enzyme application to asymmetric synthesis has been long recognized, it is only recently that these catalysts have attracted attention.

The enzymes used for this reaction may show a high degree of substrate specificity in catalysing the transformation of their natural substrate. They often accept a wide range of structurally related compounds; for example, carboxyl esterase enzymes are able to work on phosphate ester molecules. Enzymes derived from animals cannot be used for drug production, as they are prohibited in the pharmaceutical industry, because of the possible contamination by animal-related viruses or prions. These enzymes, however, can be produced in transgenic plants and utilised by the pharmaceutical industry after extraction and purification, because the risk of contamination is negligible. Studies on rabbit liver esterase enzymes proved that only one isoenzyme has the ability to react with the appropriate molecule to produce an ester molecule with the desired chirality.

The protein was sequenced and the correct sequence was pooled out from a cDNA library for cloning into the pTSI plant transformation cassette and introduction into the wheat genome using the micro-projectile bombardment method. Several hundreds of immature inflorescence-derived calli were bombarded and more than a dozen plants were regenerated after callus selection. Samples were taken from the leaves and the integration of the sequence of the gene of interest was confirmed by PCR (Jenes, unpublished results). The next generation is being grown in a controlled environment for further studies.

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Corresponding author: L. Tamás
E-mail: tamasl@ludens.elte.hu

Plasticity of plant regeneration ability in bread wheat (*Triticum aestivum* L.) cultivars

Klára Mészáros[1], Ildikó Karsai[1], Linsheng Zhang[2], Tibor Kiss[1], Magdolna Dőry[1], László Láng[1], Zoltán Bedő[1], László Sági[1]

[1] Agricultural Research Institute of the Hungarian Academy of Sciences, Martonvásár, Hungary
[2] Northwest A & F University, College Life Science, Yangling, Shaanxi, China

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Abstract

Callus induction and plant regeneration experiments were carried out with four different explants of 17 winter wheat and three model spring wheat cultivars: anther, immature embryo, mature scutellum and matured seed.

The characteristics of plant regeneration of the tested cultivars in the four *in vitro* systems were different. The range of the regeneration capacity was the widest in the case of whole mature seed (8.5-95%) coupled with the highest average value (56.1%). There was no significant difference between the efficiency of immature embryo and mature scutellum based regeneration. In the case of immature embryo the 25% of varieties showed excellent regeneration (over 30%), 30% good (between 30-10%) and 45% poor regeneration capacity under 10%. The mature scutellum based regeneration resulted in 44.44% excellent, 33.33% good and 22.22% poor regenerated cultivars. Although the range of regeneration of the tested cultivars was wider in the case of mature scutellum (0-63%) the average value was lower than in the case of immature embryo (0.9-59.4%). The mature scutellum based plant regeneration proved to be similarly efficient and genotype independent as immature embryo based regeneration, but much cheaper. The anther based regeneration proved to be the less efficient method, There wasn't any cultivar over 30% plant regeneration, 4% with good (between 30-10%), and the regeneration of the majority of genotype were under 10%. In general, plant regeneration from whole mature seed was significantly the highest among the methods (at p= 0.001 level).

Based on these results the best genotype-culture system combinations were determined for each cultivar. Our data suggest that the majority of cultivars have good response in at least one culture system, but nine (45%) of the tested cultivars were successful in two or more culture systems. These versatile cultivars could represent an essential component in improving the efficiency of the transgenic programs.

Introduction

Generation of transgenic plants is one of the central research areas for plant biotechnology and crop genomics. Cereals, including wheat are among the most important crop plants and thus efficient transformation technologies represent an essential prerequisite for high-throughput applications in genetic improvement as well as in functional genomics research. Such efficient transformation platforms are currently based on *in vitro* tissue culture systems capable of producing high numbers of regenerants in a short period of time. Although the good *in vitro* reaction of a cultivar is not equal with good transformability, but it is an essential requirement. The *in vitro* reaction is a complex trait depending strongly on the culturing methodology applied, on the *in vitro* conditions and on the genotype, therefore

improving this character by crossing is almost impossible. An other way would bee to introduce genes to the update cultivars by mediation of efficiently tarnasformable model cultivars. The disadvantages of this method are the time-consuming procedure to eliminate the adverse characters of model variety. The most useful approach seems to be the identification of economically important cultivars showing good regeneration ability in tissue cultures and their direct transformation.

There is a complex interaction of numerous factors that determine the frequency of plant regeneration in wheat tissue culture such as the explant source and age (Lu et al. 1984; Luhrs and Lörz 1987; Wu et al. 2003), genotype (Andersen et al. 1987; Fennell et al. 1996; Machii et al. 1998; Varshney and Altpeter 2001), growing conditions of donor plants (Barnabás et al. 1988), and many elements of *in vitro* culture including the composition of synthetic media (Jähne-Gärtner and Lörz 1996). The range of successfully regenerated explants in wheat is almost as broad as the type of existing tissues: immature anther (Ouyang et al. 1973; Holme et al. 1999) and isolated microspore (Mejza et al. 1993; Tuvesson and Öhlund 1993), immature embryo (Sears and Deckard 1982; Lörz et al. 1998; Varshney and Altpeter 2001; Ward et al. 2001; Wu et al. 2003) and isolated scutellum (Magnusson and Bornmann 1985; Nehra et al. 1994), immature inflorescence (Ozias-Akins and Vasil 1982; Chauhan et al. 2007), leaf base (Wernicke and Milkovits 1984; Wang and Wei 2004), mature embryo (Lazar et al. 1983; Bartok and Sagi 1990; Filippov et al. 2006) and shoot tip (Wernicke and Milkovits 1986;Viertel and Hess 1996).

As a typical example of the interplay between the above factors, the role of explants and genotypes can primarily be mentioned. Explants that are highly regenerative in a broad range of wheat genotypes are not numerous; essentially immature embryos and anthers proved effective and reproducible in independent studies (Lazar et al. 1984; Andersen et al. 1987; Jones et al. 2005). Even then, there are many genotypes that perform well with one of the two explants only (Machii et al. 1998; Özgen et al. 1998). It is therefore not surprising that several studies were conducted in order to screen for genotypes with high *in vitro* response for one or both types of explants (Andersen et al. 1987; Machii et al. 1998; Özgen et al. 1998; Varshney and Altpeter 2001). However, a common shortcoming of these studies has been that they investigated either a high number of genotypes for reaction with one explant type (Fennell et al. 1995; Varshney and Altpeter 2001) or a low number of genotypes (2-6) in both above mentioned explants (Özgen et al. 1998).

There is a differential genetic background behind the *in vitro* reaction of genotypes. Several regions on homoeologous chromosome groups 1, 2, 4 and 5 were identified as responsible for callus induction or plant regeneration from anthers (Szakács et al. 1988) and immature embryos (Agache et al. 1988; Mano et al. 1996; Ben Amer et al. 1997; Jia et al. 2009). Each step of the regeneration process is controlled by different chromosome regions, more and more accurately mapped by molecular markers (Torp et al. 2001). The 1R and 5R rye chromosomes in wheat background influenced the anther and immature embryo culture response of several genotypes (Agache et al. 1989; Machii et al. 1998; Varshney and Altpeter 2001; Dobrovolskaya et al. 2003). In the East European breeding pool the most common translocation is 1BL/1RS derived from the Petkus rye (Schlegel and Korzun 1997). More than 50% of wheat varieties registered in Hungary between 1978 and 1999 carried the 1BL/1RS translocation (Köszegi et al. 2000). A locus involved in the regeneration capability of wheat anther cultures is also located in this rye chromosome arm, and has a positive effect on embryo initiation and green plant regeneration (Torp et al. 2001). The positive effect of this chromosome region on plant regeneration from immature embryo is not unambiguous (Langridge et al. 1991; Machii et al. 1998; Varshney and Altpeter 2001) and appears to depend on interactions with the specific genetic backround (Langridge et al. 1991).

Another bottleneck of applying both anther and immature embryo culture is the cost of continuously supplying explants with reproducible quality. Usually growth chambers (phytotron) and/or a greenhouse is required, which consume huge amounts of energy for growing the donor plants. In addition, the suitable developmental stage of the donor plant for culture is also strictly limited (Luhrs and Lorz 1987). Therefore, mature embryo as an alternative explant is currently emerging for wheat tissue culture because the seeds as a constant source of mature embryos can be produced in the field at a low cost and remain available more unlimited in time (Özgen et al. 1998; Delporte et al. 2001; Filippov et al. 2006). In addition, due to their quiescent state, mature embryos are physiologically more uniform than alternative immature tissues (Delporte et al. 2001). A potential risk attached to the use of field-harvested seeds, however, is the high incidence of microbial contamination.

It is therefore highly desirable to investigate (to generate novel data on) the plant regeneration ability of a high number of genotypes in diverse range of tissue culture systems. This type of studies yield valuable information on the specific performances of individual genotypes in a particular culture system and/or may identify ones with universally good *in vitro* reaction. Such versatile genotypes, if commercially widespread or prospective, can then be directly utilized in transgenic improvement programs. To this end, whe have tested here 20 (17 winter and three spring) commercial and model wheat genotypes in four culture systems (explants: anther, immature embryo, mature scutellum, and mature seed) and report on the frequency of their embryogenic callus induction and plant regeneration in these culture systems. Our data suggest that the majority of cultivars have good response in at least one culture system, but some are more versatile and the regeneration of these cultivars is successful in two or more culture systems.

Materials and methods

The experiments were carried out with 17 winter wheat and three model spring wheat cultivars (Table 1). The winter wheat cultivars were all developed and registered by the Agricultural Research Institute of HAS, Martonvásár, Hungary. Four different explants (anthers, immature embryos, mature scutella, and mature seeds) were collected from field-grown donor plants at the respective developmental stages and tested for callus induction and plant regeneration.

Anthers containing microspores in mid-uninucleate stage were collected as described by Karsai et al. (1994). Spikes were sterilized in 0.1% (w/v) $HgCl_2$, and 2000 anthers were inoculated for each cultivar in eight replications onto the basal induction medium MN6 (Chu et al. 1990). Callus induction was carried out for 30 d in the dark at 29ºC. Then, embryogenic structures were counted and transferred to 190-2 regeneration medium (Zhuang et al. 1984). Plant regeneration took place for 30 d at 26ºC with 16-h light and 8-h dark photoperiod regime (PAR light intensity: 20 μmol m^{-2} s^{-1}). The embryo induction frequency was determined as the number of embryos/number of anthers isolated and green plant regeneration expressed as the number of green plants/number of isolated anthers.

Plant regeneration from immature embryos was carried out as described by Jones et al. (2005). The immature seeds were surface-sterilized with 70% (v/v) ethanol for 2 min, then for 15 min in 10% (w/v) Domestos with a few drops of Tween 20 followed by three rinses with sterile distilled water. Two-hundred immature embryos of about 1.5 mm size were isolated from each cultivar and placed onto inoculation medium in six replications. After 3 d of incubation, all embryos were transferred and maintained on induction medium for 10-15 d in the dark at 24°C. The embryogenic calli, 0.5 cm in size, were transferred to regeneration

medium for 3 weeks. The green plant regeneration rate was determined in the ratio of isolated explants.

In the case of mature scutellum explants and whole mature seeds a similar route was followed. The seeds were surface-sterilized as in the case of immature embryos. Then for mature scutellum as explant source, 200 mature embryos were isolated and the embryo axes were discarded. The scutella with the axis side down were placed in four replications onto semi-solid MS medium supplemented with 2.5 mgl^{-1} dicamba. In the case of the mature seed explant source, the embryos of two hundred whole mature seeds were aseptically wounded with a scalpel without detaching them from the seed as described by Filippov et al. (2006). Prepared seeds were placed with the furrow downwards onto the same induction medium than in the case of isolated mature scutella. The explants were incubated for 5 d in the dark at 24°C. The embryogenic calli were transferred for regeneration onto plant growth regulator-free MS medium. After 3 weeks, the green shoots were counted and the plant regeneration frequency was calculated in the ratio of isolated explants.
The 1BL/1RS translocation of cultivars was previously defined by SDS-Page, C-banding and *in situ* hybridization (Köszegi et al. 2000; Szakács et al. 2004).

Statistical analysis of the data was performed with SPSS Statistics version 16.0 program (SPSS Inc., Chicago, IL, USA). The statistical differences of the plant regeneration capacity among the cultivars were ranked according to Duncan's multiple range test (Duncan 1955).

Table 1. Wheat cultivars used in this study

Cultivar	Pedigree	T1BL/1RS	Growth habit
BOBWHITE26	AURORA//KALYAN/BLUEBIRD/3/WOODPECKER	NO	Spring
CADENZA	AXONA/TONIC	NO	Spring
CHINESE SPRING	LV-WEST-SICHUAN/LV-SICHUAN	NO	Spring
FATIMA2	FUNDULEA-29/LOVRIN32	YES	Winter
MV 16	MV4/KAVKAZ//P4089/3/ZLATNA-DOLINA/ARTHUR-71/4/RUBIN	YES	Winter
MV BÉRES	ERYT352/MAGDALÉNA	YES	Winter
MV CSÁRDÁS	JUBILEJNAJA-50/FUNDULEA-29//MVMA	YES	Winter
MV EMESE	MVMA/MV12//F2098W2-21	NO	Winter
MV HOMBÁR	FLEMING/MATADOR	NO	Winter
MV KOLO	MIRONOVSKAJA-OSTISTAJA/ATAY85//ALFÖLD	NO	Winter
MV MAGDALÉNA	JUBILEJNAJA-50/FUNDULEA-29//MVMA	YES	Winter
MV MAGVAS	CARMEN/MACVANKA-2//MVMA	NO	Winter
MV MAMBÓ	KALÁKA/MV16//F2076	NO	Winter
MV MARSALL	MV15-91/KALÁKA//MV15-91/FATIMA-2	YES	Winter
MV PÁLMA	F797/MV08-82//MV15	YES	Winter
MV PALOTÁS	MVMA/MV8//F2098W2-21	NO	Winter
MV REGIMENT	GA901273-46-I/F6038W12-1	NO	Winter
MV SUBA	ERYT1778/MAGDALÉNA//MAGDALÉNA	NO	Winter
MV SÜVEGES	MIRONOVSKAJA-29/MAGDALÉNA//MAGDALÉNA	NO	Winter
MV TOBORZÓ	ERYT336/MAGDALÉNA	NO	Winter

Results and discussion

The anther culture derived haploid plant production can be useful for transformation experiments, because it provides an opportunity to produce homozygous plants in one step. Comparing the four regeneration systems, the weakest reaction was however observed in

anther cultures. The average induction rate of haploid embryogenic structures was 21.1% and ranged from 97% in ‘Mv Pálma’ to 5.1% in ‘Mv Magdaléna’. The green plant regeneration capacity ranged between 20.5% and 0.1% in ‘Mv Béres’ and ‘Mv Magvas’, respectively (Figure 1a). The tested cultivars could be divided in four statistically distinct groups (Table 2, column Anther). There were four cultivars in the first two groups with regeneration ability of over 10%. One further cultivar, ‘Mv Emese’ was still significantly better (8.4%) than the majority (15) of the tested cultivars, which showed less than 5% of regeneration frequency in this system (Figure 1.a). Noticeable effects of genotype on wheat anther culture have been observed, in agreements with the published results. Whereas green plants were regenerated from all tested cultivars in our experiment, the rate of regeneration was very low. Only 20% of tested wheats produced more than 10% green plants. This rate was similar to the result of Andersen et al. (1987) and Kim and Baenziger (2005). On the contrary, Machii et al. (1998) and Orlov et al. (1993) could regenerate green plantlets from only 25% of the tested bread wheats, the majority of cultivars failed to produce any green plant. These findings underline that the level of response with unselected wheat material remains unpredictable. If well responding genotypes are rare, the direct usefulness of anther culture technique is limited (Andersen et al. 1987). Based on our results and published data, plant regeneration from anthers seems to be less efficient and more genotype dependent. There have been some cultivars with good ability to produce green plants, but the majority of the tested genotypes had very poor reaction (Figure 3), which makes them useless in the anther-based *in vitro* regeneration systems.

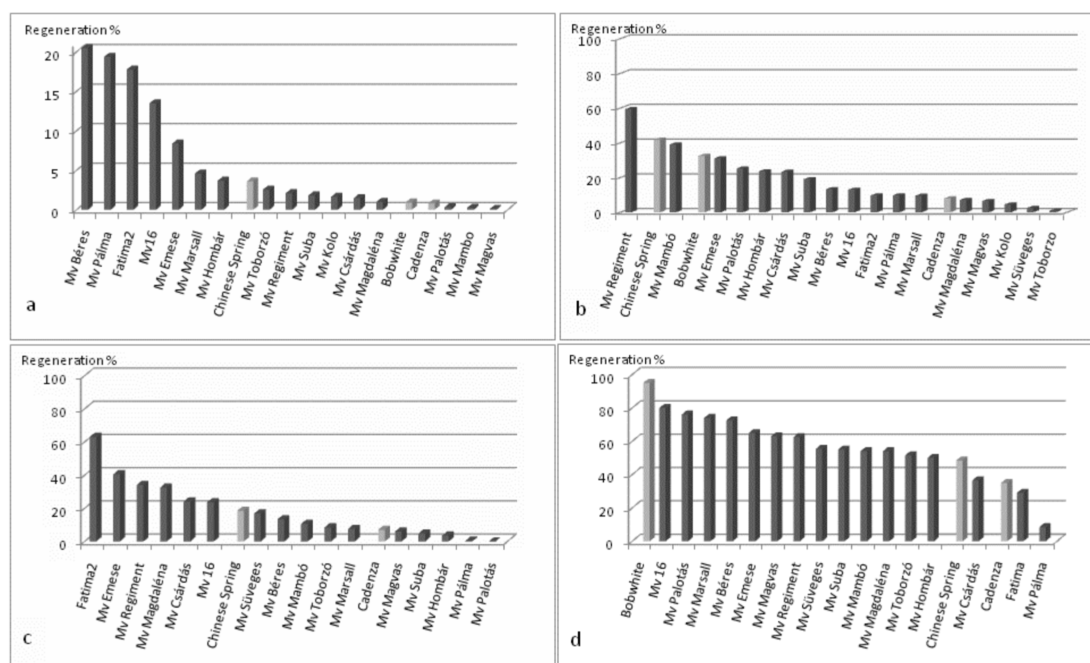

Figure 1. Green plant regeneration frequency from cultured anthers (a), immature embryos (b), mature embryos (c) and whole mature seeds (d) of 20 wheat cultivars. Dark columns are winter type, light columns are the spring type.

In the case of immature embryo explants, there were no significant differences among the cultivars in the callus induction frequency (average: 96.7%), this results being similar to those published for other cultivars (Fennell et al. 1995; Machii et al. 1998;Varshney and Altpeter 2001). ‘Mv Csárdás’, ‘Mv 16’, ‘Mv Regiment’ and ‘Chinese Spring’ produced calli with a rate of 100%. The lowest callus induction (79.5%) was observed in ‘Mv Magdaléna’.

The plant regeneration capacity of the tested cultivars ranged from 59.4% of ‘Mv Regiment’ to 0.9% of ‘Mv Toborzó’ with an average of 20.6% (Figure 1b). The 20 cultivars formed 10 significantly different groups (Table 2, column Immature embryo), but the separation between these groups was not distinct due to overlaps between them, emphasising the quantitative character of this trait. ‘Mv Regiment’ regenerated significantly better than any of the other cultivars (Figure 1b). There were four more cultivars in the best regenerating group (59-31%), although two of them overlapped statistically with the moderately regenerating group (‘Bobwhite26’, ‘Mv Emese’). Six cultivars were in the moderately regenerating group ranging between 25% of ‘Mv Palotás’ to 13% of ‘Mv16’. The regeneration frequency of the remaining nine cultivars was under 10%. There was no difference between the regeneration capacity of spring and winter wheat varieties. In the case of European cultivars (Varshney and Altpeter 2001), the plant regeneration calculated in the ratio of isolated explant was lower than in our experiment, but the frequency of the regenerable genotypes was the same. The lower green plant production, observed by Varshney and Altpeter (2001), can be explained with the longer induction period they used, which was three weeks, on the contrary we applied two weeks of induction time. The regeneration capacity of younger calli is better. Increasing the callus induction phase leads to decreased plant regeneration (Varshney and Altpeter 2001). ‘Bobwhite26’ was one of the common tested cultivars and was found to have moderate regeneration by Fennell et al. (1995) while in our experiment it was one of the most responsible genotype with its 35% regeneration rate. As a result of the 20.6% average plant regeneration frequency and the high occurrence of well regenerated cultivars, the immature embryo based regeneration seems to be more efficient and less genotype dependent.

The callus induction rates were more variable in the mature scutellum culture than in the case of immature embryos. The average frequency was 84.3% with a range of 100% (‘Mv Emese’) and 35.5% (‘Mv Pálma’). About 60% of the cultivars had more than 90% of callus induction. The range of plant regeneration frequency was between 63% of ‘Fatima2’ and 0-0.5% of ‘Mv Palotás’ and ‘Mv Pálma’ (Figure 1c). Despite of the high maximum value of plant regeneration, the average rate was rather low (17.5%). The cultivars could be divided into 10 overlapping groups (Table 2, column mature scutellum). There were four cultivars (‘Fatima2’, ‘Mv Emese’, ‘Mv Regiment’, and ‘Mv Magdaléna’) which exerted more than 30% of plant regeneration (Figure 1c). The regeneration frequency of ‘Fatima2’ was significantly higher than that of any other cultivar ($p = 0.05$). The plant regeneration frequency of six cultivars was between 30% and 10%, whereas eight cultivars showed less than 10% of regeneration capacity. In our experiment, there was no significant difference in callus initiation between the tested cultivars, but pronounced variation has been observed in green plant regeneration The small impact of genotype on callus induction but its strong effect on plant regeneration was reported previously in the case of bread wheat and other *Triticeae*, (Özgen et. al 1998; Zale et al. 2004; Ruiming and Wang 2008). Contradiction in the genotype effect on callus induction was observed by Bi et al. (2007). Due to the significant genotypic variation in plant regeneration efficiency, lines with significantly higher plant regeneration ability could be identified (Zale et al. 2004). This kind of a cultivar was ‘Fatima2’. Based on these results, the mature scutellum based plant regeneration proved to be similarly efficient and genotype independent as immature embryo based regeneration, but much cheaper.

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Figure 2. Callus induction and plant regeneration from whole mature seed. After the callus induction the axis was removed to avoid of shoot germination. (bar: 1 cm)

The callus initiation was slow in the case of whole mature seed explants. After 5 days the average callus induction was 70.7%, but despite of the 17.3% average infection rate it reached an 94.2% average value when the calli were finally transferred onto the regeneration medium (Figure 2). The range of callus induction was between 100% of ‘Bobwhite26’ and ‘Chinese Spring’ and 21% of ‘Mv Pálma’. More than half (52.6%) of the wheat cultivars showed callus induction over 90%. In our experiment the callus induction time was 12 days. Filippov and co-workers (2006) found a positive effect of even longer period of callus induction, but our observations were opposite. The separation of cultivars based on the regeneration frequency was more distinct, than in the case of immature embryos and mature scutella (Table 2, column whole mature seed). Average plant regeneration frequency was 56.1%, ranging between 95% of ‘Bobwhite26’ and 8.5% of ‘Mv Pálma’ (Figure 1d). ‘Mv 16’ was the best regenerating winter wheat with 80% regeneration frequency. The majority of the cultivars (89.4%) had a regeneration capacity over 30%. One cultivar, ‘Fatima2’, showed moderate regeneration frequency (between 30-10%) and only one cultivar was below 10%. Although there was no significant correlation between callus induction and plant regeneration of the tested cultivars, ‘Mv Pálma’ was the poorest from both aspects. In studying durum wheat, high regeneration efficiency was also achieved in mature embryo culture, although the callus induction was poorer (Özgen et al. 1996). Testing 12 bread wheat cultivars, 16%, 34% and 50% showed excellent, good and poor regeneration, respectively (Özgen et al. 1998), these frequencies being lower than those reached in our experiments. The main problem with whole mature seed based regeneration system is the higher contamination rate, but in spite of this fact, this explant source seems to be the less genotype dependent and the plant regeneration is more efficient.

Comparing the four plant regeneration systems, the correlation between the haploid embryogenic structures or callus induction and plant regeneration was never significant (in anther r=0.04, immature embryo r=0.37, mature scutellum r=0.30, whole mature seed r=0.22). With the one exception of anther culture, the variation in the callus induction ability between the cultivars was smaller than the difference in plant regeneration. Testing androgen and somatic tissue culture response of F1 population, significant difference has been found using anther explant, but there was no variation in this character in the case of immature embryos (Agache et al. 1988). Similar observations were made in other wheat and barley experiments (Machii et al. 1998; Varshney and Altpeter 2001; Halámková et al. 2004). In our experiments there were no significant differences between the callus induction frequencies originating from immature embryo, mature scutellum or whole mature seed cultures.

In evaluating the possible associations between the regeneration ability of a given genotype in various tissue culture systems we found that there was no significant correlation between the plant regeneration rates in the four tested *in vitro* methods (anther-immature embryo r=-0.25, anther-mature scutellum r=0.29, anther-mature seed r=-0.20, immature embryo-mature scutellum r=0.17, immature embryo-mature seed r=0.16. mature scutellum-mature seed r=-0.08). It is confirmed by the result of other experiments studying the connection between the regeneration systems (Lazar et al. 1987; Machii et al. 1998; Özgen et al. 1998; Chauhan et al. 2007).

The lack of correlation between the different explant based regeneration methods could be explained by the different genetic regulations. Several chromosome regions were identified being responsible for callus induction or plant regeneration. One of the most known chromosome region is the 1BL/1RS wheat rye translocation (Agache et al. 1989). The cultivars having excellent regeneration ability in androgen regeneration system was found to carry the 1BL/1RS translocation (Varshney and Altpeter 2001). In our experiments a highly significant correlation was identified between the plant regeneration frequency from anther culture and 1BL/1RS translocation (r=0.64 at p=0.001 level), the presence of the translocation

resulting in excellent plant regeneration ability. In spite of this, not all the genotypes carrying the 1BL/1RS translocation produced good regeneration ability. Regeneration frequency of Mv Emese was significantly higher than the majority of tested cultivars, but not contains translocation. Moreover this is one of the best regenerated genotype in all system. In addition, the 1BL/1RS translocation had no impact on the other regeneration systems, or it practiced rather a slight negative effect on the regeneration from immature embryo (r=-0.32) although it did not reach the significant level. The impact of the rye genes or gene segments was found to be depended on the genetic background (Langridge et al. 1991) and on the donor rye genotype (Dobrovolskaya et al. 2003). The poor androgenic reaction of 'Mv Magdaléna', 'Mv Marsall', and 'Mv Csárdás' could be explaned by the lack of positive interaction between the genetic background and 1BL/1RS translocation. Not unambiguous is the positive effect of these chromosome regions on plant regeneration from immature embryo (Langridge et al. 1991; Machii et al. 1998; Varshney and Altpeter 2001). In our experiments, neither of five best regenerated cultivars from immature embryo explant have 1BL/1RS translocation. The 1BL/1RS translocation was introduced into the Hungarian breeding pool with the varieties 'Avrora', 'Kavkaz', 'Skorospelka 35' and 'Bezostaya 2' from 'Petkus' rye. (Bedő et al. 1993). In the previous studies and our experiment there is no information of the other chromosome regions which has a role on plant regeneration, and the regulation of plant regeneration from anther and from immature embryo are known as independent characters. The difference among the genetic composition of tested cultivars could explain the various effect of 1BL/1RS translocation in the four regeneration systems and the contradiction with the previously published results.

The characteristics of plant regeneration of the tested cultivars in the four *in vitro* systems were different (Figure 3). The range of the regeneration capacity was the widest in the case of whole mature seed (8.5-95%) coupled with the highest average value (56.1%). Plant regeneration of 90% of tested cultivars were over 30%, and 5-5% of cultivars were between 10-30% and under 10%. The second most efficient system was the immature embryo based regeneration where 25% of varieties showed excellent regeneration (over 30%), 30% good (between 10-30%) and 45% poor regeneration capacity under 10%. The mature scutellum based regeneration resulted in 44.4% excellent, 33.3% good and 22.2% poor regenerated cultivars. This distribution of regeneration capacity was in good agreement with previously published results (Fennell et al. 1995; Machii et al. 1998; Varshney and Altpeter 2001). Although the range of regeneration of the tested cultivars was wider in the case of mature seeds (0-63%) the average value was lower than in the case of immature embryos (0.9-59.4%). It was primarily due to the lower numbers of efficiently regenerable genotypes in this system. The anther based regeneration proved to be the least efficient method (Figure 3.). There was not a single cultivar with over 30% plant regeneration, 4% was good (between 30-10%), and the regeneration of the majority of genotype was under 10%.

In general, plant regeneration from whole mature seed was significantly the highest among the methods (at p=0.001 level). The continuous distributions of the plant regeneration frequencies of the varieties in all the four methods underline the quantitative character of plant regeneration.

The different genetic backgrounds behind the various *in vitro* reaction of a genotype suggest that the ideal regeneration system of a given genotype may be specific. Development of tightly linked molecular markers with these regions would make it possible to predict the *in vitro* reaction of the cultivars.

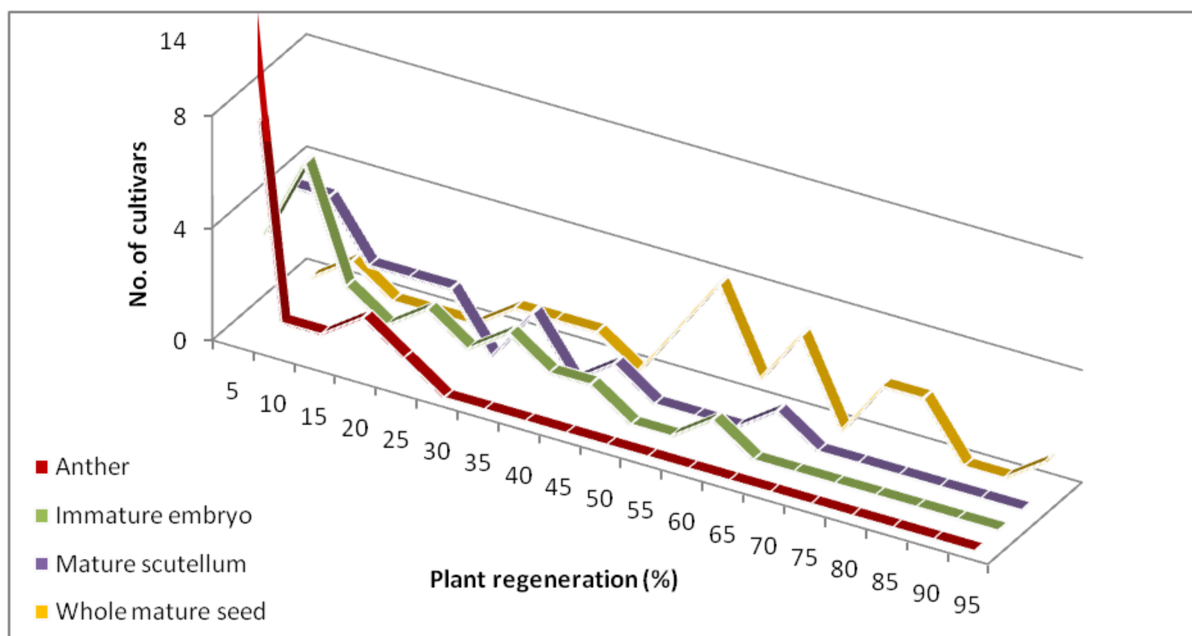

Figure 3: Plant regeneration frequency of 20 wheat cultivars from four different explants.

Based on our results the best genotype-regeneration combination for these cultivars was determined (Table 2). Ninety percent of the tested cultivars had higher than 30% regeneration capacity in at least one regeneration system. Based on the number of efficient regeneration systems, the tested cultivars could be divided into three different types. The first class was not the best one in any system, but had good or excellent regeneration capacity in at least three out of the four regeneration systems. 'Mv Regiment' and 'Mv Emese' were in this group. The regeneration ability of these cultivars was over 30% in the case of immature and mature scutellum and whole mature seed explants, but 'Mv Regiment' has poor regeneration from anther. The regeneration ability of 'Mv Emese' was significantly better than the majority of the tested cultivars in the androgenic regeneration system, too. The cultivars of the second group were the best one or had more than 30% regeneration frequency in one or two regeneration systems but had poor regeneration ability in the other systems. Three of them, 'Fatima2', 'Mv Béres' and 'Mv 16' were the best regenerated cultivars using anther explants, and the regeneration ability were over 30% using whole mature seed explant, expect of 'Fatima2' with excellent regeneration in mature scutellum. 'Bobwhite26', 'Chinese Spring' and 'Mv Mambo' had excellent regeneration capacity in the case of immature embryo and whole mature seed explants. 'Mv Magdaléna' was also in the second group. It has high regeneration frequency in mature scutellum and whole mature seed based regeneration systems.

The cultivars in the third group were excellent only in one system and had poor regeneration ability in the other systems. The regeneration ability of 'Mv Pálma' was the best in the androgenic system. Further nine cultivars from this group had excellent regeneration ability in the case of whole mature seed explant. 'Mv Kolo' was the only one which has poor regeneration in all the tested systems.

In summary, we identified the optimal regeneration system for 20 bread wheat cultivars, and determined three different reaction types. Two of tested cultivars, 'Mv Regiment' and 'Mv Emese,' proved to be the most versatile. The plant regeneration of these genotypes was over 30% in three regeneration systems. Further, seven cultivars had excellent *in vitro* performance from two different explants, and 10 (50% of cultivars) showed more than 30% plant regeneration only in one regeneration system. Regarding the efficiency of the tested systems, plant regeneration from whole mature seed proved to be the most efficient and

least genotype dependent. These results suggest that the range of genotype could be broadened for the transformation experiments by identifying the ideal *in vitro* regeneration system of a cultivar in question, but the increase of transformation efficiency might be achieved by using versatile genotypes.

Table 2. The regeneration ability of 20 wheat cultivars in four different culture systems

Group	**Cultivar**	**Efficient culture system**	**Anther**		**Immature embryo**		**Mature scutellum**		**Whole mature seed**	
1	Mv Regiment	+++	2.1	d	59.4	a	34.0	bc	62.5	bcde
1	Mv Emese	+++	8.4	c	31.1	cd	40.5	b	64.9	bcde
2	Fatima2	++	17.7	a	9.8	hij	63.0	a	29.0	h
2	Mv Béres	++	20.5	a	13.3	fgh	13.3	efghi	72.5	bc
2	Mv 16	++	13.5	b	13.0	ghi	23.6	def	80.0	b
2	Bobwhite26	++	0.9	d	35.5	bcd	NT		95.0	a
2	Mv Mambo	++	0.2	d	39,1	bc	10.5	fghij	54.0	bcde
2	Chinese Spring	++	3.6	d	41.8	b	18.5	efg	48.5	efg
2	Mv Magdaléna	++	1.0	d	7.1	hij	32.5	bcd	54.0	ef
3	Mv Pálma	+	19.3	a	9.8	hij	0.5	j	8.5	i
3	Mv Palotás	+	0.3	d	25.2	de	0.0	j	76.0	b
3	Mv Csárdás	+	1.5	d	23.3	de	24.0	cde	36.5	fgh
3	Mv Marsall	+	4.6	d	9.5	hij	7.5	ghij	74.0	bcd
3	Mv Magvas	+	0.1	d	6.5	hij	6.0	hij	63.0	bcde
3	Mv Hombár	+	3.7	d	23.6	ef	3.5	ij	50.0	efg
3	Mv Suba	+	1.8	d	19.0	efg	4.8	hij	55.0	def
3	Mv Süveges	+	NT		2.5	ij	16.8	efgh	55.5	cdef
3	Mv Toborzó	+	2.5	d	0.9	j	8.4	ghij	51.5	efg
3	Cadenza	+	0.8	d	8.0	hij	7.0	ghij	35.0	gh
	Mv Kolo	-	1.6	d	4.5	hij	NT		NT	
	Averages		**5.5**		**20.6**		**17.5**		**56.1**	

Efficient culture system means over 30% regeneration frequency(in case of anther culture over 10%). Different letters indicate significantly (P>5%) different regeneration ability within the same column. NT, not tested. Cultivars containing 1BL/1RS translocation are underlined. Entries within the same column followed by the same/different letter(s) are not significantly different from each other.

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