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Genetic transformation of an elite commercial wheat variety through *Agrobacterium tumefaciens*

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Abstract

Wheat (*Triticum aestivum* L.) is the leading food grain crop of Pakistan that contributes 2.7 percent to the GDP. However, the crop is significantly affected by *Barley yellow dwarf virus* (BYDV). To combat with, introduction of virus-derived gene(s), through genetic engineering, is a powerful tool to confer resistance. Wheat is considered recalcitrant to be transformed genetically. The success regarding genetic transformation is almost limited to the cv. Bobwhite. Bobwhite, however, is not an isogenic line and may be used as a transgenic crossing material. Crossing for a transgenic trait from Bobwhite may be complicated by a linkage drag. Alternatively, targeting elite variety for transformation will be more economical. Considering transformation of an elite variety "Sehar-2006" via *Agrobacterium tumefaciens* (LBA4404), optimization of protocol harboring binary plasmid construct (pING71/BYDV-CP19) with *npt*II marker gene has been discussed.

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Introduction

Barley yellow dwarf virus (genus *Luteovirus*) is an economically important plant virus infecting cereals (Ali *et al.*, 2014; Ali *et al.*, 2013). Globally, natural-yield losses attributable to BYDV range between 11 and 33 % (Wang and Zhou, 2003) that have been intensified up to 86 % in some areas (Wu *et al.*, 2011). BYDV-PAV, a type-species, is a widespread isolate/serotype causing losses of 17 % in wheat, 15 % in barley, and 25 % in oats, worldwide (Dupré *et al.*, 2002). The disease outbreaks have been witnessed in USA (Domier, 2009), China (Wang and Zhou, 2003), Poland (Trzmiel, 2006), Spain (Achon *et al.*, 2006), and the Czech Republic (Kundu *et al.*, 2009). True resistance against the virus has not been found in the primary and secondary gene pools of wheat. To deal with, transgenic resistance may be one of the appropriate virus-control methods, although wheat transformation is lagging behind by several factors.

Wheat, being food for over 35 % of the world population (Khurana *et al.*, 2009), is the second largest crop that covers approx. 17 % of the world's cultivable land (Atchison *et al.*, 2010; Jones *et al.*, 2005). In Pakistan, it contributes 2.7 % to the GDP. A commercial elite variety Sehar-2006 covers maximum area, in Pakistan (Hussain *et al.*, 2013). Wheat is considered recalcitrant to *in vitro* culture and regeneration into whole plants (Pérez-Piñeiro *et al.*, 2012) that contribute to its low genetic transformation efficiency. Consequently, genetic transformation of wheat is limited to either Chinese Spring or Bobwhite (Janakiraman *et al.*, 2002; Zhao *et al.*, 2013). Bobwhite, however, is not an isogenic variety. It is a heterogenous set of distinct breeding lines (Jones, 2015), a generic name referring to all sister lines – demonstrating great variability for agronomic traits – from the cross 'Aurora'//'Kalyan'/'Bluebird 3'/'Woodpecker'. Bobwhite, spring wheat, was produced in the early 1970s, by CIMMYT bread wheat program (Warburton *et al.*, 2002). Pellegrineschi *et al.* (2002) identified 'SH 98 26' as super-transformable Bobwhite line with over 60 % transformation efficiency. Alternatively, several Bobwhite lines have successfully been utilized in transgenic wheat programs, because of their excellent regeneration ability (Weeks *et al.*, 1993). Backcrossing for the transgenic traits from Bobwhite, unadapt germplasm, into elite cultivars could be complicated by linkage drag.

Contrary to the Bobwhite, transformation of commercial elite varieties is limited and ranged between 0.2 to 2 % only (Varshney and Altpeter, 2002). This study thus focuses on targeting a commercial wheat variety to optimize genetic transformation protocol via *A. tumefaciens*. A plasmid modified with coat protein gene of BYDV-PAV (a plasmid construct for transgenic resistance), was used to genetically engineer an elite variety.

Materials and methods

Virus source, amplification and cloning

To amplify BYDV coat protein gene (ORF3), wheat plant sample (M19) naturally infected with BYDV (leaf yellowing symptoms) was collected from Islamabad, and total RNA was extracted using TRIzol reagent (Thermoscientific). RT-PCR was used to amplify coat protein (600 bp) from the plant using CP(+)(Liu *et al.*, 2007) with *kpn*I site as a forward primer and Lu4 (Robertson *et al.*, 1991) with *pst*I restriction site as a reverse primer. The amplified product was cloned into pTZ57R/T (Thermoscientific) and confirmed through restriction digestion and sequencing in both orientations from Macrogen (Seoul, Korea).

Binary construct and Agrobacterium

To create binary construct, the CP gene was sub-cloned under CsVMV (*Cassava vein mosaic virus*) promoter into a binary plasmid vector pING71 (with *Pst*I and *Kpn*I restriction sites). The sequence was confirmed with restriction digestion and sequencing. The binary vector pING71 has neomycin phosphotransferase gene II (*npt* II) as a selectable marker (Fig. 1.). *Agrobacterium tumefaciens* cells LBA4404 transformed with the binary constructs were used for plant transformation.

Explant selection

Wheat commercial elite cultivar, Sehar-2006, was

grown under greenhouse conditions at 24 to 28 °C. Fifteen days after heading stage, spikes were harvested (7-14 days post-anthesis) and sterilized. Briefly, spikelets were removed from the spikes. The embryos were excised after rinsing spikelets with 70 % ethanol for 5 min and 20 % store-bought bleach for 20 min.

Co-cultivation

Sterile immature embryos (IEs) were excised and collected in 1.5 ml sterile Eppendorf tubes (25 embryos per tube) having co-cultivation liquid media. Excess media was removed and the embryos were incubated at 43 °C / 3 min followed by 25 °C / 2 min. 1 ml of bacterial culture (MGL media; OD_{550} = 0.4) was added and incubated for 15 min at RT. The embryos were then transferred onto a semisolid co-cultivation media plate and stored in dark for three days at RT.

Regeneration

After three days, IE were transferred to callus induction media with their scutellum placed up on the media following Singh *et al.* (2012). After two weeks on callus induction media, the calluses were transferred to regeneration transition media. The shoot bearing calluses were then transferred to rooting media (either composition 1 or 2; Table 1). The plantlets bearing roots were then shifted to the soil for hardening. For confirmation of transgene, leaf samples were collected and total DNA was isolated using DNA isolation kit (Thermoscientific). For amplification of transgene 100 to 200 ng DNA was used with the same primer pair, CP(+) and Lu4, described above.

Results and discussion

Barley yellow dwarf virus is an economically important phytopathogen that claims high yield losses in wheat. Development of resistant genotypes, through genetic engineering, is one of the most appropriate control method. However, wheat is known as a recalcitrant plant to be transformed efficiently. Factors influencing successful *Agrobacterium*-mediated wheat transformation has been frequently investigated by many researchers, to achieve high transformation efficiency. Furthermore, transforming commercial wheat, rather than model genotype such as cv. Bobwhite, has much potential for market to explore. The present study focuses on the *Agrobacterium*-mediated transformation of a commercial wheat variety.

Table 1. Media composition for *Agrobacterium* mediated wheat transformation.

Medium Type	Composition 1 (1 litre)	Composition 2 (1 litre)
MGL Media (pH 7.5)	2.5 g Yeast Extract, 5 g Tryptone, 5 g NaCl, 5 g Mannitol, 1.16 g Monosodium Glutamate, 0.25 g KH_2PO_4, 0.1 g $MgSO_4$ $7H_20$, 1 µg Biotin	Same as composition 1
Co-cultivation Media (pH 5.8)	4.3 g MS salts, 30 g Maltose, 1 g Casein Hydrolysate, 0.30 g Myo-inositol, 0.65 g Proline, 1 mg Thiamine HCl, 2.5 mg Dicamba, 2 g Gellan Gum	Same as composition 1
Callus Induction Media (pH 5.8)	4.3 g MS salts, 30 g Maltose, 0.10 g Myo-inositol, 1 mg Thiamine HCl, 2 mg 2,4-D, 50 mg Kanamycin, 300 mg Timentin, 2 g Gellan Gum, 0.5 mg Nicotinic Acid, 0.5 mg Pyridoxine HCl	4.3 g MS salts, 20 g Maltose, 0.75 g Glutamine, 0.1 g Myo-inositol, 1 mg Thiamine HCl, 2.5 mg 2,4-D, 50 mg Kanamycin, 300 mg Timentin, 2 g Gellan Gum, 0.165 g $CuSo_4$, 0.1 mg BAP
Regeneration Media (pH 5.8)	4.3 g MS salts, 30 g Maltose, 1 mg Thiamine HCl, 0.16 mg $CuSo_4$, 1 mg BAP, 300 mg Timentin, 50 mg Kanamycin, 2 g Gellan Gum, 0.5 mg Pyridoxine HCl, 0.5 mg Nicotinic Acid, 1 mg IAA	4.3 g MS salts, 30 g Maltose, 1 mg Thiamine HCl, 0.16 mg $CuSo_4$, 1 mg BAP, 300 mg Timentin, 50 mg Kanamycin, 2 g Gellan Gum, 1 g Casein Hydrolysate, 0.25 g Myo-inositol, 0.69 g Proline, 2.5 mg 2,4-D
Rooting Media (pH 5.8)	4.3 g MS salts, 30 g Sucrose, 1 mg Thiamine HCl, 2 g Gellan Gum, 0.5 mg Pyridoxine HCl, 0.5 mg Nicotinic Acid, 0.16 mg $CuSo_4$, 1 mg IAA, 1mg IBA, 1 mg NAA	2.7 g MS salts, 20 g Maltose, 2.5 mg Thiamine HCl, 2 g Gellan Gum, 0.1 g Myo-inositol, 0.75 g Glutamine, 300 mg Timentin, 50 mg Kanamycin

Initially, coat protein (CP) gene was amplified and cloned into pTZ57R/T. Sequence analysis showed that the sequence determined to be 600 bp, as complete CP, was an isolate of BYDV-PAV (Data not shown). The sequence was submitted to the GeneBank [accession number: KT198976]. For the development of plasmid construct, coat protein gene (600 bp) of BYDV was sub-cloned into binary vector pING71 using *pst*I and *kpn*I restriction endonuclease sites. The binary construct (pING71/BYDV-CP19) was verified with restriction digestion and DNA sequencing and was used to transform *Agrobacterium tumefaciens* (LBA4404). A single colony harboring the binary construct was used for wheat transformation.

Table 2. Procedure, duration and developmental stages of the transgenic plants from immature embryos.

Experiment Components	Co-cultivation medium	Callus Induction medium	Regeneration medium	Rooting medium
	48 hours (dark)	*2-4 weeks* (dark)	*2-4 weeks* (light)	*2 weeks* (light)
+ pING71/BYDV-CP + Kanamycin	Growth of Agro. and IEs	Callus formation	Green shoots develop	Roots develop
- pING71/BYDV-CP + Kanamycin	-do-	Callus formation	White shoots emerge	Roots develop
+ pING71/BYDV-CP - Kanamycin	-do-	Callus formation	Green shoots develop	Roots develop
+ pING71 + Kanamycin	-do-	Callus formation	Green shoots develop	Roots develop

'+' sign represents the presence while '-' sign represents the absence of a component/antibiotic.

Healthy wheat plants grown in the greenhouse were used for collection of spikes at 7 to 14 days post-anthesis. Spikelets were separated and sterilized. IEs were then excised in a sterile condition. The IEs were incubated at 43 °C / 3 min followed by 25 °C / 2 min is known to increase transformation efficiency (Gurel *et al.*, 2009).

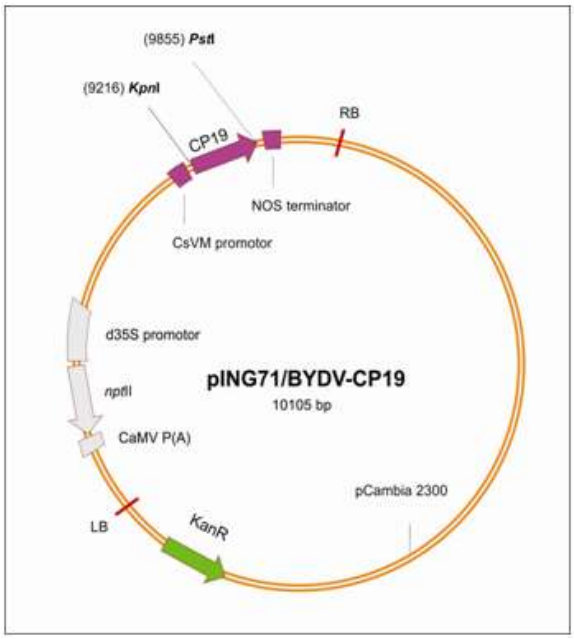

Fig. 1. Map of pING71/BYDV-CP19 construct. BYDV CP gene has been cloned at *kpn*I and *pst*I sites under the promoter (CsVM) of Cassava vein mosaic virus.

Two media compositions (Table 1) were compared to regenerate the wheat variety effectively.

Since transgenic plants are phenotypically indistinguishable from non-transgenic plants, the binary vector (pING71) contained *npt*II gene as a selectable marker within the expression cassette, the antibiotic kanamycin was used to select the transgenic plants. Upon selection on media supplemented with kanamycin, non-transformed explants bleached (i.e. turn white), while the transformed calli bear dark green shoots (Table 2). The antibiotic marker gene (*npt*II) used as a selectable marker for wheat transformation, has previously been employed by several investigators (Ding *et al.*, 2009).

IEs were cultured on media without any antibiotic, in order to know the response of explants to the media, which lead to their successful proliferation (Fig. 2.). IEs were also cultured on antibiotic (kanamycin) supplemented media, to know the response of explants to the antibiotic selection. Calluses were produced with no difference from the calluses cultured without any antibiotic. However, the calluses

on selection media produce white (discolored/bleached) shoots (Fig. 3.). Whereas, calluses with no antibiotics give rise to green shoots (Fig. 2.). IEs after co-cultivation with binary construct *Agrobacterium* were transferred to callus induction media supplemented with 50 mg l^{-1} kanamycin (Fig. 4A.). The IEs transformed, with disarmed *A. tumefaciens* LBA4404 having either binary plasmid vector or construct, start producing calli on the antibiotic supplemented callus induction media (Fig. 4B.). Formation of embryogenic calli (emergence of shoots) starts within the first two weeks on regeneration media followed by shoot sprouting (Fig. 4C.). The plantlets produced were transferred to rooting media. Roots were produced within two to four weeks (Fig. 4D.). The plantlets bearing roots were shifted to the pots containing soil in the growth room with 26 ^{o}C temperature and 16/8 hours photoperiod. The plants appeared to complete hardening within the two weeks in the glass house.

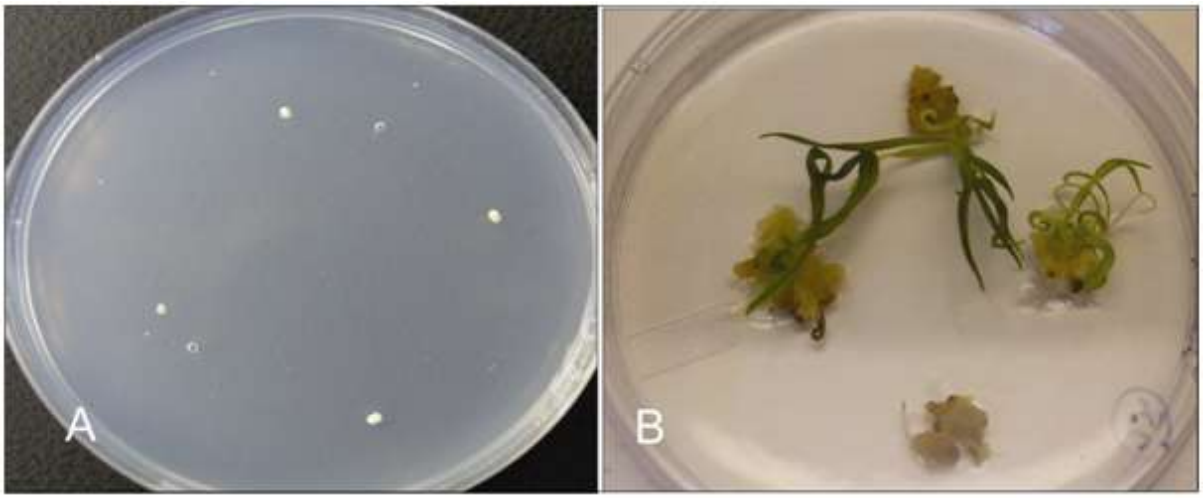

Fig. 2. Response of non-transformed explants to regeneration media without antibiotic. **A**) IEs placed on callus induction media, **B**) shoots proliferate from the successfully grown calli shooting transition media.

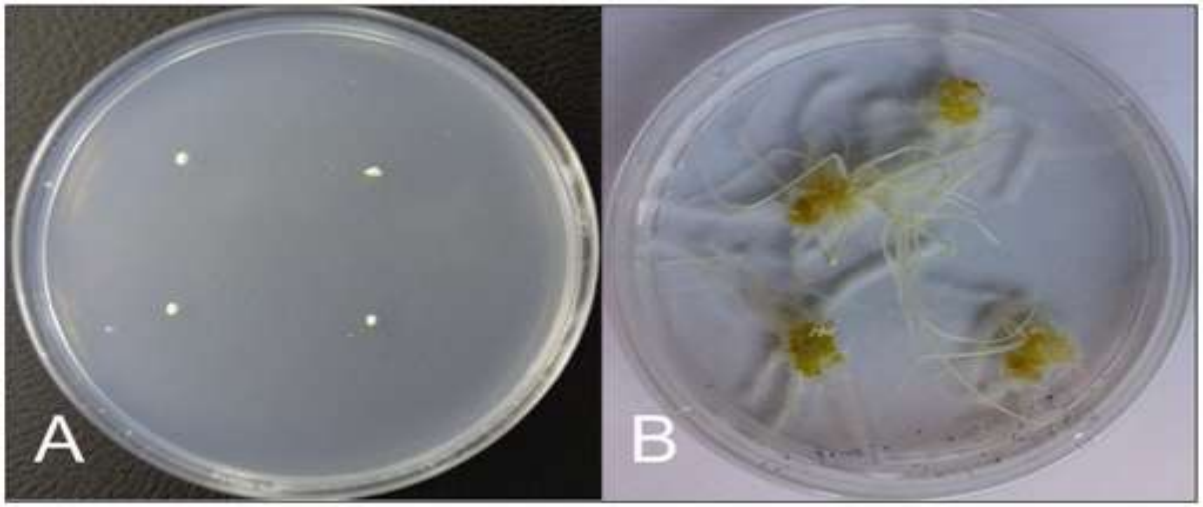

Fig. 3. Response of non-transformed explants to antibiotic kanamycin. **A**) IEs placed on kanamycin added media, **B**) White shoots emerge from the non-transformed calli on kanamycin added regeneration media.

IEs, as an explant, is considered as an ideal stage for wheat transformation (Jones, 2015; Pellegrineschi *et al.*, 2002; Varshney and Altpeter, 2002; Weeks *et al.*, 1993). The IEs exhibited swelling followed by callus initiation. Callus induction media in composition 1 showed better callus formation that contained 2 mg l^{-1} 2,4-D. Moghaieb *et al.* (2010) suggested that callus formation was optimum when the use of growth

regulator (2,4-D) was in the range of 1-2 mg l^{-1}. Our results are in congruence with the earlier report. The success of wheat transformation is dependent on regeneration of the genotype. Explants derived calli sub-cultured on regeneration media developed into embryogenic calli. The nodular structures were then differentiated into adventitious shoots. Comparison of the two different regeneration media showed that the composition 1 was good enough to produce transgenic plantlets. Green healthy shoots transferred to rooting media given in composition 1 initiate rooting more efficiently. The seedlings were hardened and shifted to the green house for seed production (Fig. 4E.). PCR amplification also confirmed the transgenic status. Amplification was visualized (Fig. 4F.) for the transgenic plants selected on the media, using BYDV CP gene specific primers.

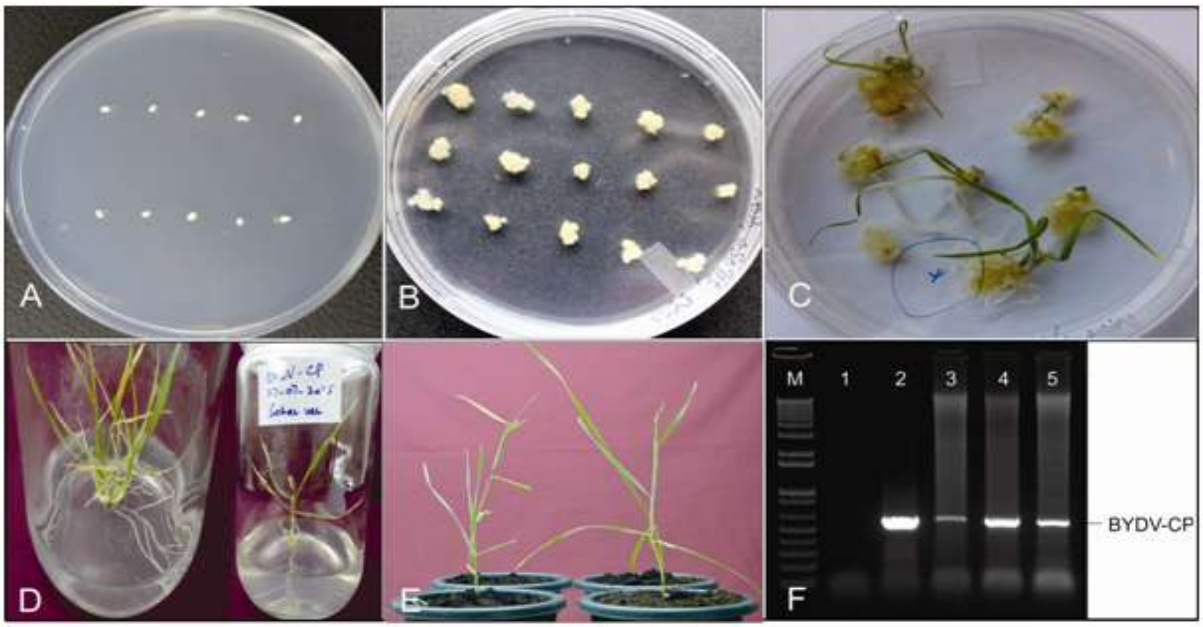

Fig. 4. Genetic transformation of wheat immature embryos. **A**) immature embryos on co-cultivation media, **B**) Callus induction, **C**) regeneration of calli, **D**) rooting transition, E) Hardening of the plantlets, and F) confirmation of transgene through PCR (well M shows 1 kb marker, 1 shows negative control, 2 shows positive control, 3-4 are amplifications from transgenic plants).

Genetic transformation of wheat is limited may be due to the ploidy level or otherwise to cv. Bobwhite. This study describes a procedure for efficient transformation of a commercial elite variety "Sehar-2006" to express coat protein (CP) gene of *Barley yellow dwarf virus*, using *Agrobacterium tumefaciens*.

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Authors' Contribution

MA conducted the research. MT and SH supervised the research. All authors endorsed the manuscript.

References

Achon MA, Serrano L, Ratti C, Rubies-Autonell C. 2006. First detection of *wheat dwarf virus* in barley in Spain associated with an outbreak of Barley Yellow Dwarf. Plant Disease **90,** 970-970. http://dx.doi.org/10.1094/PD-90-0970A.

Ali M, Hameed S, Tahir M. 2014. Luteovirus: insights into pathogenicity. Archives of Virology **159,** 2853-2860. http://dx.doi.org/10.1007/s00705-014-2172-6.

Ali M, Tahir M, Hameed S, Ashraf M. 2013. Coat protein based molecular characterization of Barley yellow dwarf virus isolates identified on oat plants in Pakistan. Acta Virol **57,** 383-5. http://dx.doi.org/ 10.4149/av_2013_03_383.

Atchison J, Head L, Gates A. 2010. Wheat as food, wheat as industrial substance; comparative geographies of transformation and mobility. Geoforum **41,** 236-246. http://dx.doi.org/ 10.1016/j.geoforum.2009.09.006.

Ding L, Li S, Gao J, Wang Y, Yang G, He G. 2009. Optimization of Agrobacterium-mediated transformation conditions in mature embryos of elite wheat. Molecular Biology Reports **36,** 29-36. DOI: http://dx.doi.org/10.1007/s11033-007-9148-5.

Domier LL. 2009. Barley Yellow Dwarf Viruses. In Desk Encyclopedia of Plant and Fungal Virology, B. W. J. Mahy and M. H. V. V. Regenmortel, eds. (Elsevier), 279-285 P.

Dupré P, Henry M, Posadas G, Pellegrineschi A, Trottet M, Jacquot E. 2002. Genetically engineered wheat for *Barley yellow dwarf virus* resistance. Barley Yellow Dwarf Disease: Recent Advances and Future Strategies, 27.

Gurel S, Gurel E, Kaur R, Wong J, Meng L, Tan HQ, Lemaux PG. 2009. Efficient, reproducible *Agrobacterium*-mediated transformation of sorghum using heat treatment of immature embryos. Plant Cell Rep **28,** 429-44. http://dx.doi.org/10.1007/s00299-008-0655-1.

Hussain M, Rafiq M, Akhtar L, Tariq A, Ahmad S, Aslam M, Nadeem M, Zubair M. 2013. Release of high yielding wheat variety AaS-2011 resistant to stem rust (UG-99) in Pakistan. The Journal of Animal and Plant Sciences **23,** 1115-1124

Janakiraman V, Steinau M, McCoy SB, Trick HN. 2002. Recent advances in wheat transformation. In Vitro Cellular & Developmental Biology. Plant **38,** 404-414. http://dx.doi.org/ 10.2307/23321149.

Jones HD. 2015. Wheat Biotechnology: Current Status and Future Prospects. In Recent Advancements in Gene Expression and Enabling Technologies in Crop Plants (Springer), pp. 263-290. http://dx.doi.org/10.1007/978-1-4939-2202-4.

Jones HD, Doherty A, Wu H. 2005. Review of methodologies and a protocol for the Agrobacterium-mediated transformation of wheat. Plant Methods **1**, 229-232.

Khurana P, Chauhan H, Desai SA. 2009. Wheat. In Compendium of Transgenic Crop Plants (John Wiley & Sons, Ltd). http://dx.doi.org/10.1002/9781405181099.k0103.

Kundu J, Jarošová J, Gadiou S, Cervená G. 2009. Discrimination of three BYDV species by one-step RT-PCR-RFLP and sequence based methods in cereal plants from the Czech Republic. Cereal Research Communications **37,** 541-550.

Liu F, Wang X, Liu Y, Xie J, Gray S, Zhou G, Gao B. 2007. A Chinese isolate of barley yellow dwarf virus-PAV represents a third distinct species within the PAV serotype. Archives of Virology **152,** 1365-1373. http://dx.doi.org/10.1007/s00705-007-0947-8.

Moghaieb RE, El-Arabi NI, Momtaz OA, Youssef SS, Soliman MH. 2010. Genetic transformation of mature embryos of bread (*T. aestivum*) and pasta (*T. durum*) wheat genotypes. GM Crops **1,** 87-93. http://dx.doi.org/10.4161/gmcr.1.2.11172.

Pellegrineschi A, Noguera LM, Skovmand B, Brito RM, Velazquez L, Salgado MM, Hernandez R, Warburton M, Hoisington D. 2002. Identification of highly transformable wheat genotypes for mass production of fertile transgenic plants. Genome **45**, 421-430.

Pérez-Piñeiro P, Gago J, Landín M, Gallego PP. 2012. Agrobacterium-mediated transformation of wheat: general overview and new approaches to model and identify the key factors involved. Transgenic Plants-Advances and Limitations. Rijeka, Croatia: Intech Open Access Publisher, 3-26.

Robertson NL, French R, Gray SM. 1991. Use of group-specific primers and the polymerase chain reaction for the detection and identification of luteoviruses. Journal of General Virology **72,** 1473-1477.

Singh S, Tan H, Singh J. 2012. Mutagenesis of barley malting quality QTLs with Ds transposons. Functional & Integrative Genomics **12,** 131-141. http://dx.doi.org/10.1007/s10142-011-0258-8.

Trzmiel K. 2006. Barley yellow dwarf virus-MAV (BYDV-MAV) and Barley yellow dwarf virus-PAV (BYDV-PAV) on maize in Poland. Phytopathologia Polonica, 53-56.

Varshney A, Altpeter F. 2002. Stable transformation and tissue culture response in current European winter wheats (*Triticum aestivum* L.). Molecular breeding **8,** 295-309. http://dx.doi.org/10.1023/A:1015240901016.

Wang X, Zhou G. 2003. Identification of a protein associated with circulative transmission of Barley yellow dwarf virus from cereal aphids, Schizaphis graminum and Sitobion avenae. Chinese Science Bulletin **48**, 2083-2087.

Wang X, Zhou G. 2003. Identification of a protein associated with circulative transmission of Barley yellow dwarf virus from cereal aphids, Schizaphis graminum and Sitobion avenae. Chinese Science Bulletin **48,** 2083-2087.

Warburton M, Skovmand B, Mujeeb-Kazi A. 2002. The molecular genetic characterization of the 'Bobwhite' bread wheat family using AFLPs and the effect of the T1BL. 1RS translocation. Theoretical and Applied Genetics **104,** 868-873.

Weeks JT, Anderson OD, Blechl AE. 1993. Rapid production of multiple independent lines of fertile transgenic wheat (Triticum aestivum). Plant Physiology **102,** 1077-1084.

Wu B, Blanchard-Letort A, Liu Y, Zhou G, Wang X, Elena SF. 2011. Dynamics of molecular evolution and phylogeography of *Barley yellow dwarf virus*-PAV. PloS One **6,** e16896. http://dx.doi.org/10.1371/journal.pone.0016896.

Zhao X-Q, Nie X-L, Xiao X-G. 2013. Over-expression of a tobacco nitrate reductase gene in wheat (Triticum aestivum L.) increases seed protein content and weight without augmenting nitrogen supplying. PloS one. http://dx.doi.org/10.1371/journal.pone.0074678.