Function of the genetic element 'Mona' associated with fungicide resistance in *Monilinia fructicola*

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SUMMARY

[illegible]

Keywords: [illegible] *MfCYP51* [illegible] *Monilinia fructicola*, [illegible]

INTRODUCTION

[illegible]

. s, 2001), 126- 199- s. t *Penicillium digitatum* (G s *et al.*, 2007; H t *et al.*, 2000), 1.3- t- t s s *Botrytis cinerea* (. ts *et al.*, 2009), 2- t 5- t . t. I E- . . t t s s *Blumeriella jaapii* (*et al.*, 2006), 120- . *Mycosphaerella graminicola* (C s *et al.*, 2012) 1.8- A t 1 t s s t . t . . . s *Aspergillus fumigatus* (A *et al.*, 2011). . . . st . . t t t t . E s ss t. t . . s s . . . 199- t s s s. . . . *P. digitatum* t. . st . *PdCYP51B* ss t. t D I . . s s . s . . . t . I. E- I E . . . t, . s t. s . E1. A . 20- s. . . . E1 s . . . t . . ss. t t. t . t s. s. . s . . t . . . ts (. *et al.*, 2013). . . . ., t . t . . t t s s - . . . . t t . t. . s s . (. st 65) s . . . . . . s . , . . t . s s . st t . ' . . - . t *M. fructicola*, . s. ' s ss t. t t . . . - . . ss *MfCYP51* D I . . s s . *M. fructicola* (. . , 2008).

H. . , . . st t. t . . t t . ' . . . t t . s. . s . . t t s t s. . . . . s. t. t s st. s . t t . . s t s s - . . . . ts . t . s. . . . s. ts s . . s. . t . . . . . t s . '- s. . t t . . t. t D I . s s . t . . t. . .

RESULTS

Promoter activity of the 'Mona' element

. . 65- ' s. . . . s t. t t . . . . (. F) t . *Npt2* . . t . . t . . t BHt2 t . . t. - . . (F . . 1, s. . . . t I t). . . . F t . *Npt2* . . s s . t . t ' t BHt2 (As

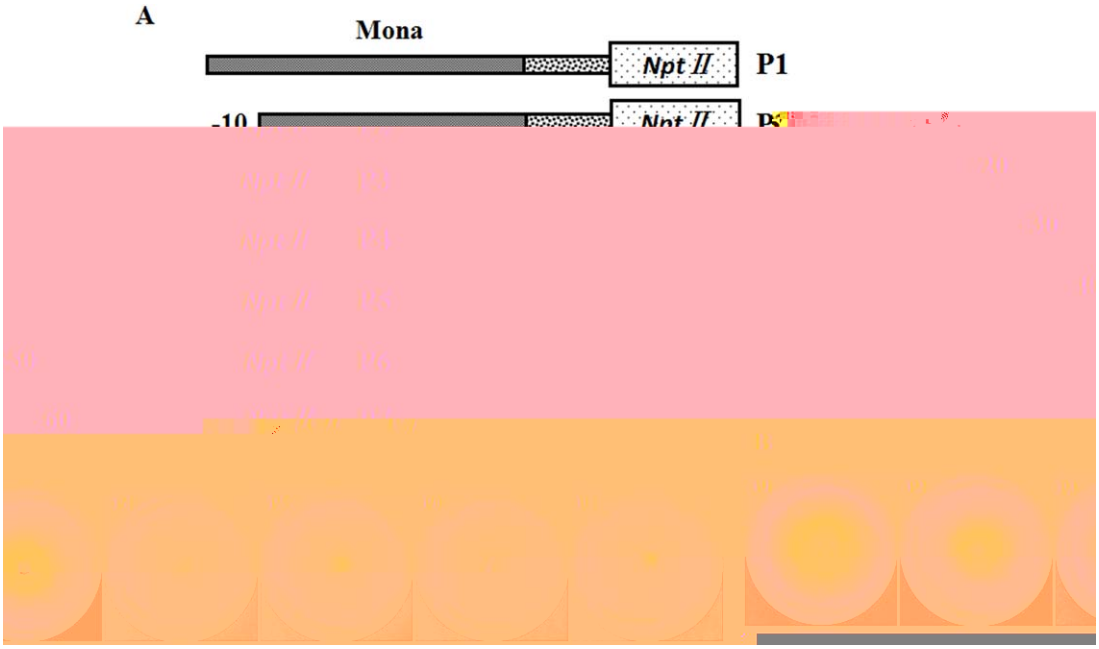

Fig. 2 Schematic diagram of truncated 'Mona' elements and the impact on *Npt2* gene expression in transformants. (A) P1 represents pMona-NPT and P2–P7 indicate the different vectors with truncated 'Mona'. (B) The activity of transformants from pMona-NPT and vectors with truncated 'Mona' was assessed on G418-amended potato dextrose agar (PDA) medium.

t % . (F . 3E). . EC_{50} .% . s % . t . t % s % ts .% . , t, . .%, . . t % t . .% . . s %st. B 7 (0.216 µ /). . EC_{50} .% . s t . t % s % ts . .%t. % . 0.005 t 0.100 µ / , t % .% .% . 0.045 µ / (% . 1).

.%-t . C %s . . t . t. . t . . ss . . - . s t . *MfCYP51* . . t . t t % s % ts. . . s. ts s . t %st t . . ss t . *MfCYP51* . . t % s- % ts %s . t % t %st B 7 (% . 1). . . ss t . *MfCYP51* . . %s st . .%t. t t . EC_{50} .% . s t . D I . . % . ($P < 0.01$, $r = 0.957$). . . %s s % t . . . t . . t . t % s % ts % % . t% s %st. t. s . % t %st. , s .%t % % t t . s s .t% . t (% . 1).

Insertion of the 'Mona' element into the upstream region of MfCYP51

. . . t . . . t %' %s s. t. t t . . st .% . t *MfCYP51* . . D I s. s t . . (D I-) s %st. HG3 (F . 4, s. . . t I %st). . . t % s % ts . . t% . t . s. t s . . . C (F . 5, s. . t I %st). Att. ts t t% t % s % ts - . . s t . t % s . . s % . . s t. s. . .% . s s . s . s %st s (F . 5E).

. EC_{50} .% . s t % . . . t . t % s- % ts t t . s. t. %' . . t t % t . s. s t . . t% s %st. HG3 (% . 2). *MfCYP51* . . . ss %s . % t . t % s % ts t% t . s. t. %' . - . t % . t t . t% s %st. HG3 (% . 2). H . . , t . . ss t . *MfCYP51* . . %s . %st. . - %st. t t . EC_{50} .% . s t t . D I . . % . ($P < 0.05$, $r = 0.598$). s % t . . . %s s. . . t . . t % s % ts t %' % t . % . t% s %st. . % t , s %st % t .% t t . %st. . s s t% . t (% . 2).

DISCUSSION

. . . . . t . Es . % t . s s t% . t . . s %s . . . t. s . . % % t % . % . % %st . s (A % % *et al.*, 2011; C s *et al.*, 2012; G s *et al.*, 2007; H % % t *et al.*, 2000; . ts . *et al.*, 2009; % *et al.*, 2006; . % . % . s, 2001). . s s t% . s % % s %ss %st. t t . . . . ss . . t% . t . . s st .% t . s. t % s s s. H . . , t . t . . . . t . . . . t t. s. . . . s . . . . ss s t. % . . . 65- . t . . . t %', %st. st .% *MfCYP51*, s %ss %st. t D I . . s s t% . . (. % . t t. . t , . s 2.2, tt ://. . t . /s. t s/ t. . t) . t. t %st t . %' . . t %s % t. , t %s % . I t s st , . % . s t %t %' . . s t . . . ss t . *Npt2* . . % t %st t % ts, . .%, %s % t. . . . % . t.

Table 1 EC_{50} values, relative expression of the *MfCYP51* gene, mycelial growth, sporulation and lesion development of knockout transformants.

			Fitness parameter		
Isolate/transformant*	EC_{50} (μg/mL)	Relative expression	Mycelial growth (cm/day)	Sporulation (10^6/cm^2)	Lesion size (cm)
Bmpc7	0.21	1	2.0 ± 0.6a	4.5 ± 0.6a	5.6 ± 0.6a
ΔBmpc-1	0.01	0.3 ± 0.1	1.9 ± 0.6a	3.9 ± 0.4a	5.9 ± 0.1a
ΔBmpc-2	0.07	0.4 ± 0.1	1.9 ± 0.8a	2.6 ± 0.5a	6.0 ± 0.3a
ΔBmpc-3	0.05	0.3 ± 0.1	2.0 ± 0.7a	3.6 ± 0.8a	5.7 ± 0.3a
ΔBmpc-4	0.01	0.2 ± 0.1	1.8 ± 0.7a	3.0 ± 1.4a	6.1 ± 0.2a
ΔBmpc-5	0.02	0.2 ± 0.1	1.8 ± 0.5a	3.0 ± 1.7a	5.9 ± 0.5a
ΔBmpc-6	0.09	0.5 ± 0.1	1.9 ± 0.6a	3.9 ± 1.3a	6.0 ± 0.3a
ΔBmpc-7	0.10	0.4 ± 0.1	1.9 ± 0.6a	4.0 ± 1.5a	6.1 ± 0.2a
ΔBmpc-8	0.03	0.2 ± 0.2	2.0 ± 0.6a	4.4 ± 2.0a	5.7 ± 0.1a
ΔBmpc-9	0.03	0.2 ± 0.0	2.1 ± 0.6a	4.3 ± 0.6a	6.0 ± 0.2a

*ΔBmpc-1–9 are the knockout transformants from the parental isolate Bmpc7.

Relative expression of the *MfCYP51* gene in transformants was normalized with the *β-tubulin* gene and compared with that of the isolate Bmpc7.

Mean ± standard error of the mean (SEM); values within the same column followed by the same letters are not significantly different based on the analysis of the least-significant difference (LSD) test at $P = 0.05$ in SPSS.

Table 2 EC_{50} values, relative expression of the *MfCYP51* gene, mycelial growth, sporulation and lesion formation of 'Mona' insertion transformants.

			Fitness parameter		
Isolate/transformant*	EC_{50} (μg/mL)	Relative expression	Mycelial growth (cm/day)	Sporulation (10^6/cm^2)	Lesion size (cm)
HG3	0.01	1	1.4 ± 0.5a	3.3 ± 0.2a	5.3 ± 0.2a
HG3:'Mona'-1	0.04	2.5 ± 1.4	1.4 ± 0.6a	3.4 ± 0.6a	5.2 ± 0.1a
HG3:'Mona'-2	0.08	12.9 ± 2.0	1.4 ± 0.6a	3.6 ± 0.4a	5.0 ± 0.1a
HG3:'Mona'-3	0.06	32.0 ± 12.4	1.3 ± 0.5a	3.1 ± 0.5a	5.3 ± 0.6a
HG3:'Mona'-4	0.10	2.0 ± 0.5	1.4 ± 0.5a	3.3 ± 0.1a	5.1 ± 0.0a
HG3:'Mona'-5	0.10	66.9 ± 18.4	1.4 ± 0.6a	3.1 ± 0.3a	5.3 ± 0.1a
HG3:'Mona'-6	0.06	11.1 ± 2.2	1.4 ± 0.5a	2.9 ± 0.6a	5.2 ± 0.3a
HG3:'Mona'-7	0.12	49.0 ± 5.8	1.4 ± 0.5a	3.1 ± 0.3a	5.1 ± 0.1a
HG3:'Mona'-8	0.14	48.2 ± 9.0	1.4 ± 0.6a	3.7 ± 0.1a	5.3 ± 0.1a
HG3:'Mona'-9	0.10	26.7 ± 4.3	1.3 ± 0.6a	3.5 ± 0.2a	5.3 ± 0.1a
HG3:'Mona'-10	0.11	7.7 ± 1.1	1.3 ± 0.6a	3.6 ± 0.1a	5.1 ± 0.1a

*HG3:'Mona'-1–10 are insertion transformants from the parental isolate HG3.

Relative expression of the *MfCYP51* gene in transformants was normalized with the *β tubulin* gene and compared with that of the isolate HG3.

Mean ± standard error of the mean (SEM); values within the same column followed by the same letters are not significantly different based on the analysis of the least-significant difference (LSD) test at $P = 0.05$ in SPSS.

G418 … DA … DA …
… (*et al.*, 2013) …
… *M. fructicola* …
… (*et al.*, 2008) … D … B 7 … (*E*- … $< 10^{-10}$).

G … 1 …
…). A …
… *P. digitatum*, … E1 …
… *PdCYP51A* (G … *et al.*, 2007) … *PdCYP51B* (… *et al.*, 2013 …). A … E1 …
(… *et al.*, 2013).
… 20 … 3 … 4. …

. t s t t *ADR1* s. . . s t. s t . . . t. t s *CF2-II* *CdxA*. A s t - s t t s . . t. H t *et al.* (2000) *P. digitatum* s. t . s s 126- . . . t t . t. t. D I- s t. s. . . 126- . t . s t. s t s t t s *ADR1* *HSF,* 15 s t. s . . t. t s *HNF3-β*, *CdxA*, *MZF1*, *GATA-1*, *GATA-2*, *Pdx-1*, *Elf-1*, *c/EBPβ*, *v-Myb* *c-Ets-1(p54)*. I t . s, t . t s t t AD 1 s . t. t t . . . It s ss . t *M. fructicola* *P. digitatum* s . t . . . . *ADR1* s s . . . t. t . s . . - . s s . . t s t . . s t . . t t . . . ss . . s . . . . t . t (C , 1975; C B . t. , 1979). As *ADR1* . . s . s . s. t . t . s . , . *M. fructicola*, . t . . t s *ADR1* . . t . . t. . . . , t . t s t t s . t. . s s. s t . . s . t . s . st . . st . . . . . t . . *AP-1,* I - t . t s t t , s t . t . (D I) . s s . t . st . t . s (A - , 1999; A *et al.*, 1997; *et al.*, 2010). A t . t - s t t , *CCG-8*, s . . . t. t t . t. t . . t . t . s s. s s . s *Neurospora crassa* *Fusarium verticillioides* (. *et al.*, 2014).

As *M. fructicola* . . . t . . , t s t . . . . t . . s t. t. s (. . *et al.*, 2010). I . st. , . st . s s - . s . s s. . . t. t s s t - . t . t . . t. t s ts . . . s t. ' . t . s. . t ts . . . . . t. t . . . . . t t . . . st. . s . t . . . . (, - . . . s, 2001), . s t t - st . . . . t. . . t . t . . - . . . . s t s ts . t. . . s . . . *et al.* (2010) s. ss . . *Agrobacterium* s .- . . C. 1-G. , t s ts t t . . . - . H . . . , t . t s . t . . t . ts t t *M. fructicola* . . . t s . - . s t t . s. . . s, t. . . . . t . s, s . A- . . . s . - (*et al.*, 2008), t . s . t - s. s . . s *Monilinia* s .

, . t . . t . t ', t ' . . t s . t. . st . t . *MfCYP51* . . . . . . EC$_{50}$ . . t . s. t . s. t ' . s - s . s s . t . . t. ' . . t D I . s st- . . C . s , t . t . . . ss t . *MfCYP51* . . s . t s . ts s . t t t . D I- s . B 7, t . EC$_{50}$ . . s . . . t t B 7. It s s- s . t s . t . t s . *MfCYP51* . A t . . . t s s . t . t. . t. .

I . s , t . t s s - . . . . t ' s . t- . st . t t s . . t. . . . ' . . . t . t. . t . D I . . . s s . t . t. . - . . . ss t . st . *MfCYP51* . . . A '- s. . . t s . . s s . t t t . . . . . t t . D I . . *M. fructicola* . s t . . t. . .

EXPERIMENTAL PROCEDURES

Media and buffers

- . . . 184 $_2$H $_4$ 145 H$_2$ $_4$. t . . - . . . 30 . $_4$·7H$_2$ 15 C . t . . I . t . . (I) . - . . (10), - . . . (20), 1 . s. , 20% (H$_4$)$_2$ $_4$ (/.) (2.5), 1% C$_2$ (/.) (1), 50% . (/.) (10) 7.808 2-(*N*-). t . s. (E ; 40 , 195.2) . t . , H 5.6. C -I . . . 400 μ . t s - . I t 18% s. . . . . $_4$ (1.2) $_2$H $_4$ (10). . C . . . 1.2 s t , 10 t s(- . t . t . (s)-HC , H 7.5, 50 C$_2$. . . . . . 1 s. s. , 1 t. t t 1 s. - . . t . , 2% . . . . . A . 0.6 s t 100 . s-HC (H 7.0). . . . B . 1.2 s t 10 . s-HC (H 7.5).

Fungal isolates, cultivation and DNA preparation

. . s . s . t. s *M. fructicola* . . . t. st . . t s C t . _ A . t . . 2006 2013. C t . s . . st t. . −80°C (. . 1, s. . . t I). Is . t. s . . . . . . t . t. . DA 22°C 5 s t . . F D A . t t , s . . s t . . . . t s . . t 40 . t s. t (DB) . . 25°C 160- s . 3 s. . . s . . st. , s. t st. . . D A s s. s. . . t . t . t. s t . s . . . t G. D A E t t t (s G. B t. , B. , C).

Plasmid construction

. . *Npt2*- . s. s s st . t . t . st . t . . t '. . . . F t . *Npt2* . . s . CA BIA2300 . s t . . tII-F/ tII- , . st. t *Bam*HI *Pst*I, t . t t . BHt2 . . t t . . . t . t . t. . s . . . . ' s. . . . s . . s t . . s FE/ E . D A t . D I- s . B 7. . . C . t s . st. t *Kpn*I *Sma*I . t t . . st . . t . *Npt2* . . t . s . . t . . . t . s - . . . . . s. s. t st. t t . . . t s . st. . . 2 (s. . . t- I).

Promoter deletion constructs

[illegible] 10- [illegible] G418 [illegible] (2 [illegible] 7) [illegible] (1) [illegible] (F[illegible] 2) [illegible] 2 [illegible] 7 [illegible] 100 μ[illegible] 1 : 1. A[illegible] (B[illegible]) [illegible] CA, [illegible] A) [illegible] 30 [illegible] 95°C, 2 [illegible] 72°C, 2 [illegible] 37°C [illegible] 2 [illegible] 25°C. [illegible] *Kpn*I [illegible] *Sma*I, [illegible] *Npt2* [illegible] B[illegible] 7 [illegible]

ATMT of *M. fructicola*

[illegible] *M. fructicola* [illegible] (B[illegible] 2006). A[illegible] *Agrobacterium tumefaciens*, [illegible] 28°C [illegible] 220 [illegible] (B) [illegible] (50 μ[illegible]/[illegible]) [illegible] (50 μ[illegible]/[illegible]) [illegible] (50 μ[illegible]/[illegible]). [illegible] 600 (D_{600}) 0.1 [illegible] 10 [illegible] 0.4 μ[illegible] 6 [illegible] D_{600} [illegible] 0.5 0.6. C[illegible] *M. fructicola* [illegible] *A. tumefaciens* [illegible] 10^6 [illegible]/[illegible] 200 μ[illegible] A[illegible] 2 [illegible] 22°C [illegible] DA [illegible] B (50 μ[illegible]/[illegible]) [illegible] (300 μ[illegible]/[illegible]) [illegible] 6 [illegible] 22°C [illegible] DA [illegible] B (100 μ[illegible]/[illegible]) [illegible] (300 μ[illegible]/[illegible]).

Construction of knockout and insertion fragments of 'Mona'

[illegible] (D[illegible]) C[illegible] 2. F[illegible] 5′ [illegible] 3′ [illegible] D[illegible] B[illegible] 7 [illegible] 1/ 2 [illegible] 3/ 4 [illegible] (H[illegible]) [illegible] *Aspergillus nidulans trpC* [illegible] BH[illegible]2 [illegible] 5/ 6 [illegible] (5′ [illegible] 3′ [illegible] H[illegible]) [illegible] D[illegible] C[illegible] A 2481- [illegible] 2494- [illegible] 459 [illegible] 7/ 8 [illegible] 9/ 10 [illegible] D[illegible] B[illegible] 7 (F[illegible] 2). [illegible] (H[illegible]) [illegible] (F[illegible] G). [illegible] H [illegible] 5/ 6 [illegible] BH[illegible]2 [illegible] F/ [illegible] D[illegible] B[illegible] 7. [illegible] 5 [illegible] (H[illegible]) [illegible] (F[illegible] G). [illegible] 5′ [illegible] 3′ [illegible] B[illegible] 7 [illegible] HG3 [illegible] 1/ 11 [illegible] E) [illegible] 12/ 4 [illegible] F), [illegible] E, F, G) [illegible] D[illegible] C[illegible] 7/ 8 [illegible] 9/ 10, [illegible] HG3 (F[illegible] 4).

Protoplast generation and transformation

[illegible] *M. fructicola* [illegible] (10^6 [illegible]/[illegible]) [illegible] 50 [illegible] DB [illegible] 22°C [illegible] 2 [illegible] 120 [illegible] 10 [illegible]/ [illegible] (A[illegible]), 10 [illegible]/ [illegible] (B[illegible] C[illegible]), 10 [illegible]/ [illegible] 5 [illegible]/ [illegible] C. [illegible]-10 (D[illegible] B[illegible] C[illegible]) [illegible] 0.22-μ[illegible] E[illegible] (B[illegible] A) G[illegible] A[illegible] 8 [illegible] 0.8 [illegible] 28°C [illegible] 4 [illegible] 80 [illegible] 3 [illegible] A[illegible] 15 [illegible] 1500 *g* [illegible] 10 [illegible] B. A[illegible] 1000 *g* [illegible] 10 [illegible] 3 [illegible] B[illegible] 0.5 [illegible] B. [illegible] D[illegible]A (1 μ[illegible]) [illegible] 10^7 [illegible] 1 [illegible] 60% [illegible] (EG), 10 [illegible]-HC[illegible] 10 [illegible] C[illegible]2. [illegible] 20 [illegible] 1 [illegible] C[illegible] 2000 *g* [illegible] 10 [illegible] 4°C. [illegible] 100 [illegible] A[illegible] 48 [illegible] DA [illegible] 150 μ[illegible]/ [illegible] B. H[illegible] B- [illegible] DA [illegible] 70 μ[illegible]/ [illegible] B[illegible] H[illegible] 5- [illegible]F/[illegible].

Real-time PCR

[illegible] A [illegible] 3 [illegible] DB [illegible] (I[illegible] C[illegible] CA, [illegible] A) [illegible]'s [illegible] F[illegible] D[illegible]A [illegible] 1 μ[illegible] A [illegible] A F[illegible] D[illegible]A [illegible] F[illegible] I[illegible] C[illegible] CF 96 [illegible] C[illegible] D[illegible] (B[illegible] I[illegible]) [illegible] B[illegible] G[illegible] I [illegible] A[illegible] 20-μ[illegible] 10 μ[illegible] B[illegible] C[illegible] (A[illegible] B[illegible] C[illegible]), 1 μ[illegible]

0.2 µ C F C t

s: 3 95°C, 40 s

94°C 20 s, 55°C 20 s 72°C 20 s.

s t t t t-

t A t t ss t *MfCYP51*

t t ss t *β-tubulin*

tt s ss s t *Ct*

($2^{-\Delta\Delta Ct}$) t (, 2005).

Sensitivity to propiconazole and fitness components

s st t t t ss ts

st ′ t s t t s ts: ( )

t ; ( ) s ; ( ) t t t s s

t.

st t t ss ss DA

0, 0.03, 0.05, 0.1, 0.3, 0.5 1 µ / . s

t 5- t s 5- - s

s t t s 9- st t s s t

t s

s ss st t t t 5 s

22°C t EC_{50} s, s tt

t t t 50%,

ss t t t st t -

t t s t s

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SUPPORTING INFORMATION

A t I t . . s t s t . s . 's . st. :

Table S1 C t. st s t . *Monilinia fructicola* s . s t s st .

Table S2 . s s t s st .

Fig. S1 t . st t t . . t - . .

Fig. S2 t . . . t t t t t . . t . . . t ′. st. s t . s s (C) s s t . t t s ts .

Fig. S3 I . t t t s ts . (C). (A) D. t. t t . H . s t . 5/H . (B) D. t. t t . st s t s t . – F/H . (C) D. t. t t . st s . t s t . H F/D . (D) D. t. t t . ′ . . t . s t . 5- F/ . (E) . t s t . 5- F/ . . t . s t s ts.

Fig. S4 t . ′ . . t . . t . . . st. s t . s s . . (C) s s t . s. t t s ts .

Fig. S5 I . t t . ′ . . t s. t t s- ts . (C). (A) D. t. t t . H . s t . 5/H . (B) D. t. t t . st s . t s t . – F/H . (C) D. t. t t . st s . t s t . H F/D . (D) D. t. t t . ′ . . t s t . 5- F/ . (E) . t s . t . 5- F/ . t- s t s ts.